MICROBIOLOGICAL TRANSFORMATIONS OF TERPENES

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by
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CONTENTS

LIST OF ABBREVIATIONS

IR	Infrared
LAH	Lithium aluminium hydride
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatogram
UV	Ultraviolet
VPC	Vapour phase chromatogram
ATP	Adenosine triphosphate
CoA	Coenzyme A
En	Enzyme
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
NAD	Nicotinamide-adenine dinucleotide
NADH	Reduced form of NAD
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced form of NADP

The serial numbers of the compounds in Chapter I and the rest of the thesis belong to two different series.

CHAPTER I

INTRODUCTION

INTRODUCTION

The present investigations have as their main objective the study of the mechanisms and stereochemical features involved in the transformations of terpenes by moulds with the aid of hydroaromatic model substrates, as well as a few simple monoterpenic hydrocarbons. Since the microbiological transformations of terpenoids may be regarded as a special case in the general behaviour of microorganisms towards hydrocarbons, it may be worthwhile to recapitulate at the outset briefly the main trends observed in the fermentation of hydrocarbons in general with microorganisms. A more detailed summary will follow on the microbiological oxidation of terpenes, the main subject of this dissertation.

Degradation of hydrocarbons by microorganisms

The ability to utilise hydrocarbons as carbon and energy sources is a general characteristic of a wide variety of microorganisms. It is a common experience to find that many bacteria, yeasts and fungi chosen at random can degrade most of the alkanes, isoalkanes and olefines. A striking phenomenon that is repeatedly encountered in this field is that many organisms, although not originally selected for their special ability to utilise hydrocarbons, are found to be even more versatile in their capacity for metabolizing diverse hydrocarbon substrates, than those which are specially isolated to degrade hydrocarbons by enrichment culture techniques (McKenna et al. 1962: Foster 1962).

A review of literature would indicate that the metabolism of hydrocarbons by microorganisms has been studied for the past seventy years from diverse objectives, ranging from the mechanisms of degradation of a particular compound by a specific strain, to the possible utilisation of hydrocarbons - one of the cheapest carbon sources - for obtaining proteins, carbohydrates, fats and other nutritionally important compounds through microbial means (ZoBell 1946, 1950; Happold, 1950; Beerstecher, 1954; Raymond and Davis 1960; Davis and Raymond, 1961; Fuhs 1961; Champagnat 1962, 1963, Shiio et al, 1963; Yamada et al 1963; Aaon, 1964; Darlington 1964; Johnson 1964; Miller et al 1964).

The absence of large quantities of hydrocarbons among the debris of decomposing plants is a priori evidence that these compounds undergo decomposition in nature in all excepting certain peculiar environments such as source bed of petroleum where conditions favour their preservation (Chibnall 1934; Chibnall and Piper 1934; Sanders 1937). This is of considerable importance in the carbon cycle of nature. That this decomposition of hydrocarbons is brought about by soil microbes, has been established long ago and the capacity of isolated organisms to attack all types of hydrocarbons - aliphatic, alicyclic, olefinic, aromatic and naphthenic in all physical states - solid, liquid and gaseous - has been demonstrated through several decades of investigation.

Regarding the microbiological decomposition of hydrocarbons two opposing trends are clear. (1) It is a common experience

to find that the higher homologues of simple paraffins are more readily attacked by a larger variety of microorganisms than the lower gaseous paraffins. The higher oxidisability of the bigger paraffins has been ascribed to two factors, viz. (i) the presence in the bigger hydrocarbons a larger number of vulnerable points of microbial attack and (ii) the reduced thermodynamic stability with increase in size in these compounds (Zobell 1946, 1950; Johnson 1964).

(2) The greater oxidisability of the higher homologues is, on the other hand, partly offset by their decreasing solubility with increasing chain length and complexity (Johnson 1964).

As regards the simplest hydrocarbon, methane, inspite of its abundant formation in swamps, peatbogs, soils etc., the atmospheric concentration remains at a low level. Urbain, as early as 1901 (Urbain 1901) postulated that methane was either oxidised by ozone in the upper atmosphere or probably by bacteria associated with green plants. His observations were perhaps influenced by the earlier discovery (Miyoshi, 1895) that a strain of Botrytis cinerea degrades paraffin hydrocarbons. A few years later Rahn (1906) demonstrated that the soil moulds including a strain of Penicillium glaucum could not only decompose paraffins, but also utilised them as the sole source of carbon for growth.

Systematic studies on this subject were initiated by Tausz and Peter (1919) who demonstrated that a strain, designated as Bacterium aliphaticum liquefaciens, attacked several olefinic and naphthenic hydrocarbons but failed to utilize benzene, toluene

or xylene. This organism, however, grew profusely on hydroaromatic or alicyclic compounds such as cyclohexane, methyl cyclohexane, 1,3-dimethyl cyclohexane and 1,3,4-trimethyl cyclohexane. This strain and another, "Bacterium aliphaticum", seemed to prefer the unsaturated hydrocarbons caprylene, $C_{10}H_{18}$, and octene, C_8H_{16}, to the saturated caprane, $C_{10}H_{12}$, and octane, C_8H_{18}, as growth substrates. It is a common experience to find that the presence of a double bond in the hydrocarbon molecule makes it more susceptible to microbial attack. Haag (1926) noticed that the higher the iodine number of paraffin waxes, the more readily they were broken down by a Mycobacterium sp., as measured by carbon dioxide production. Lantzsch (1922) had earlier presented some evidence that this was not true of cyclic compounds, since a strain of Actinomyces oligocarbophilus which readily assimilated aliphatic hydrocarbons failed to attack cyclic aromatic compounds such as benzene.

The decomposition of aromatic hydrocarbons by soil microbes is, however, by no means, a rare phenomenon and Matthews (1924), Tattersfield (1927), Gray and Thornton (1928), Jacobs (1931) and Sen Gupta (1931) reported on the occurrence of bacteria which could oxidise readily benzene, toluene, xylene and other aromatic hydrocarbons. Zobell et al (1943) reported on the isolation from marine sediments of bacteria which grew on aromatic hydrocarbons such as benzene, toluene, xylene, naphthalene and anthracene, but not as readily as on the paraffinoid hydrocarbons of comparable molecular weights. Similar results were also reported by Strawinski and Stone (1940) for enrichment cultures of soil

bacteria which attacked naphthalene, biphenyl, tetralin, decalin and butyl benzene, but not as fast as they metabolised the acyclic paraffins.

Regarding the oxidisability of long chain hydrocarbons, Hopkins and Chibnall (1932) made the observation that a strain of Aspergillus versicolor showed good growth on n-tricosane, $C_{23}H_{48}$, fair growth on n-heptacosane, $C_{27}H_{56}$, n-nonacosane $C_{29}H_{60}$, and n-triacontane, $C_{30}H_{62}$. A farther increase in the chain length in the substrate molecule retarded the growth, as the organism grew feebly on n-tetratriacontane, $C_{34}H_{70}$, but not on n-pentatriacontane, $C_{35}H_{72}$.

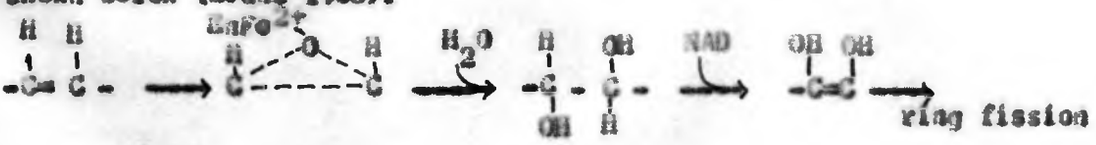
In the olefinic hydrocarbons the position of the double bond in the molecule also influences to some extent the effectiveness of the compound as a carbon source. n-1-Pentene $CH_2{=}CHCH_2CH_2CH_3$ supported the growth of a mixed culture far more effectively than n-2-pentene, $CH_3{-}CH{=}CHCH_2CH_3$ (Strawinski, 1943). No such generalisation could be made about alicyclic or aromatic compounds. While naphthalene was found to be metabolized more readily than the saturated decalin, cyclohexene on the other hand, proved to be much superior to benzene as a carbon source for these organisms (Strawinski, 1943). In general, the increased susceptibility of unsaturated hydrocarbons to microbial attack may possibly, at least in part, account for the absence of olefinic hydrocarbons in petroleum (Zobell, 1942, 1949). However, other explanations are equally admissible to account for this lack of olefines, since it is known that hydrogenase-producing anaerobes can saturate double

bonds in compounds such as octene and hexadecene.

It is, however, not possible in every case to correlate on the structure of a given hydrocarbon with its acceptibility as substrate by microorganisms in general, due to the unpredictable specificity of enzymes in hydrocarbonoclastic microorganisms. A case in point will be discussed in connection with the microbiological transformations of terpenes.

Another generalisation which appears to hold true in a large number of cases is that the branched chain hydrocarbons are usually more susceptible to microbial attack than their straight chain isomers (Strawiaski, 1943). However the data obtained by Thijsse and Zwilling-De Vries (1959) on this point are contradictory to this general behaviour. They found that a Pseudomonas growing well on n-pentane did not metabolise 4-methyl pentane and 5-methyl hexane. Increasing the chain length by one more carbon as in 6-methyl heptane, however, rendered the hydrocarbon acceptable to this organism as a growth substrate. Matthews (1924) claimed that in the aromatic hydrocarbons methyl substitutions on the benzene ring increased the susceptibility of the compounds to bacterial degradation, presumably due to the greater potential energy (ΔH) of the substituted benzenes. Following this argument, since breaking of the aromatic ring in benzene yields 800 Kcal. in comparison to 1400 Kcal. given by pinene, it is expected that pinene will support growth more readily than benzene (Zobell, 1946).

Regarding the pathways for degradation of the aromatic rings a wealth of information is available from the study of degradation of phenolic compounds and aromatic acids. In all cases known so far, the oxidation is initiated by molecular oxygen as an obligatory oxidant. The microorganisms are believed to elaborate by induction a whole sequence of enzymes which convert aromatic substrates first to ortho and para disubstituted derivatives, followed by a cleavage of the ring to aliphatic acids (Happold 1950, Stanier 1955, Dagley et al 1960, Fuhs 1961, Hogoff 1961, Hayaishi 1962, Evans 1963, Hayaishi, 1964). These ring-fission products are further metabolised and then funnelled into the Krebs cycle through a variety of pathways depending on the organism as well as the cultural conditions. In most of these pathways the cleavage of the aromatic ring can be considered to be a distinctive step. Instances, however, are also known where microorganisms only modify aromatic compounds either by hydroxylation or by elimination of substituent group without causing ring-fission. A common experience in such cases is the dihydroxylation reaction through a perhydroxylation reaction usually leading to the trans dihydro diols which then undergo dehydrogenation to catechols with or without ring fission according to the scheme shown below (Evans 1963):

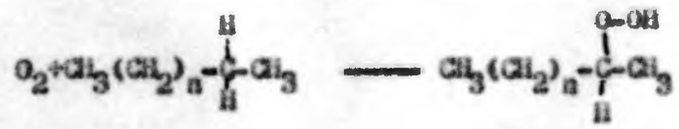


This sequence of reactions has been demonstrated in cell-free extracts from hydrocarbon decomposing organisms (Dagley et al 1960,

Rogoff, 1961, Evans, 1963).

The effect of substituents on aromatic rings has been the subject of several recent investigations and the results have been summarised by Evans (1963). The following trends are observed: methyl ethers are demethylated to give the hydroxy compounds. Methyl groups are oxidised to carboxylic acids but chloro, nitro and sulphonic acid groups remain unaffected unless their removal is obligatory for ring fission. Decarboxylation of carboxyl groups and oxidative elimination of aliphatic side chains are also observed.

It is worthwhile to compare the microbiological oxidation and thermal oxidation of hydrocarbons where such data are available. It is known that the thermal oxidation of a paraffin at 100-200° tends to initiate at a beta carbon atom. In the alkylated naphthalenes, however, the target of this oxidation is usually the carbon of the ring to which the side chain is attached, but in alkylated benzenoid compound the attack is usually on the position adjacent to ring (Zaidens 1946). The initial oxidation product is presumed to be a hydroperoxide in all hydrocarbons such as:



Paraffin Hydroperoxide

Davis and Raymond (1961) have described an interesting case where a Nocardia species which normally grows on alkanes can bring about the oxidation of alkyl substituted alicyclic or aromatic hydrocarbons only when the latter are present as co-substrates

along with alkanes. Usually the alkyl substituted cyclic hydrocarbons are oxidised to aliqyclic acids. However, compounds such as n-acayl benzene and n-dodecyl benzene with sufficiently long alkyl side chains attached to the aromatic ring support excellent growth of this organism in mineral salt medium even in the absence of alkanes. In these cases ω -oxidation of the alkyl substituents was followed by a progressive β-oxidation. Of particular interest in this connection are the observations that cyclohexylacetic and phenylacetic acids with a two-carbon residual side chain are resistent to further oxidation by this Nocardia, although cyclic acids with one, three or other odd-carbon side chains were readily oxidised and utilised for growth. p-Cymene was converted by this mould to cumic acid if added to the medium along with n-alkanes.

While it seems fairly certain now that alkanes are oxidised by ω-oxidation followed by β-oxidation, the nature of primary attack at the terminal methyl group is still an open question. There are apparently two mechanisms for the primary attack on the terminal methyl group of the alkanes. The first of these involves the action of a mixed function oxidase and the participation of molecular oxygen. Isotopic studies with O^{18} have demonstrated that there is an incorporation of labelled oxygen in the product (Stewart et al 1959 ; Foster and Foster 1963). The participation of a hydroperoxide as an intermediate has also been suggested (Finnerty et al 1962).

In addition to the aerobic oxygenation, there seems to be a second mechanism involving anaerobic hydroxylation of the

alkanes probably via unsaturation and hydration. From the observation that hydrogen was transferred from paraffinic hydrocarbon to methylene blue in Thunberg tube experiments, it has been assumed that perhaps the first step in the degradation of alkanes is the formation of unsaturated hydrocarbons (Tausz and Donath 1930, Webley and DeKock 1952, Senez and Azoulay 1961, Chouteau et al 1962, Azoulay et al 1963, Gholson et al 1963). However, the exact mechanism of anaerobic conversion of the alkanes to the corresponding alcohols is still obscure and subject to controversy (Johnson 1964).

MICROBIOLOGICAL TRANSFORMATIONS OF TERPENES

The microbiological transformations of steroids which may be regarded as special cases of oxidation of compounds of terpenoid origin have been the subject of extensive studies in the last thirteen years. This subject will not be dealt with in the present summary as many excellent reviews are available on the subject (Fried et al 1955, Eppstein et al 1956, Talalay 1957, Fischer and Wettstein 1957, 1958, Tamm 1961, 1962, Pasqualini 1963).

Sporadic reports are available in literature on the subject of microbial action on simple mono and sesquiterpenes covering a period of fifty years. Although most of these reports deal with oxygenated terpenes as substrates, it is necessary to present a brief review, as the starting material for these studies may be regarded as intermediates in the degradation of terpene hydrocarbons.

Mayer and Neuberg (1913) obtained citronellol in 59%

yield by reducing the aldehyde citronellal by bottom yeast. Neuberg and Kerb (1913) were able to convert citral to geraniol in 30% yield by the same method. Cyclocitral was, however, not affected by this strain. Another example of such transformation is the conversion of coniferyl aldehyde to coniferyl alcohol (Pauly and Feuerstein, 1929). Bolinari (1929) demonstrated the conversion of citronellal to citronellol and citronellic acid by Acetobacter xylinum.

In more recent years Neuberg (1950) while studying the phytochemical reduction of terpenoid compounds with Baker's yeast observed that 2,3 diketocamphane (1) is reduced in 63% yield to 3-hydroxy camphor (2).

1 2

There was some stereoselectivity in the reduction process, since the (+) camphorquinone gave the (+) hydroxycamphor, while the racemic dl quinone was converted to the levorotatory hydroxyketone. Transformations of some potential commercial interest were discovered by Babicka et al (1955) who found that the fermentation of citronellol, pulegol and isopulegol by a strain of Penicillium digitatum yielded menthol in good yields.

Microbiological degradation of camphor:

The first systematic studies on microbiological transformation of a terpenoid ketone, camphor (3) were initiated several years ago by Gunsalus and coworkers (Bradshaw et al, 1959) at the University of Illinois. Several strains of bacteria were found to degrade camphor. Of these two Pseudomonads C_1 and C_3 isolated from sewage sludge by enrichment culture technique utilised (+) camphor (3) as a sole source of carbon. The pathways of degradation by both these strains were similar, although some minor differences in the adaptation pattern and the relative concentrations of the fermented products were apparent (Madegnard et al 1961, Conrad et al 1965a).

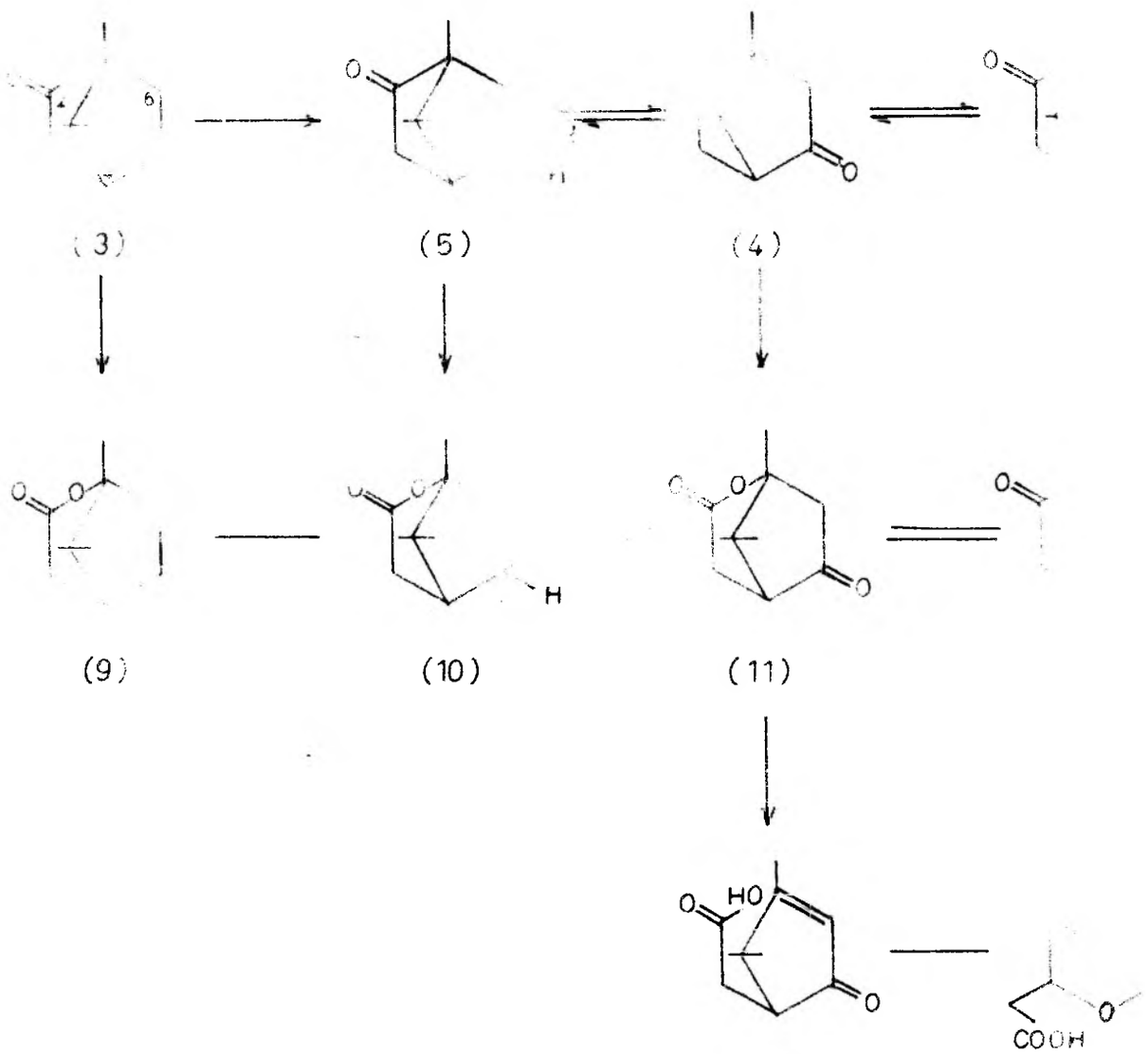
The neutral products obtained from fermentation of camphor were identified as 2,5-diketocamphane (4), 5-exo-and 5-endo-hydroxy camphor (5 and 6). Among the acidic products 3:4:4-trimethyl-5-carboxymethyl cyclopentenone (7) was isolated and identified. Complete oxidation of this acid by the resting bacterial cells was inhibited by 2,2'-bypyridine with the accumulation of a lactonic acid intermediate (8). (Fig.I)

The pathways of degradation of camphor were partially formulated as follows:

D(+)Camphor (3) ⟶ 5-hydroxy camphor (5, 6) ⟶ (4) ⟶ (7) ⟶ (8) ⟶ ?

The ring cleavage reaction of the carbocyclic rings of camphor was studied extensively (Conrad et al 1961a and 1961b, Conrad et al 1962, Bertland et al 1963, Gunsalus et al 1964, Conrad et al 1965b).

PATHWAY EGRADA



Cell-free extracts from the Pseudomonad oxidised camphor to 1,2 campholide (9) and 2:5-diketocamphane (4) to the compound (7) with NADH and oxygen in presence of added FMN. The formation of compound (7) from (4) appeared to proceed via the 1,2 lactone (11). The hydroxylactones (10,12) were also obtained as by products. The unfractionated extracts also reversibly dehydrogenated compounds (10 and 12) to (7) through (11) (Fig.I).

The interesting feature in the dissimilation of camphor by this organism is that both the carbocyclic rings are cleaved by a lactonising mechanism. The lactonising enzyme system has been purified and has been shown to consist of two distinct enzymatic activities. The overall reaction may be represented as follows:

1) $NADH + H^+ + FMN \xrightarrow{E_1} NAD^+ + FMNH_2$

2a) $FMNH_2 + E_2\ 2Fe^{3+} \longrightarrow FMN + E_2.2Fe^{2+}$

2b) $FMNH_2 + O_2 \longrightarrow FMN + H_2O_2$

3) $E_2\text{-}2Fe^{2+} + O_2 + (4) \longrightarrow E_2 2Fe^{3+} + (7) + H_2O$

$NADH + H^+ + O_2 + (4) \longrightarrow NAD^+ + (7) + H_2O$ = sum (1+2a+3)

Both the enzymes which constitute lactonising system, E_1, the FMN coupled NADH oxidase and the iron containing lactonising enzyme itself (E_2-$2Fe^{2+}$) were highly purified (Bertland et al, 1963, Conrad et al 1962) and studied in considerable detail (Gunsalus et al 1964, Conrad et al 1964, 1963b). Both the enzymes are supposed to exist as a loose complex, so that the flavin in the preparations is not autooxidised, since it is not accessible to oxygen. However, in presence of an inhibitor such as 2-2 dipyridyl, the flavin becomes autooxidisable. The lactonising enzyme itself is a mixed function

oxidase requiring the presence of a reducing agent along with substrate. It has been postulated that the lactonisation is brought about by a mechanism analogous to Bayer-Villager oxidation of ketones to esters mediated by an enzyme-bound peracid anion on the carbonyl carbon of camphor (3), followed by a migration of the bridgehead carbon to the peroxidic oxygen of the adduct (Fig.II). In this connection it is worthwhile to recall that some microorganisms are known to lactonise ring D of the 17-oxo steroids by a similar mechanism (Jacobson et al 1964).

Apart from D+ camphor both L and DL camphor supported the growth of Pseudomonas C_1 (Le Gall et al, 1963) yielding identical products but with different specific rotations.

Fermentation of camphor by a diphtheroid strain:

A different pathway in the degradation of camphor involving the intermediate 2,6-diketocamphane (13) has been found to be present in a diphtheroid strain, T_1, isolated by enrichment on terpin hydrate. This strain has been found to grow on terpin hydrate, α-terpineol as well as camphor. This organism accumulates 6-hydroxy camphor (14) and small amounts of α-campholenic acid (15) (Fig.III) in the medium. An enzyme responsible for the dehydrogenation of 6-hydroxy camphor to 2,6 diketocamphane in presence of NAD^+ was obtained in a crystalline form from this diphtheroid strain (Kuo et al 1964). This enzyme exhibits some interesting steric requirements. (Baum & Gunsalus 1962; Kay et al 1962; Chapman et al 1963).

FIG. II.

BAEYER – VILLAGER OXIDATION OF CAMPHOR

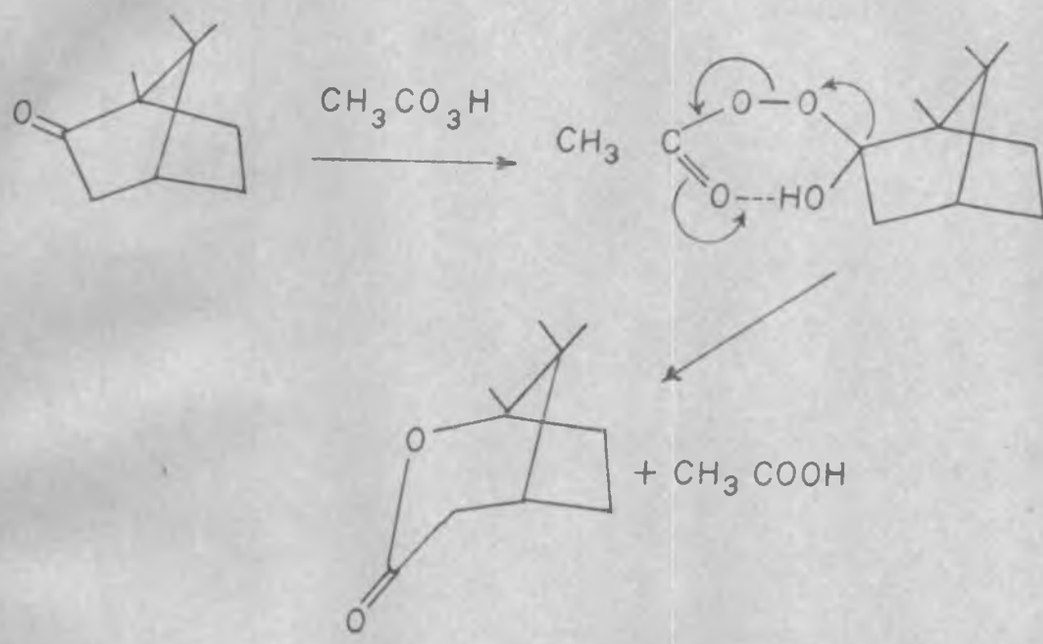
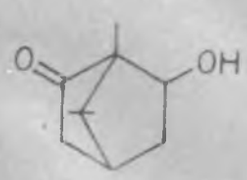


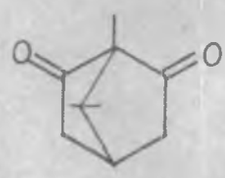
FIG. III

INTERMEDIATES IN CAMPHOR METABOLISM BY A CORYNEBACTERIUM



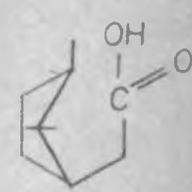
6-HYDROXY CAMPHOR

(14)



2;6-DIKETOCAMPHANE

(13)



∝-CAMPHOLONIC ACID

An interesting case of cross adaptation between terpenes and steroids have been observed in some pseudomonads. Jacobson et al (1964) found that Pseudomonas testosteroni is also capable of utilising camphor as the sole source of carbon and enzyme induction studies have revealed that the lactonising system is inducible in this bacterium either with 17-ketosteroids or camphor as inducers.

Microbial oxidation of citronellol:

Using enrichment culture techniques Werner Seubert (1960) isolated a Pseudomonas sp., Pseudomonas citronellolis, which oxidised citronellal and farnesal, as well as a number of other compounds which may be probable intermediates in the degradation of citronellol. Extending the studies of metabolism of acetate in dried cells and using labelled carbon he was able to demonstrate that the substrate, citronellol, is partially converted into an ether-soluble fluorescent pigment and a number of amino acids and other products. The nature of the fluorescent pigment has not been elucidated.

Transformation of mono and sesquiterpenic hydrocarbons:

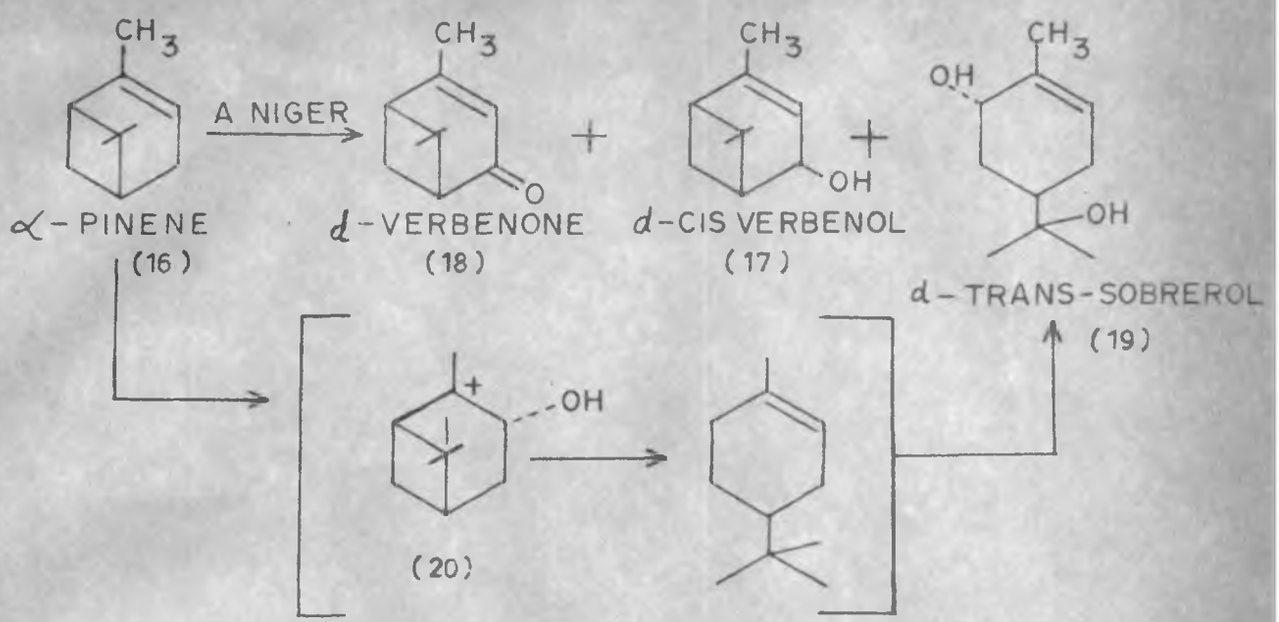
Systematic investigations on the microbial transformations of terpenoid hydrocarbons were started in this Laboratory six years ago. These investigations were stimulated by the earlier observation that the "Agar"-producing plant, Aquilaria agallocha, normally yielding sesquiterpenic hydrocarbons, produced dark patches of a sweet-smelling oleoresin, "Agar" of value to perfumery industry only when heavily infected with fungi. (Bose 1938; Sadgopal & Varma 1952; Jain & Bhattacharyya, 1959; Maheshwari et al 1963).

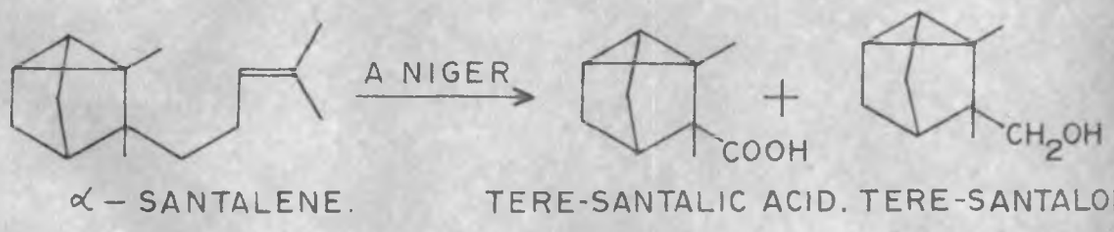
Preliminary screening studies with α-pinene (16) as a substrate led to the selection of a strain of A.niger (NCIM 612) on the basis of its proficiency to metabolise this hydrocarbon (Bhattacharyya et al 1960, Prema & Bhattacharyya, 1962a). It was found that α-pinene was completely degraded by this organism with prolonged fermentation. However by arresting the fermentation at an intermediate stage under conditions of partial utilisation of the added α-pinene, three oxygenated intermediates were obtained (+) cis verbenol (17); (+) verbenone (18) and (+) trans-sobrerol (19). The formation of verbenol and verbenone could be accounted for by an allylic oxygenation process and the formation of sobrerol was probably mediated through an attack of an electrophilic oxygen on the double bond and the epoxide intermediate (20) was postulated to undergo a prototropic rearrangement and hydration to form sobrerol (19) (Fig.IV). The α-pinene employed in these studies corresponded to a mixture of 70% d and 30% l isomers. The products, however, were optically pure and presumably derived exclusively from the d isomer. The l-isomer was also apparently metabolised, since no enrichment was observed of any of these isomers in the unreacted α-pinene recovered from the fermentation.

Subsequently, the same strain of A.niger was found to exhibit a surprising degree versatility in metabolising different terpenoid hydrocarbons. α-Santalene (21) was oxidised by the mould mainly to tere-santalic acid (22) and to traces of tere santalol (23) and a tertiary C-15 alcohol of the probable structure (24) (Prema & Bhattacharyya, 1962b) (Fig.IV) .

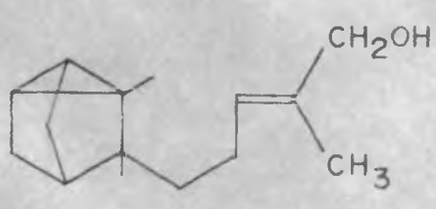
FIG. IV.

MICROBIOLOGICAL TRANSFORMATION BY A. NIGER



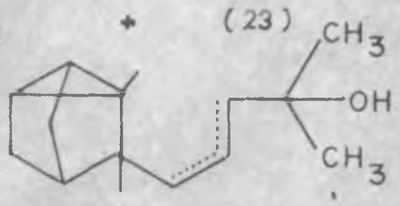


(21)



α-SANTALOL

(22) + (23)

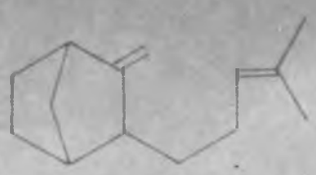


C_{15}-ALCOHOL

(24)

An interesting product, 3-nonene-2,3-dicarboxylic acid, which could be isolated only as the anhydride (25), structurally unrelated to the terpenes used as substrates, was found to accumulate in the medium, when camphene (26), β-santalene (27) and longifolene (28) were incubated with A.niger pellets (Bhattacharyya et al 1963). Since the accumulation of the anhydride (25) took place at a rate parallel to that of the disappearance of longifolene (28) from the medium, the speculation was made whether the (.)-marked fragment common to all the substrates could give rise to the (.)-marked citraconic acid moiety in the anhydride. A mechanism based on the ring-cleavage reaction through epoxydation like the one which leads to sobrerol (19) formation from α-pinene (16), was advanced by Prema (1962) for the formation of the anhydride in accordance with the above hypothesis. Later it was demonstrated that fermentation of two other hydrocarbons of non-camphenoid structure, caryophyllene (29) and δ-cadinene(30) also gave rise to the anhydride (25) (Fig.V). Further, by using sensitive detection techniques it was possible to demonstrate that this anhydride was produced even in the control experiments run without the terpenoid substrates. Radioactive tracer studies with glucose-U-C^{14} and unlabelled longifolene (28) finally established that the anhydride molecule in its entirety may be derived from glucose carbons and that there was no evidence of a preferential incorporation of any partially-degraded carbon skeleton from the terpenoids into the citraconic acid moiety of the anhydride (Bhattacharyya and Dhavalikar, 1965). From the magnitude of disappearance of the substrates during these fermentations, it was

COMPOUNDS STIMULATING THE ACCUMULATION OF ANHYDRIDE

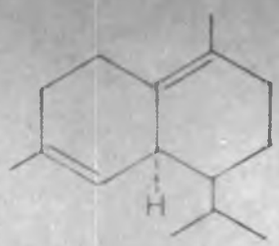


β-SANTALENE

(27)

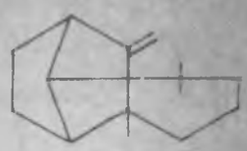
CAMPHENE

(26)



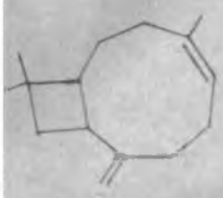
δ - CADINENE

(30)



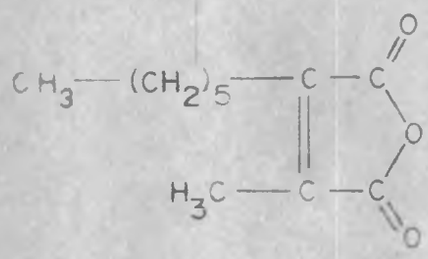
LONGIFOLENE

(28)



CARYOPHYLLENE

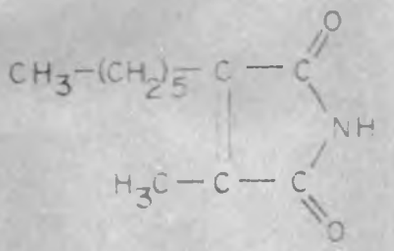
(29)



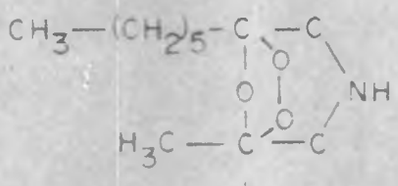
ANHYDRIDE

(25)

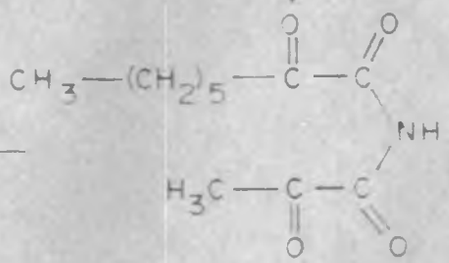
NH_3



O_3



H_2 | $Pd/CaCO_3$



HOH

$H_3C-(CH_2)_5-COCOOH$

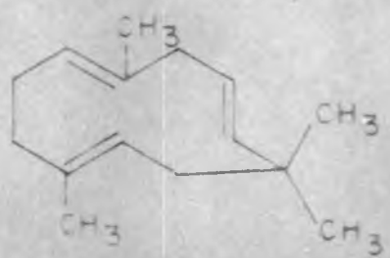
+

$H_3C-COCOOH$

COMPOUND RESISTANT TO ATTACK BY A NIGER

Δ^3 CARENE

CARANE



HUMULENE

evident that although these hydrocarbons do not take part in the synthesis of the anhydride, excepting possibly in the form of one or two-carbon fragments, they were all utilised by the fungus.

Several terpenoids such as carane (31), Δ 3-carene (32), and humulene (33), however, proved to be surprisingly resistant to attack by the mould (Prema & Bhattacharyya, 1962b).

Fermentation of terpenoid hydrocarbons by bacteria

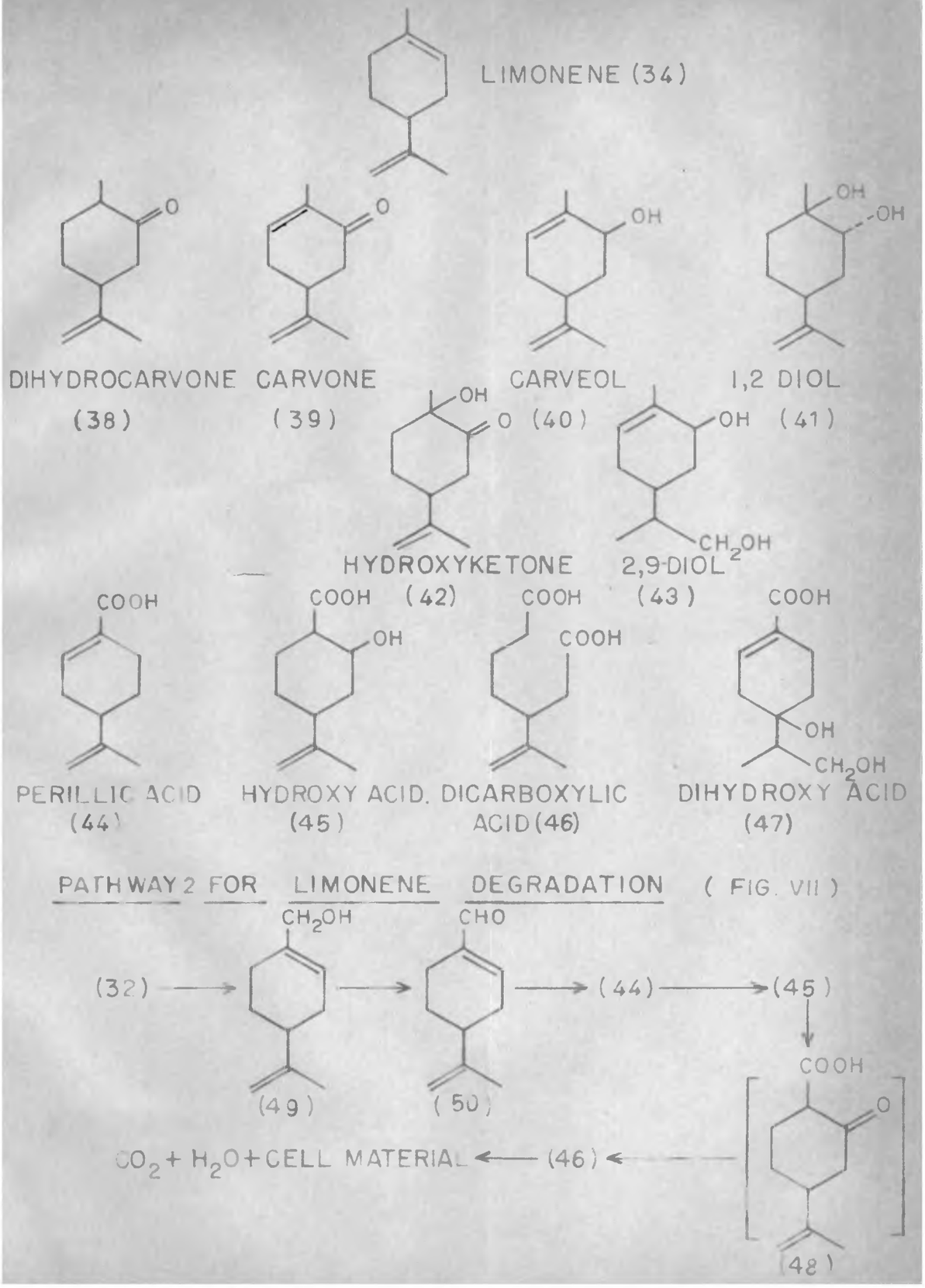
A soil Pseudomonad isolated by enrichment culture technique with α-pinene as the carbon source, exhibited a certain degree of versatility by growing equally well on limonene (34), α-pinene (16), β-pinene (35), Δ 1-p-menthene (36) and p-cymene (37). The pinene-grown cells showed simultaneous adaptation to the other hydrocarbons. But limonene-grown cells did not adapt to the pinenes without a lag phase (Shukla, 1965).

Pathways for degradation of limonene:

Incubation of limonene (34) with this pseudomonad resulted in the formation of large number of neutral and acidic transformation products among which the following were isolated and identified: dihydrocarvone (38), (+) carvone (39), (+) carveol (40), p-menth-8-ene-1, 2-trans diol (41), p-menth-8-ene-1-ol-2-one (42), p-menth-6-ene-2, 9 diol (43), (+) perillic acid (44), 2-hydroxy-p-menth-8-ene-7-oic acid (45), β-isopropenyl pimelic acid (46) and 4, 9-dihydroxy-p-menth-1-ene-7-oic acid (47) (Fig.VI). Adaptive enzyme studies revealed that none of the neutral compounds (38) to (43) was further metabolised by limonene-grown cells and are

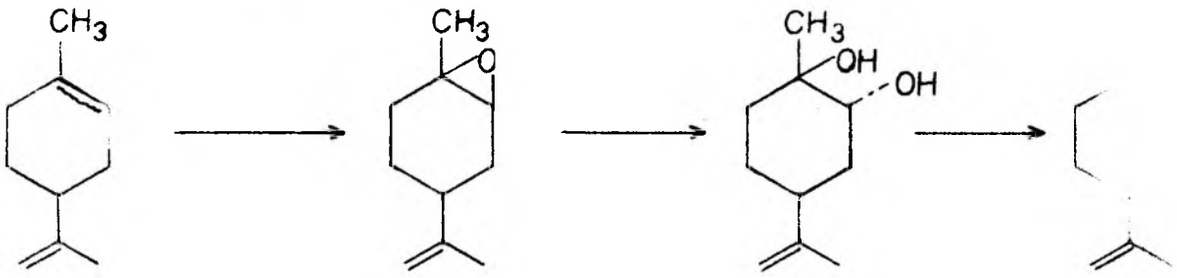
FIG. VI.

FIG. VI PRODUCTS OF BACTERIAL DEGRADATION OF LIMONENE



PATHWAY 2 FOR LIMONENE DEGRADATION (FIG. VII)

PATHWAY I

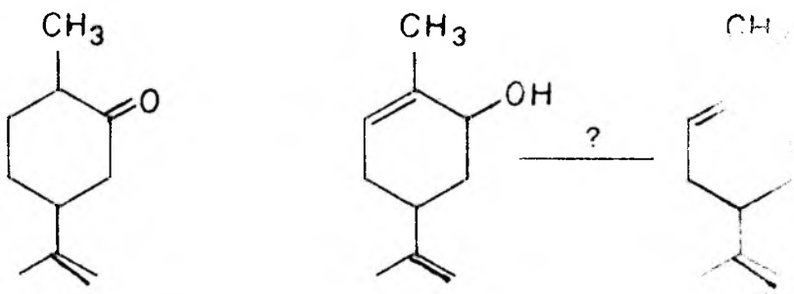


LIMONENE (34)

LIMONENE EPOXIDE

p-MENTH-8-ENE 1-2-DIOL (41)

p-MET E 1-OL-2-



DIHYDRO CARVONE (38)

CARVEOL (40)

CARVONE (39)

probably derived through limonene-1:2-oxide as an intermediate by pathway I (Fig.VII). The acidic compounds (44-46), on the other hand, are all oxidised with and without added chloramphenicol. It was thus possible to formulate pathway 2 initiated by progressive oxidation of the 7-methyl group finally leading to the cleavage of the carbocyclic ring through a base catalysed cleavage of the β-keto acid intermediate (48). Further evidence in support of the pathway 2 was obtained by fermenting limonene with glucose-grown cells which accumulated perillic acid and small amounts of the intermediate, perillyl alcohol (49). Fermentation of perillic acid (44) by cells grown on this substrate or limonene led to the accumulation of the dicarboxylic acid (46).

The hydroxylating enzyme, which leads to the oxygenation of limonene in presence of NADPH and oxygen, was found to be associated with the 100,000 g precipitate from the cell-free sonicates of limonene-grown cells. The soluble fraction (the supernatant from the above precipitate) converted perillyl alcohol (49) and perillic aldehyde (50) to perillic acid (44) in presence of added NAD and a mixture of FAD, FMN respectively. In presence of CoASH, Mg^{2+}, ATP and NAD the same extract oxidised perillic acid (44) to β-isopropenyl pimelic acid (46). (Bhattacharyya et al, 1964).

Pathways for degradation of α- and β-pinene (14,35) and l-p-menthene (36):

The product patterns obtained after fermentation of α and β-pinenes by the same strain were very similar, although they were almost totally different from that from limonene. Among the

neutral products, the presence of borneol (51) and myrtenol (52) were detected. The acidic fractions contained traces of phellandric acid (53), myrtenic acid (54), oleuropeic acid (55), 4 hydroxy-p-menthane-7-oic acid (56) (from α-pinene), 4-hydroxy-p-menth-1-ene-7-oic acid (57) (from β-pinene) and β-isopropyl pimelic acid (58). In addition two other products from the limonene degradation pathway - perillic acid (44) and the dihydroxy acid (47) were also detected in trace quantities. Besides the difference of one double bond in compounds (56 and 57) the products from α and β-pinene had different rotation e.g. α-pinene gave rise to (+)borneol and (+)oleuropeic acid whereas β-pinene gave the corresponding optical antipodes. Curiously enough, the specific rotation of the saturated dicarboxylic acid (58) was the same, irrespective of its origin from α or β-pinene.

α-Pinene-grown cells failed to oxidise borneol (51) myrtenol (52), myrtenic acid (54), oleuropeic acid (55). However phellandric acid (53) and all the compounds in the limonene pathway (Fig.VII) (49, 50, 45 and 46) were oxidised with or without chloramphenicol. From these data it was concluded that the pinenes are degraded by a complex series of pathways 1 to 6. In pathway 1 (Fig.VIII) only the 7-methyl group (or methylene) is oxidised progressively through the alcohol (52) and the aldehyde (59) to myrtenic acid (54) which is resistant to further oxidation. Pathways 2 to 6 are triggered by an initial protonation step on the 4 double bond leading to a carbonium ion (60). In pathway 2 this carbonium ion (60) undergoes a Wagner-Meerwein rearrangement to yield a new cation (61) which yields borneol. The cyclobutane

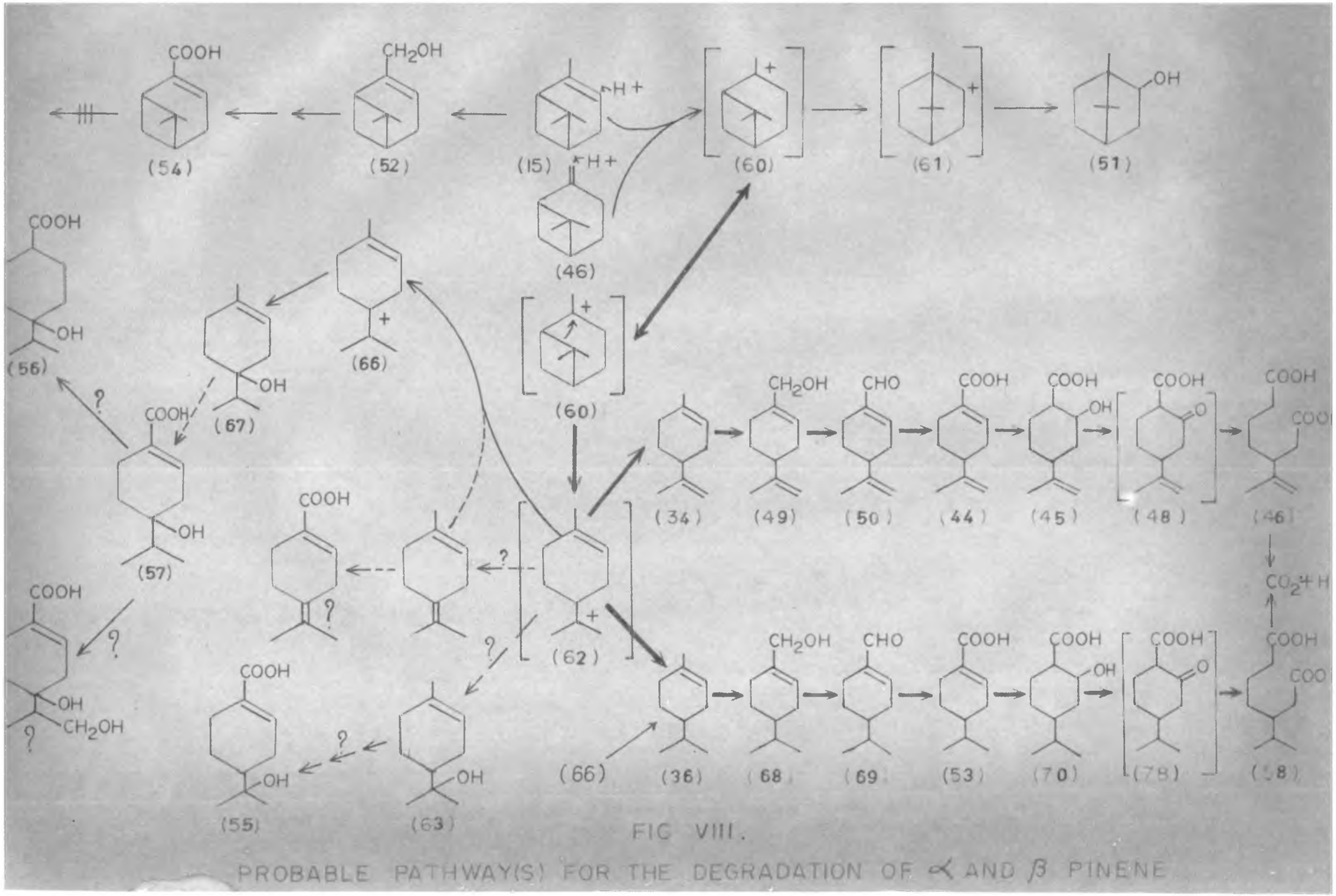


FIG VIII.

PROBABLE PATHWAY(S) FOR THE DEGRADATION OF ∝ AND β PINENE

ring in the ion (60) can also undergo a cleavage to yield (62) which in pathway 3 eliminates a proton to yield limonene which undergoes further transformations (Fig.VIII, pathway 3). A neutralisation of the carbonium ion may lead to α-terpineol (63) which according to pathway 4 is progressively oxidised at the 7-methyl group to yield oleuropeic acid (55) through the alcohol (64) and the aldehyde (65). The carbonium ion (62) can also undergo an internal hydride shift from position 4 to yield a new cation (66).

Two more pathways are possible from 66 in pathway 5 it is neutralised to form 4 terpineol (67) which yields the hydroxy acids (56) and (57) or in pathway 6, this ion (66) is stereospecifically reduced to Δ-1-p-menthene (36) which follows a pathway parallel to that of limonene involving the saturated side chains ultimately to yield β-isopropyl pimelic acid (58) through the intermediate (68), (69), (53) and (70).

Although complete enzymatic evidence in support of the above pathways is not available, Shukla (1965) has shown that the same alcohol dehydrogenase from the cell-free extracts from pinene-grown cells oxidise perillyl alcohol (49) phellandrol (68) and cumyl alcohol (74) at the same rate with NAD. The oxidation of oleuropeyl alcohol (64) is much faster, while myrtenol (52) is not oxidised. Further, the rate pattern with these different alcohols is approximately the same as that exhibited by a purified perillyl alcohol dehydrogenase (Ballal et al 1965) from limonene-

grown cells.

The aldehyde dehydrogenase also oxidises perillic aldehyde (59), phellandral (69), cumic aldehyde (75) and surprisingly enough myrtenal (59) to the corresponding acids in presence of FAD and FMN and with methylene blue as the electron acceptor at comparable rates. Here again oleuropeic aldehyde (65) is oxidised at a faster rate.

Pathways of degradation of p-cymene:

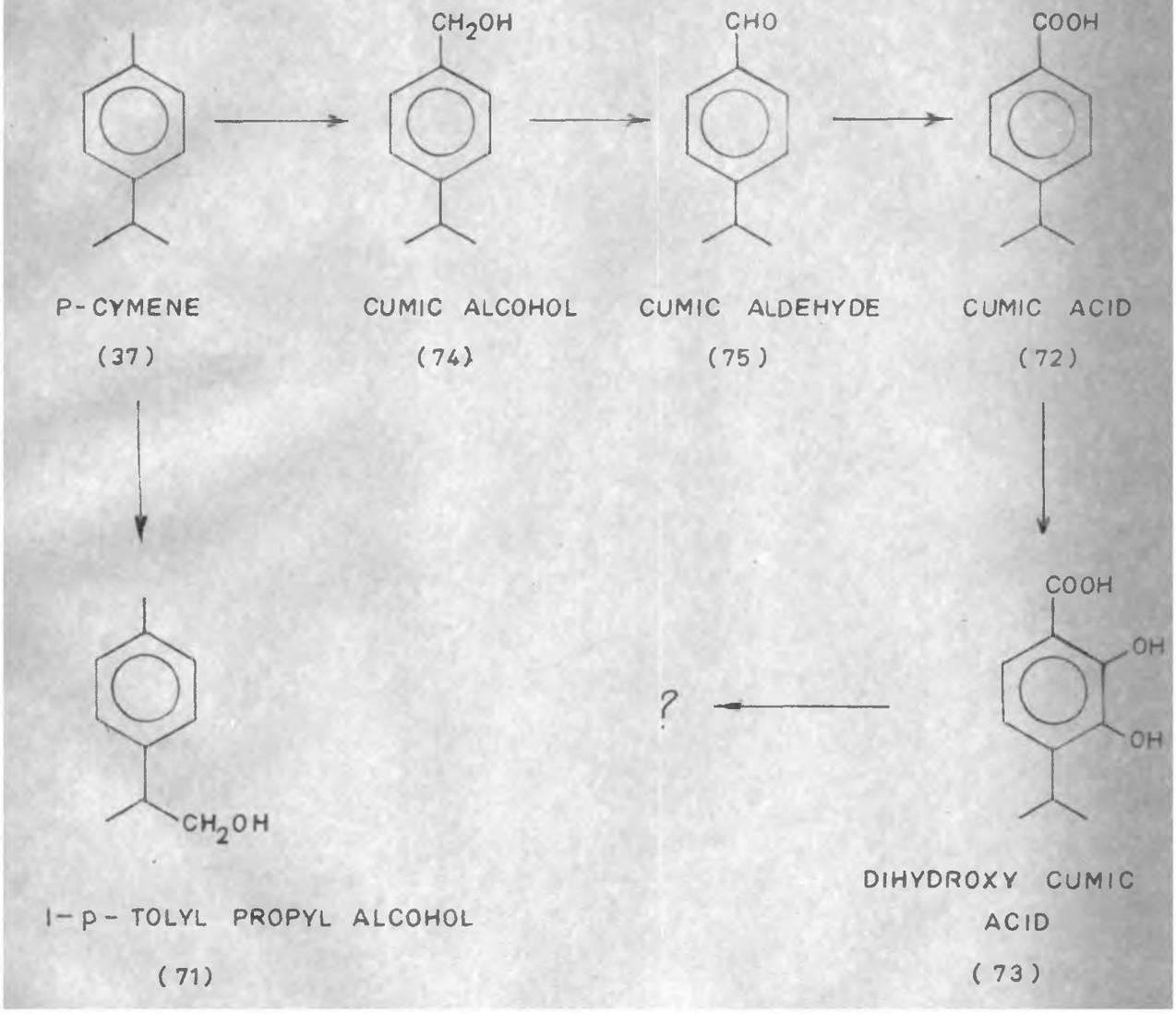
Madhyastha et al (1965) found that the fermentation of p-cymene by the same Pseudomonas leads to the accumulation of 9-hydroxy p-cymene (71), cumic acid (72) and 2,3-dihydroxy cumic acid (73) in the medium. p-Cymene-grown cells oxidise cumic alcohol (74), cumic acid (72) and dihydroxy cumic acid (73). On the basis of these data two distinct pathways are possible (Fig. IX). Upto cumic acid the bacterium follows the same pathway as with limonene (Fig. VII, pathway 2). However, from the data on hand, it appears that at the cumic acid stage a dioxygenation takes place. The aromatic ring appears to break up beyond the stage of the dihydroxy acid.

Substrate specificity studies:

From the observed behaviour of this Pseudomonas towards the terpenoid compounds and intermediates it is apparent that certain basic structural features, functional groups and stereo-chemical factors are necessary in the substrate molecule in order

FIG. IX

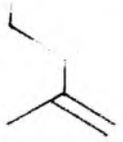
PATHWAY FOR DEGRADATION OF P-CYMENE BY A SOIL PSEUDOMONAD



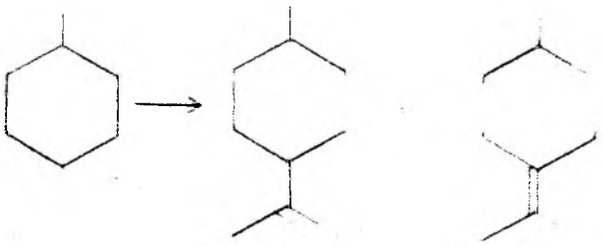
to make it acceptable to the organism. The main targets of chemical activity are (1) the 7-methyl group in all the hydrocarbons (or methylene) (2) a double bond adjacent to this methyl group (1,2 in case of limonene and 4-5 in case of α-pinene) and (3) an isopropyl side chain either free or bound in the form of a cyclobutane bridge. The organism needed the double bond at position 1 in the ring in limonene and Δ_1-p-menthene to break open the carbocyclic ring by hydration. In α- and β-pinenes the same double bond (or the exocyclic one) is utilised for opening up both the carbocyclic rings - the cyclobutane ring by protonation and the resulting p-menthene by hydration at the 7-carboxylic acid stage.

Alteration of any of these essential features resulted in a loss of growth-supporting activity. Although limonene (34) and Δ-1-p-menthene (36) were capable of supporting the growth of this organism their saturated analogue - p-menthane (76)-failed to do so. Pinane (77) and partially saturated Δ-8-p-menthene (78) also behaved in a similar way (Fig.X). However, a mixture of products (78), (79) and (80) obtained by dehydration of dihydroterpineol (81) supported a slow growth. Δ_1-7-p-Menthene (82) and Δ 1(7),8-p-menthadiene (83) were found to be good substrates. The demethylated compound, apopinene (85), was found to be incapable of supporting the growth of this organism. Camphene (26), 1-methyl cyclohexene-1 (86) and β-pinene epoxide (87) and nopinic acid (84) also failed to show a positive response. Oxygenation at position-8 in the p-menthene nucleus, as in α-terpineol (63) and oleuropeic acid (55), made the resulting compounds unacceptable

P-MENTHANE
(76)



LIMONENE Δ^1 P MENTHEN
(34)



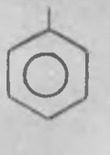
(79)

to the microorganism. Even the methylated (39) or acetylated (8 8) derivatives did not support growth. Δ-3-Carene (32) also failed to act as a growth substrate presumably because of steric reasons (Fig.X). Myrtenol (52), myrtenal (59) or myrtenic acid (54) did not serve as growth substrates presumably because the energy requirement for protonation of the double bond in these compounds to bring about the cyclobutane ring cleavage was rather high.

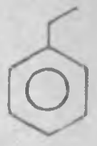
Among the aromatic substrates tested (Fig.XI) the organism failed to grow on benzene, toluene (90), ethylbenzene(91), or cumene (92). Even p-xylene (93) did not support growth. The organism grew, however, on p-ethyl toluene (94) after a lag phase. Lag phases, were also observed with p-isopropenyltoluene (101), p-isobutyl toluene (104) and p-tert-butyl toluene (105). It grew freely however on p-n-propyl toluene (100) and p-sec-butyl toluene (103). Further extensions of the side chain such as in p-3-n pentyl toluene(106) (1-methyl-4-(1'-ethyl-propyl benzene), 1-ethyl-4-isopropyl benzene (97) and 1,4-diethyl benzene (96) were not acceptable to the organism. Here also introduction of an oxygen function at the forbidden position 3 as in p-methyl acetophenone (95), p-methyl propiophenone (114) and p-tolyl dimethyl carbinol (102) resulted in a total loss of growth-promoting activity (Shukla 1966; Shukla et al, 1966). Other compounds tested (98), (99) and (107) to (116) were also inactive. The organism behaves towards these model substrates as if it has a chemical logic for degrading these compounds. It fails to accept as substrates compounds in the degradation of which this logic is not easily applicable.

FIG. XI AROMATIC MODEL COMPOUNDS

P-CYMENE (87)



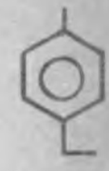
TOLUENE (90)



ETHYL BENZENE (91)

CUMENE (92)

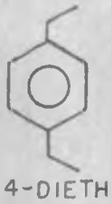
P-XYLENE (93)



1-METHY 4-ETHYL BENZENE (94)

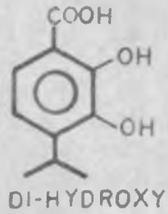
$H_3C-C=O$

p-METHYL ACETO PHENONE (95)



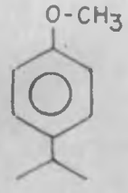
1,4-DIETHYL BENZENE (96)

CUMIC ACID (72)

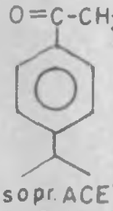


2,3 DI-HYDROXY CUMIC ACID (73)

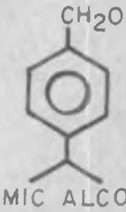
ETHYL-p-Isopr BENZENE (97)



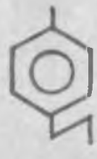
(98)



p-Isopr. ACETO-PHENONE (99)

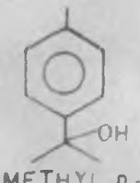


CUMIC ALCOHOL (74)

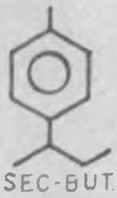


p-n-PROPYL TOLUENE (100)

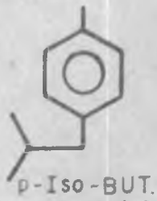
p-ISOPROPENYL TOLUENE (101)



DIMETHYL, p-TOLYL CABINOL (102)

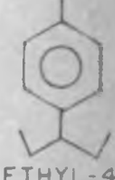


p-SEC-BUT. TOLUENE (103)

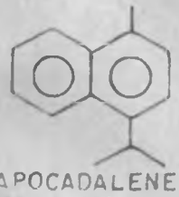


p-Iso-BUT. TOLUENE (104)

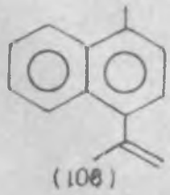
p-ter. BUTYL TOLUENE (105)



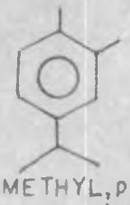
1-METHYL-4-(1-ETHYL-PROPYL)-BENZENE (106)



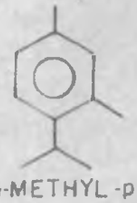
APOCADALENE (107)



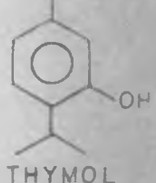
(108)



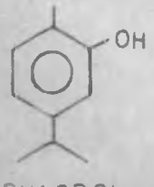
2-METHYL, p-CYMENE (109)



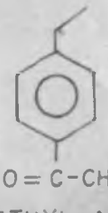
3-METHYL-p-CYMENE (110)



THYMOL (111)

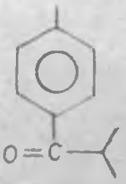


CARVACROL (112)

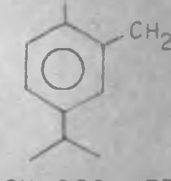


p-ETHYL, ACE-TOPHENONE (113)

p-METHYL-PRO PIOPHENONE (114)



ISOPROPYL-p-TOLYL KETONE (115)



2 CHLORO METHYL P-CYMENE (116)

PRESENT PROBLEM

From the above review it is clear that microorganisms are capable of bringing about different chemical changes in a variety of terpenoid compounds with widely different structures. These reactions include hydroxylation at different carbons, hydration without or with rearrangement, further oxidation of oxygenated functional groups, carbocyclic ring rupture and carbon-carbon bond cleavage.

In the case of the fungal transformations, the subject of this thesis, two rather apparently contradictory trends are observed: In the first place the hydroxylation and other reactions appear to be stereospecific and in the second, a considerable latitude is possible regarding the basic structure of the substrate molecule, since a wide variety of compounds are metabolised by the same mould. Even regarding the second point not all compounds are accepted by the mould since carene, carane and humulene were found to be resistant to microbial attack.

Furthermore, although with the data available it is possible to classify the basic reactions, the development of a general hypothesis from which it will be possible to anticipate the behaviour of the mould towards a given substrate molecule has so far not been possible. In other words, the observed transformations were understandable, but at the same time unpredictable.

In order to investigate more effectively the stereochemical features and the mechanisms involved in these fungal transformations, studies were undertaken with simple hydroaromatic model substrates as well as a few terpenoid hydrocarbons. The results of such studies are presented in this thesis.

Chapter II deals with the materials and methods as well as the experimental procedures involved in the fermentation and extraction of the metabolites.

The transformation of the simplest model compound, cyclohexane, by A. niger is the subject of Chapter III. Three major transformation products have been isolated and identified as 2-cyclohexene-1-one, (+) 2-cyclohexene-1-ol and (+) 3-cyclohexene-1, 2-cis diol. The structure of the diol has been arrived at by physico-chemical and degradation studies and confirmed by synthesis. A model reaction involving vanadium pentoxide and hydrogen peroxide which converts 2-cyclohexene-1-ol to the diol has been developed and the analogy of this reaction with the fungal hydroxylation of cyclohexane has been discussed.

In Chapter IVa the transformations of 1-methyl-1-cyclohexene, a model compound related at the same time to cyclohexene and p-menthene, is discussed. Three major metabolites, a hydration product, 1-methyl cyclohexane-1-ol and two oxidised products (+) 1-methyl-1-cyclohexene-6-ol and 1-methyl-1-cyclohexene-6-one have been isolated and identified.

In Chapter IVb it has been demonstrated that l-methyl-3-cyclohexene, isomeric with l-methyl-l-cyclohexene, is converted by the mould to two main products: the racemic, l-methyl-3-cyclohexene-2-ol and the corresponding ketone l-methyl-3-cyclohexene-2-one.

Chapter V deals with the studies on fungal transformations of the simplest alicyclic monoterpene, limonene. The products from fermentation have been identified as (+) carveol, (+) carvone, (+)-α-terpineol and (+) 2,8 paramenthadiene-ol-1.

The data presented in Chapter VI indicate that l-α-pinene also undergoes the same transformation as were earlier reported for d-α-pinene. Thus the presence of verbenone, cis-verbenol and trans-sobrerol has been demonstrated among the fermentation products. Among the products, the verbenol has been isolated and identified as (-) cis verbenol.

The transformations of β-pinene as described in Chapter VII, lead to the formation of three main products, (-)pinocarvone, (-)pinocarveol and (-)myrtenol.

A general discussion of the stereochemical implications and the possible mechanisms involved in the biooxygenation processes in different substrates has been included in Chapter VIII.

In the concluding Chapter (IX) a summary of the results is presented.

CHAPTER II

MATERIALS & METHODS

CHAPTER II

MATERIALS AND METHODS

Spores of *A. niger* (N.C.I.M. No.612) were maintained in sterile soil as stock culture and subcultured into potato dextrose agar (PDA) medium before use for fermentation studies.

Propagation of the culture: The culture was propagated in a medium containing potato, dextrose, and agar which had the following composition:

Potato extract	20% w/v
Dextrose	2%
Agar	2% - 3%

pH adjusted to 5.8

The above medium (5 to 6 ml) was used for the preparation of the slants used for the growth of the culture. After the medium had been prepared and steamed to form a homogenous melt, 5 to 6 ml aliquots were poured into pyrex test tubes (previously sterilised at 20 p.s.i./1 hr) and autoclaved at 15 p.s.i. for 20 min. These were now slanted and allowed to solidify. The slants were incubated as such at 28°C for 2 to 3 days to ensure their sterility. The slants were now inoculated with the spores of *A. niger* from the stock culture and incubated at 28°C for 6 to 7 days when they exhibited a maximum growth of the spores.

Medium and inoculum:

Modified Czapek Dox medium used in these studies had the following composition: (Prescott & Dunn, 1959)

Glucose C.P.	4.0%
Potassium chloride (Riedel)	0.05%
Magnesium sulphate (B.D.H.)	0.025%
Sodium nitrate (B.D.H.)	0.2%
Potassium dihydrogen phosphate (Daspha).....	0.095%
Dipotassium hydrogen phosphate (B.D.H.).....	0.005%
Ferrous sulphate (Daspha)	0.001%
Corn steep liquor (H.A.L.Poona)...	0.5%
Bacteriological yeast extract (Difco)....	0.05%

pH adjusted to 4.5 to 4.8.

The fermentations were carried out either in deep tank fermentors or in shake flasks.

The fermentors used for the growth were Chain type stainless steel equipments of 6-litre capacity provided with a stirrer and air inlet tube and three additional openings (portholes) on the top of the fermentor in order to facilitate the inoculation and addition of the substrate. The fermentors were preautoclaved for 20 p.s.i. for 1 hr before use. The medium (4 litres) was added and the fermentors containing the medium autoclaved at 15 p.s.i. for 20 min. After cooling they were inoculated with a suspension of the spores in sterile

water (5 ml for each tube) from a single PDA slant under aseptic conditions. The inoculation was conveniently done with the help of a rubber tube attached to a flask. The entire attachment was autoclaved at 20 p.s.i. for 1 hr before use.

In the case of (-) α-pinene and β-pinene the fermentations were carried out on a shaker at 220 r.p.m. with 500 ml Erlenmeyer flasks containing 100 ml of the medium.

Fermentation:

The inoculated fermentors were placed in a thermostatic bath at 28°±1°C and agitation (700 r.p.m.) and aeration (1 vol/min) with a current of sterile air were started. A good growth was usually obtained between 28-36 hr. After the mycelia had attained maximum growth, the substrate under study was added. The mode of addition of the substrate varied with its nature and in each case these conditions were established after initial trials. Usually volatile substrates were added dropwise with the help of a sterile burette to a capillary glass rod (sterilised) which was attached to the fermentor dipping below the medium through one of the openings of the fermentor. This method was adopted because of the high volatility of most of the substrates. During the course of the fermentation the air supply was cut off and the fermentor flushed for 5 min (½ vol/min) at intervals of 30 min. The rates of oxidation were studied for each of the substrates varying the concentration, time of incubation and temperature

to establish the effect of variation of different parameters on the accumulation of products. At the end of each experiment fermented products were worked up according to the procedure described later. Periodic withdrawls of aliquots from the fermented broth were made from time to time during the fermentations and examined for any possible bacterial contamination. Any contamination could be usually detected by the estimation of pH which showed a sharp rise in case of bacterial contamination and by staining broth smears by the standard Gram staining techniques. It was observed that normally the pH remained between 4.5 to 4.8 during the fermentation.

In case of l-α-pinene and l-β-pinene the fermentation was carried out in 500 ml Erlenmeyer flasks containing 100 ml of sterile Czapek dox medium. The flasks were incubated on a rotary shaker (220 r.p.m.) at 28°±1°C after inoculating with dry spores of A.niger (NCIM 612) for 24 to 29 hr to ensure good growth after which the substrate was added under aseptic conditions. Blank experiments were conducted side by side without the mould in order to study the autooxidation products formed. Besides these blanks, control experiments were also run without the substrates but otherwise using identical conditions

Substrates:

Cyclohexene was obtained by dehydration of commercial cyclohexanol (Amrut) with phosphoric acid (Org.Syn.Vol.I, G.H.Coleman and H.F.Johnstone), purified by refluxing over metallic sodium and the fraction b.p.80°-81° at 715 mm was collected. The sample was found to have all the physical

properties as reported in literature for cyclohexene.

1-Methyl-1-cyclohexene

1-Methyl-1-cyclohexene was obtained by the action of methylmagnesium iodide on cyclohexanone followed by dehydration with iodine according to the method described by Haworth (1913). The product obtained (b.p. 110°-112°C) showed all the physical properties as reported in literature (American Petroleum Institute, Infrared spectral catalog spectrograms 897-899 contributed by University of Minnesota, 1949).

1-Methyl-3-cyclohexene

1-Methyl-3-cyclohexene was obtained from dehydration of 1-methyl-1-cyclohexanol with phosphoric acid (Vogel 1938). The physical properties of the product obtained (b.p. 102°-104°C) agreed with all those reported in literature. The IR spectrum was identical with that reported in literature (American Petroleum Institute, Infrared spectral catalog spectrograms, 897-899).

(+) Limonene

Limonene used for the fermentation was either prepared by fractionation of orange oil by a batch stripping distillation process under total reflux with a Towers Fractionation column (the product was redistilled after refluxing over metallic sodium, b.p. 172°-174°C/710 mm, purity 99% by VPC. Alternatively it was obtained from Fluka (VPC purity 99%) and redistilled over metallic sodium.

l-α-Pinene

l-α-Pinene was prepared by refluxing l-β-pinene with gum resin for 24 hr and purified by fractionation of the product under total reflux by a batch stripping distillation, b.p.153°-154°C/711 mm, $\alpha_D = -39.6^\circ$ (VPC showed 99% purity).

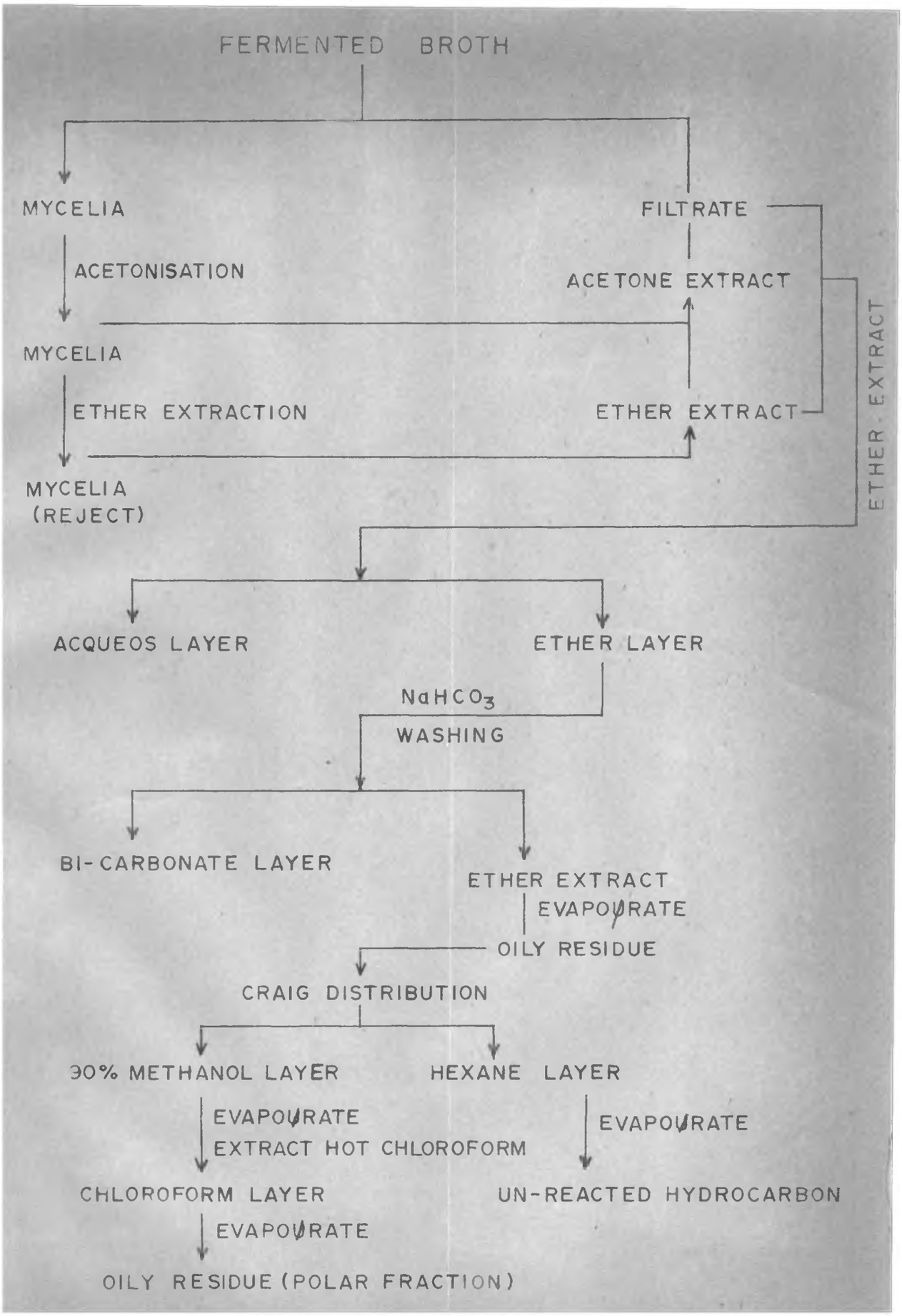
β-Pinene

The sample of β-pinene was prepared from the technical grade material (Fluka, 90% VPC pure) by fractionation through an efficient spinning band column. The fraction boiling between 162°-163° was collected and found to be 99.5% pure by VPC. $\alpha_D = -18.6^\circ$.

(+)Carvone was obtained through the courtesy of Dr S. C. Bhattacharyya, Essential Oils Division of this Laboratory. Samples of (+)cis verbenol, verbenone and (+)trans sobrerol were obtained by fermentation of (+)α-pinene according to Prema & Bhattacharyya (1962a). (+) Perillyl alcohol was supplied by Mr N. R. Ballal of this Laboratory.

Extraction and separation of metabolites:

After the fermentation the fungal mat was filtered and converted with hot acetone and the acetone was decanted after 4-5 min of stirring. The extraction was repeated twice more and the pellets were then extracted with ether (thrice). The filtered broth was extracted three times with 1/3 volume of ether. The acetone and the ether extracts were pooled together.


FERMENTED BROTH
MYCELIA
FILTRATE
ACETONISATION
ACETONE EXTRACT
MYCELIA
ETHER EXTRACTION
ETHER EXTRACT
ETHER EXTRACT
MYCELIA
(REJECT)
ACQUEOS LAYER
ETHER LAYER
NaHCO3
WASHING
BI-CARBONATE LAYER
ETHER EXTRACT
EVAPORATE
OILY RESIDUE
CRAIG DISTRIBUTION
30% METHANOL LAYER
HEXANE LAYER
EVAPORATE
EXTRACT HOT CHLOROFORM
EVAPORATE
CHLOROFORM LAYER
UN-REACTED HYDROCARBON
EVAPORATE
OILY RESIDUE (POLAR FRACTION)

and washed with ½ volume of 5% aqueous sodium bicarbonate in order to remove any of the acidic components as their sodium salts. The bicarbonate layer was then acidified with conc.HCl and the acidic products were recovered by three extractions with equal volumes of ether. The neutral products obtained after evaporation of the neutral ether layer were then subjected to a modified four transfer Craig distribution, between petroleum ether (b.p.40°-60°C) and 90% aqueous methanol using 100 ml of each solvent. All the petroleum ether layers were passed through the four methanolic layers in succession and pooled and evaporated to get the non-polar fraction. The methanol layers were also combined, evaporated, diluted with water and extracted with chloroform to obtain the polar fractions. For the more volatile substrates the evaporation was done through a Vigreaux type fractionating column to minimise losses due to evaporation. The non-polar and polar fractions obtained from the above separation were further purified by chromatography over alumina, fractional distillation and/or preparative thin layer chromatography according to the procedure described for each substrate in the corresponding experimental section. The separation of the components was monitored by TLC and VPC. The hydrocarbon blanks and the controls run without the hydrocarbon substrates were also processed in an identical manner. Here the extracts were examined on TLC to detect autooxidation products and normal fungal metabolites.

Physical methods

The infrared spectra were recorded either in the Perkin-Elmer Model 221 or Infracord Model 137 with sodium chloride optics either as a liquid film or in nujol mull. Ultraviolet absorption spectra were recorded in a Beckman Model DU spectrophotometer in 95% ethanol as solvent unless otherwise mentioned. Optical rotations were determined on a Hilger Standard polarimeter using 1 dm tube. The rotations of the samples were either taken as such or in chloroform or ethanol solutions. The n-m-r spectra were recorded in carbon tetrachloride solution at 60 m.c. with a Varian Associate Model A60 Spectrometer using tetramethylsilane as the internal reference standard.

Chromatographic Methods

Thin layer chromatography was used for detecting the purity of the samples as well as for monitoring the separation of different components. Silicic acid (E.Merck) 200 to 400 mesh and Plaster of Paris (E.Merck) in the proportion of 85% to 15% were used. The spraying reagent was concentrated sulphuric acid or a mixture of nitric acid and sulphuric acid (20:80) in some cases. The sprayed plates were heated in an oven at 110°-160°C for development of spots. Preparative Thin Layer Chromatography was carried out on 25 x 25 cm plates. A mixture of plaster of Paris and silicic acid (34 to 36 gm) was poured as a slurry on to the plates placed horizontally on a square trough and the

plates allowed to set. After drying in air the plates were activated in the oven for 6 to 8 hr at 110°C. The chambers used for the development of the plate were ≠ 30cm x 30cm x 30cm cubic aquarium tanks in which the solvent system (petroleum ether + 10% ethyl acetate) was poured 1 hr prior to the experiment and the chambers allowed to saturate with the vapours of the solvent. The plates were then streaked with the sample under consideration with the help of a fine capillary and placed in the chamber on a glass rack above the solvent layer and allowed to saturate for 1 hr before being dipped in the solvent. The solvent front was allowed to run upto a distance of 20 cm. The plates were dried and once again dipped into the developing solvent. This operation was repeated once again in order to get compact bands. Better resolutions were usually obtained by multiple runs. The plates were now dried and portions of the plate (1.0 cm) on either side were marked out. The middle portions of the plates were covered with rectangular glass plates and the strips on both the sides were sprayed with conc. sulphuric acid and developed by careful heating the sides of the plate over a bunsen burner. The strips showed the development of coloured zones. The corresponding zones were marked out in the undeveloped middle portion with the help of a fine needle, then scraped out and eluted with ether to get the desired component.

Gas chromatography was carried out on a Griffin Tetlock Model G Mark III or a Perkin-Elmer Vapour Aerograph

Analytical Model (A350) using hydrogen as the carrier gas. The columns were of the P type (succinic acid polyester of polyethylene glycol).

Column chromatography

Neutral alumina was obtained from the Fine Chemicals Project of this Laboratory and standardised into Grade I and II by Brockmann's method (1941). The solvent systems used in the elution of column chromatography were pet-ether (40°-60°), pet-ether + 1% ether, pet ether + 2% ether, pet-ether + 5% ether, pet ether + 10% ether, pet ether + 15% ether, pet ether + 20% ether, pet ether + 25% ether, pet ether + 50% ether, 100% ether, ether + 1% methanol, ether + 2% methanol, methanol ether + 5% methanol, 100% methanol.

CHAPTER III

FERMENTATION OF CYCLOHEXENE

CYCLOHEXENE

DISCUSSION

From the observed behaviour of *A. niger* NCIM 612 towards the different types of terpenoid hydrocarbons as discussed in the introductory Chapter (I), it is evident that the hydroxyl group is introduced into the substrate molecules by three different ways:- (a) neutralisation of a carbonium ion, (b) oxygenation in a position allylic to a double bond and (c) oxygenation on a double bond usually with a rearrangement. The data, however, could not be rationalised into a working hypothesis by which it would be possible to predict the course of hydroxylation in a given compound in this fungal system.

In order to study further the rationale and the stereochemical factors involved in the microbial hydroxylation processes, simple alicyclic model compounds were employed as substrates. The present chapter deals with the results obtained with the simplest model compound, cyclohexene (1).

The choice of cyclohexene as a model compound instead of the saturated cyclohexane, was dictated from two major considerations. In the first place it was evident from the experience with different terpenoid substrates that the observed fungal hydroxylations were oriented around a double bond in the molecule (either at the allylic position or at the double bond itself). In the second, any stereoselectivity in the

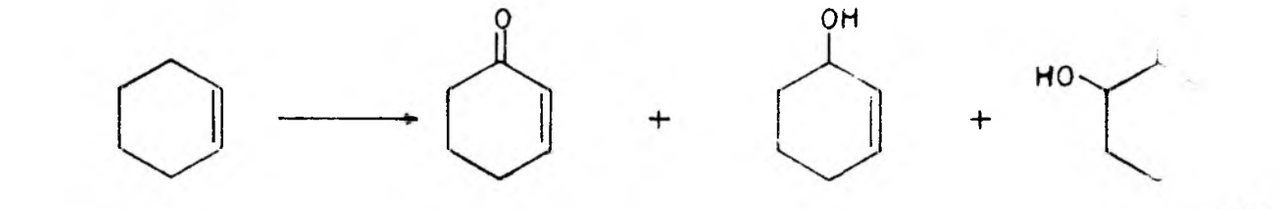
hydroxylation process will be more easily detectable in the cyclohexene nucleus as all the cyclohexenols, unlike cyclohexanol, are capable of existence in optically active forms.

The conditions for the fermentation of cyclohexene (I) were established in preliminary trials. The shake flask methods were found to be unsatisfactory for carrying out the transformation because of the ready volatility of the substrate. The fermentations were finally conducted in closed systems in Chain-type stainless steel fermentors of 6-litre capacity with periodic displacement of the enclosed air. Prior to the addition of cyclohexene, the inoculum, however, was grown for 30 hr on the modified Czapek medium in the form of pellets with full aeration and agitation. It was found that the optimum level of cyclohexene for obtaining the maximum amount of oxygenated products was around 0.6% (Table I) and that with 0.5% substrate levels the optimum time of incubation was 2 hr (Table 2). Beyond this period both the substrate as well as the oxygenated products disappeared from the medium.

Transformation products (Fig.I).

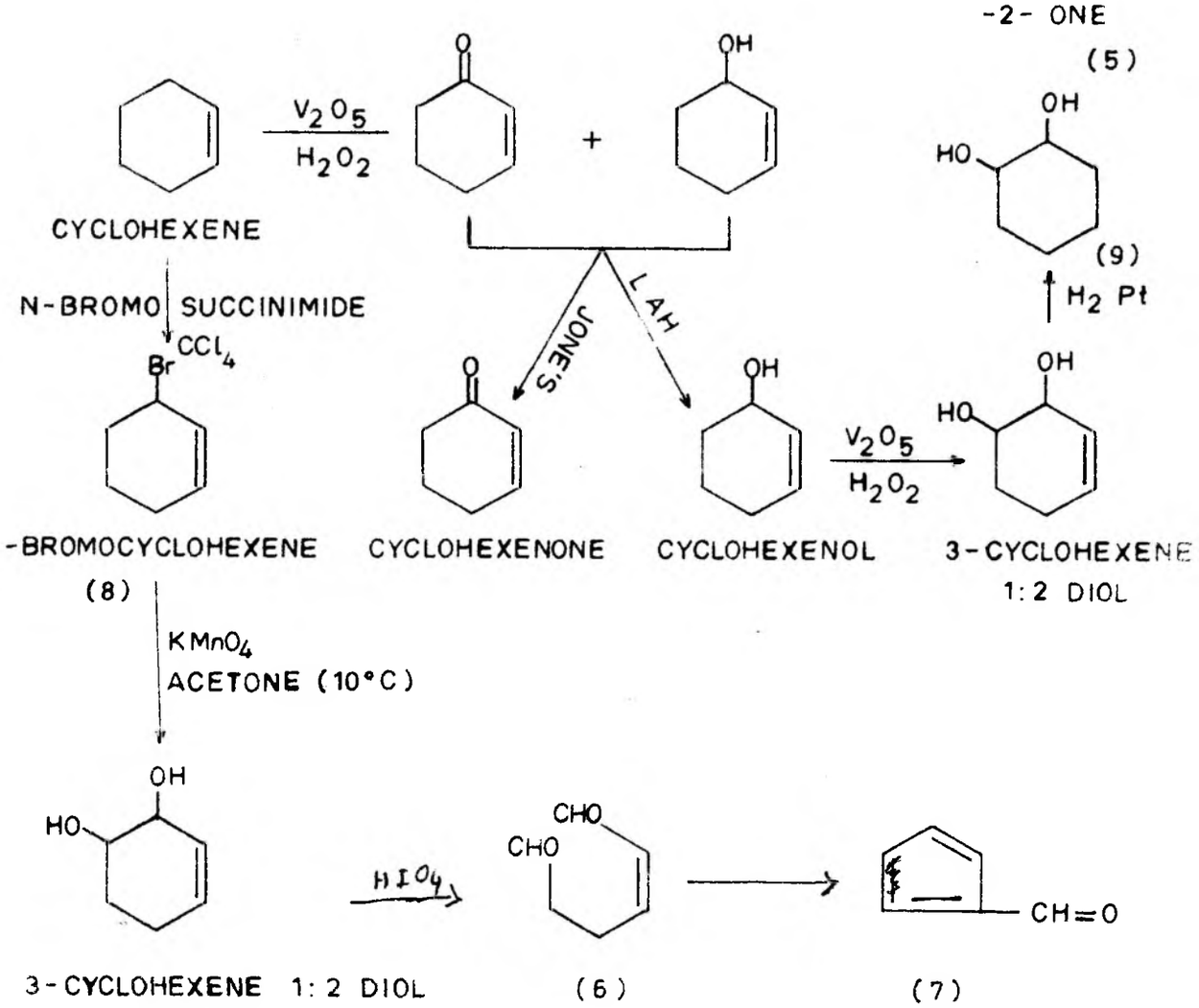
The products after fermentation were extracted in the usual manner (Chapter II) with some additional precautions to minimise the losses due to evaporation and were separated into neutral and acidic fractions. The acidic fraction did not yield any metabolite. The neutral fraction was partitioned in the manner described earlier (Chapter II) into non-polar and

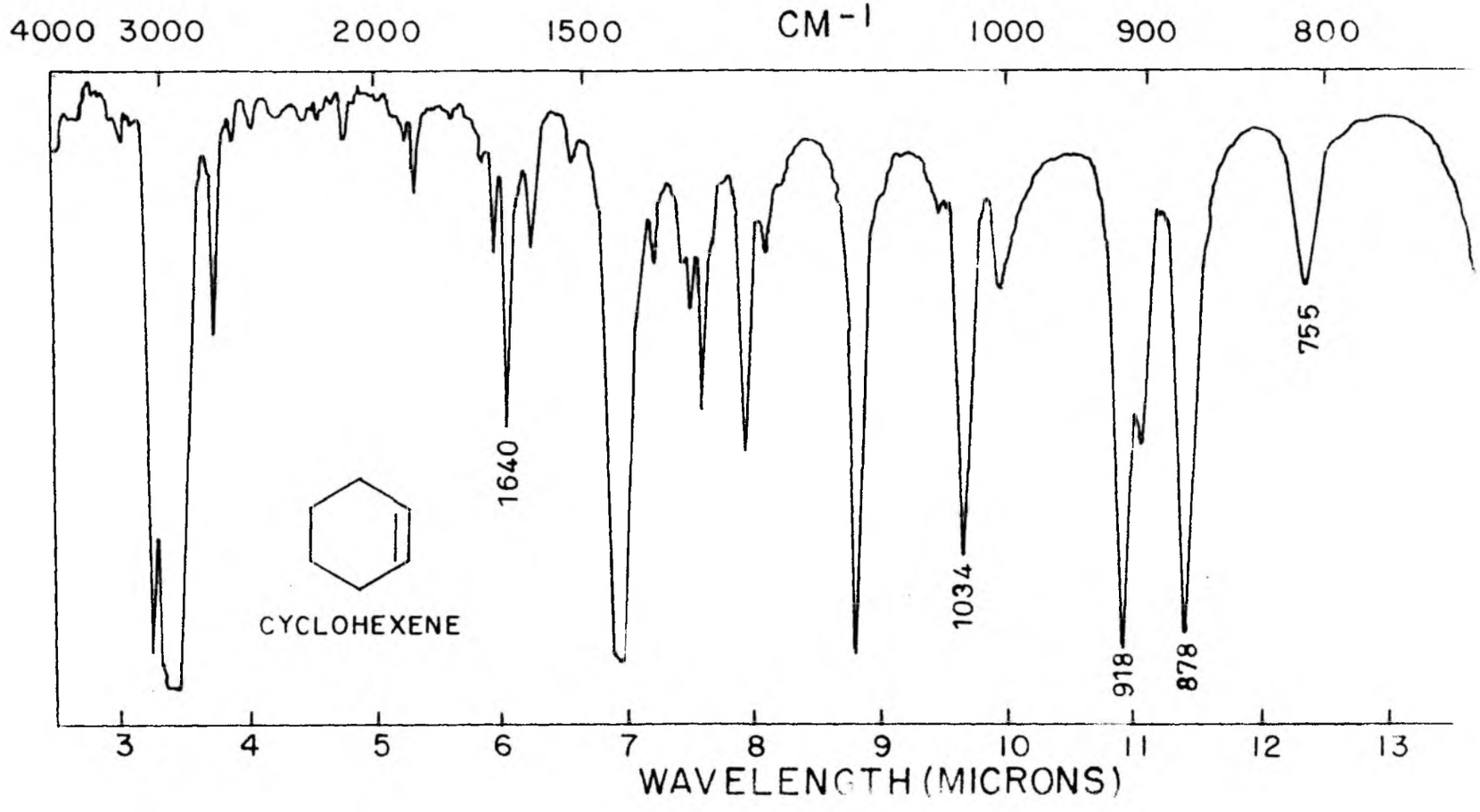
TRANSFO



CHEMICAL SYNTHESIS OF CYCLOHEXENONE , CYCLOHEXENOL

3-CYCLOHEXENE 1:2 DIOL




4000 3000 2000 1500 CM-1 1000 900 800
1640
1034
918
878
755
CYCLOHEXENE
3 4 5 6 7 8 9 10 11 12 13
WAVELENGTH (MICRONS)

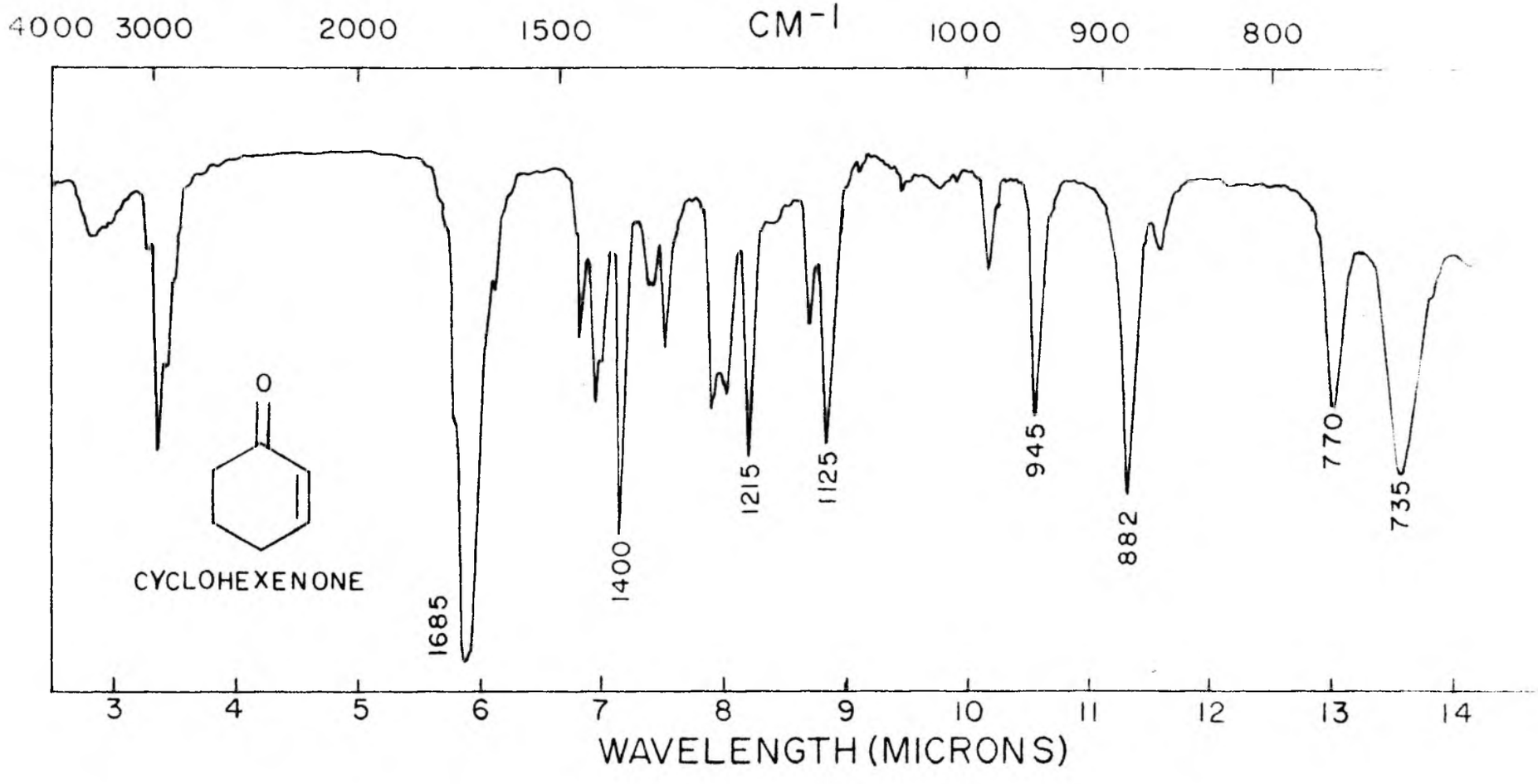
polar sub-fractions by distribution between light petroleum ether and methanol. The non-polar sub-fraction yielded after distillation unreacted cyclohexene and mould lipid. Three main components were detected in the polar sub-fraction by TLC. Although the faster moving component (2) could be separated from the slower moving mixture (3 and 4) by either chromatography on an alumina column or by bisulphite treatment which converted compound 2 to an adduct, the components 3 and 4 could not be resolved by any common physical methods. The separation of all three components was finally achieved by the use of preparative thin layer chromatography.

Ketone (2)

Compound 2 analysed for C_6H_8O and exhibited UV and IR(Fig. IV) absorption bands corresponding to an α,β unsaturated alicyclic ketone (λ max 224.5 nm; ϵ max 10,300; ν max 1685 cm^{-1}). It gave a 2,4-dinitrophenyl hydrazone, m.p. 162°-163°C. From these properties it was possible to characterise compound 2 as 2-cyclohexene-1-one. The mould metabolite (2) and an authentic sample of cyclohexenone had superimposable IR spectra (Fig. IV) and the mixed melting point of the corresponding 2,4-dinitrophenylhydrazones showed no depression.

Alcohol (3)

Compound (3), $C_6H_{10}O$, was a monohydroxylated compound containing a double bond (IR ν max 3330, 1650 cm^{-1}, Fig. V).

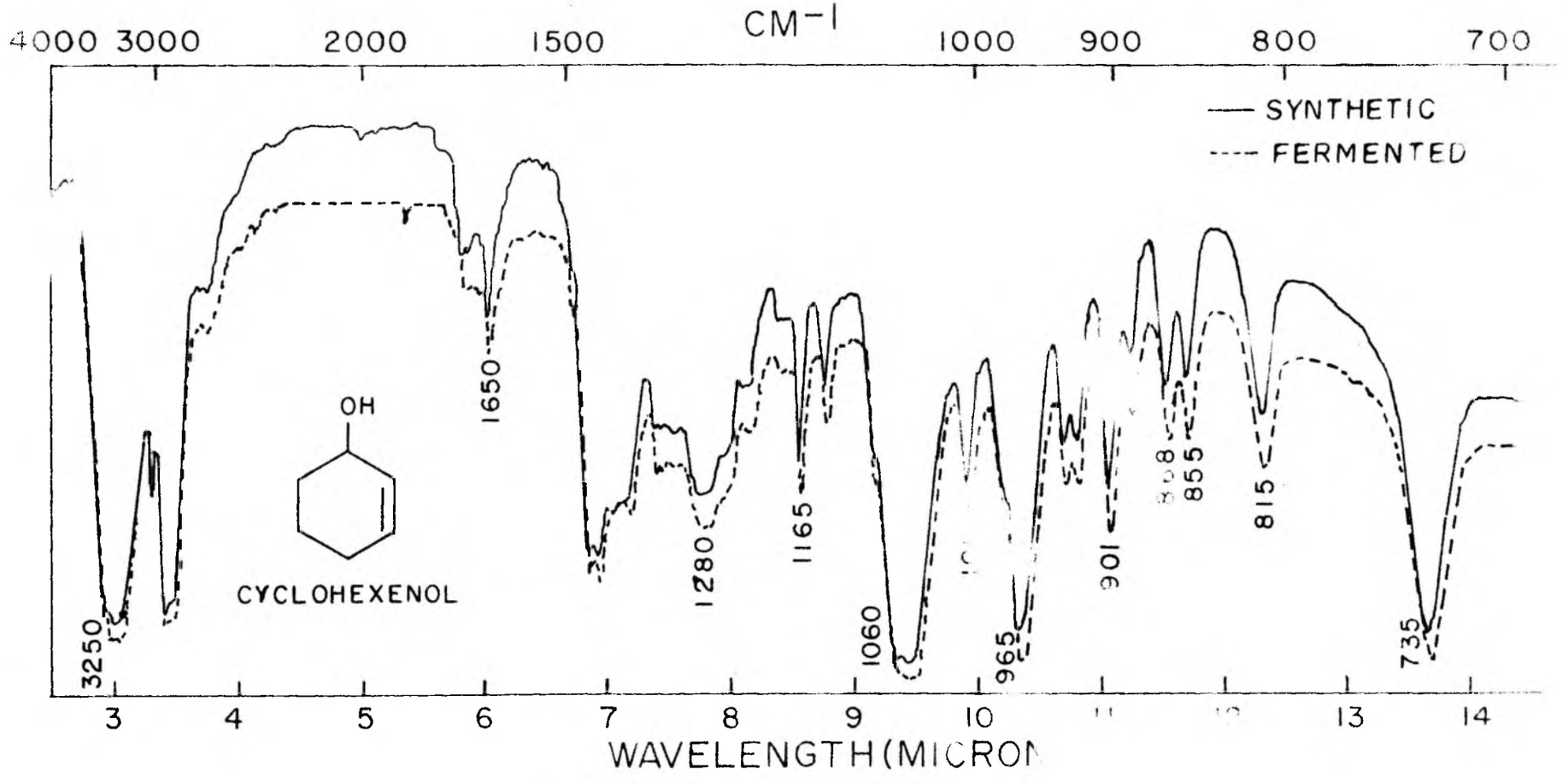

4000 3000
2000
1500
CM-1
1000
900
800
O
CYCLOHEXENONE
1685
1400
1215
1125
945
882
770
735
3
4
5
6
7
8
9
10
11
12
13
14
WAVELENGTH (MICRONS)

The n-m-r spectrum (Fig.VI) indicated the presence of a cisoid disubstituted double bond (a finely-coupled signal of two proton intensity at 4.23 τ), an allylic proton on a carbon carrying the hydroxyl (split signal at 5.9τ), two other allylic protons (broad signal at 8.1τ), four methylenic protons (broad band at 8.35τ) and a hydroxyl proton (a sharp signal at 6.68τwhich disappeared after the addition of D_2O). Compound (3) was optically active $[\alpha]_D$ + 11.7. From the spectral data the alcohol (3) was identified as (+) 2-cyclohexene-1-ol and this identification was confirmed by a spectral comparison with a synthetic sample of dl 2-cyclohexene-1-ol. This compound was easily acetylated to give a monoacetate which analysed for $C_9H_{12}O$. The infrared spectrum showed bands at 1740 and 1245 cm^{-1} (Fig.VII) due to the presence of the ester group. The n-m-r spectrum indicated the presence of the acetoxy methyl (a strong signal at 8.13 τ of three proton intensity) (Fig.VIII), a cisoid disubstituted double bond (finely coupled signal of two proton intensity at 4.35τ) a proton on the carbon carrying the acetylated hydroxyl at 5.95τ. Two more allylic methylene protons appeared as broad band at 8.1 $\tau$and four other methylenic ring protons at 8.35τ.

On manganese dioxide oxidation the alcohol (3) was converted to cyclohexenone (2).

Compound 4

Compound 4 was also optically active, $[\alpha]_D$ = +16.7. It analysed for $C_9H_{10}O_2$ and exhibited a strong hydroxyl absorption


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
SYNTHETIC
FERMENTED
OH
CYCLOHEXENOL
3250
1650
1280
1165
1060
965
901
855
815
735
3 4 5 6 7 8 9 10 13 14
WAVELENGTH (MICRON

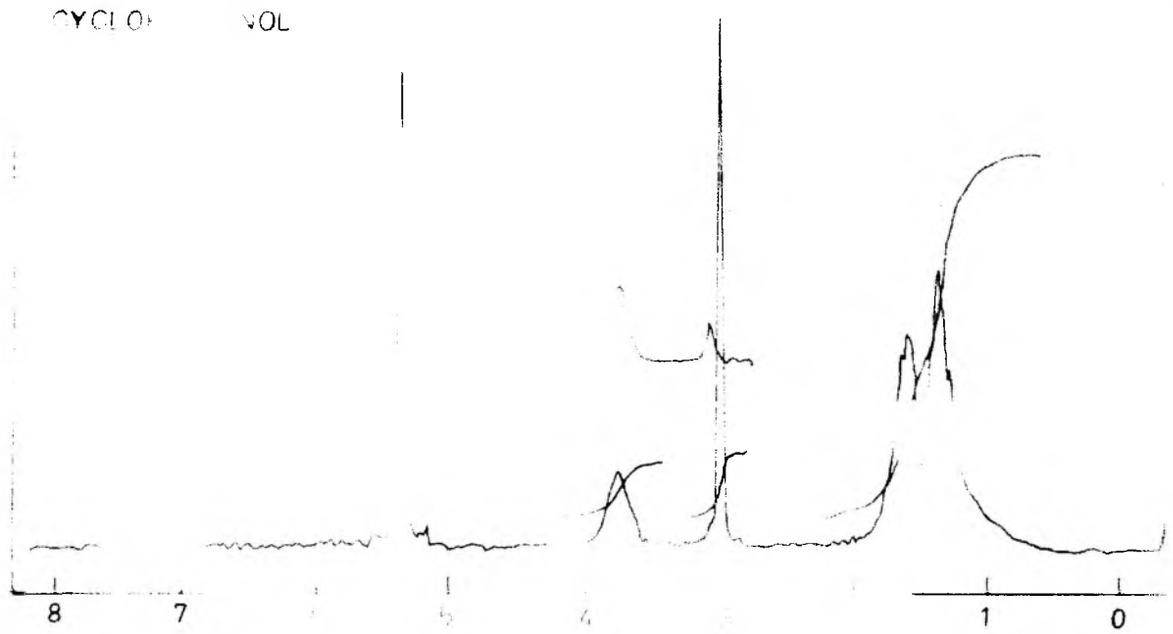


FIG. VI

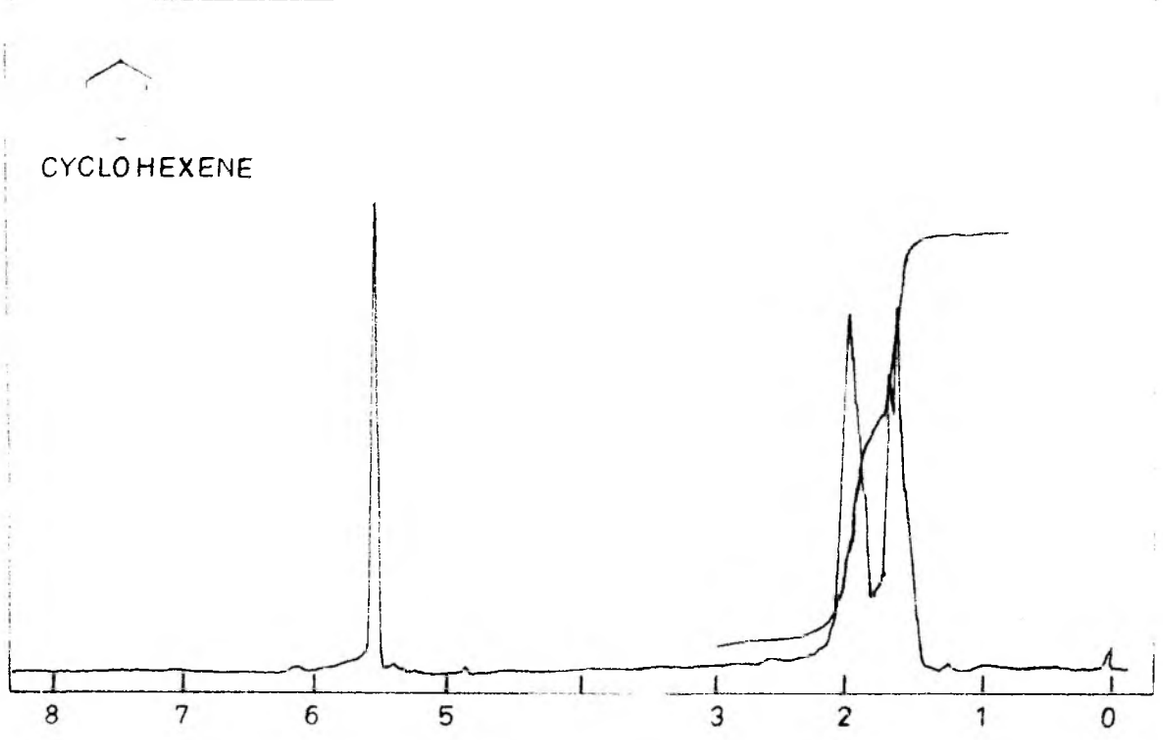


FIG. III.

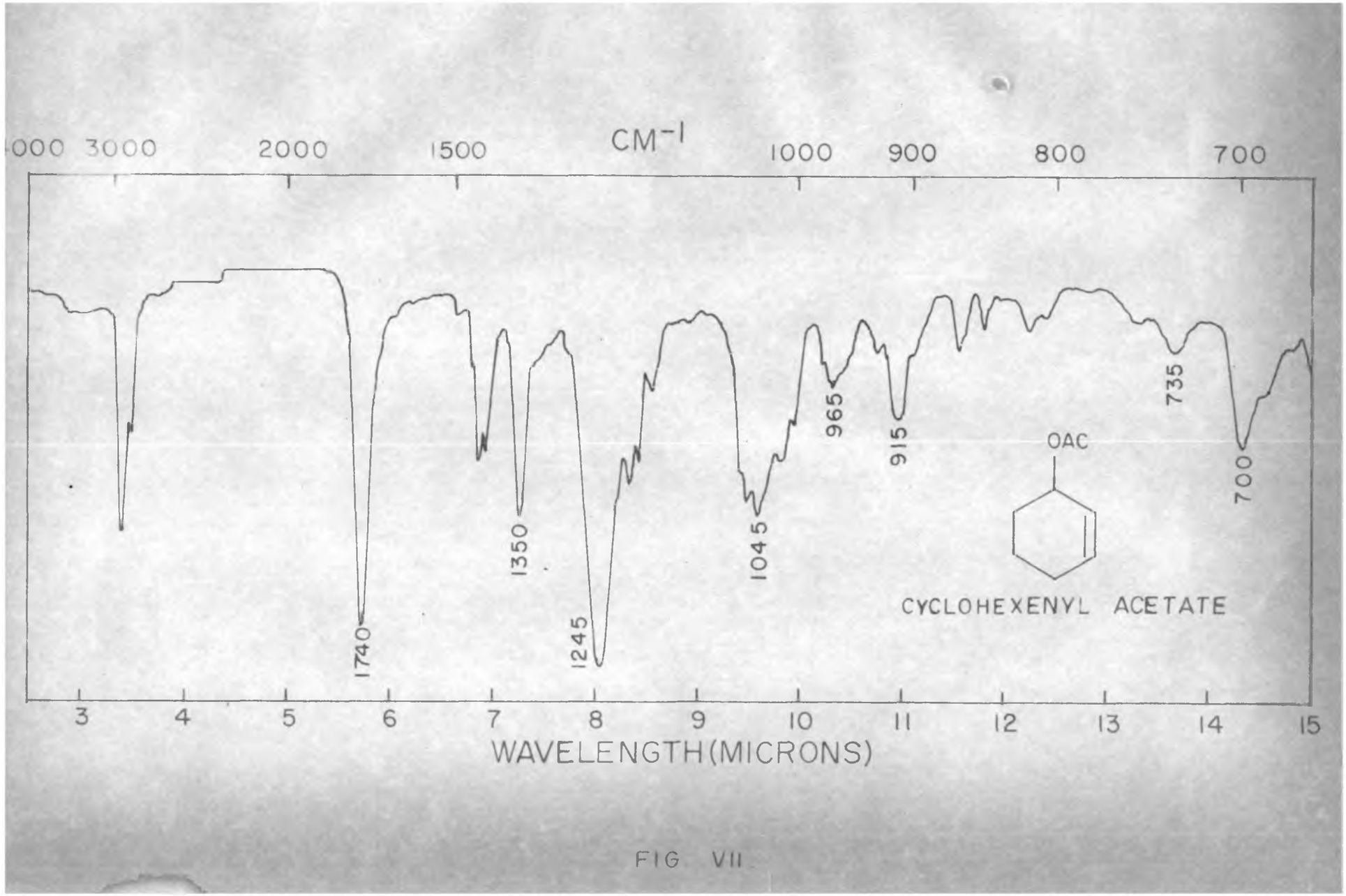
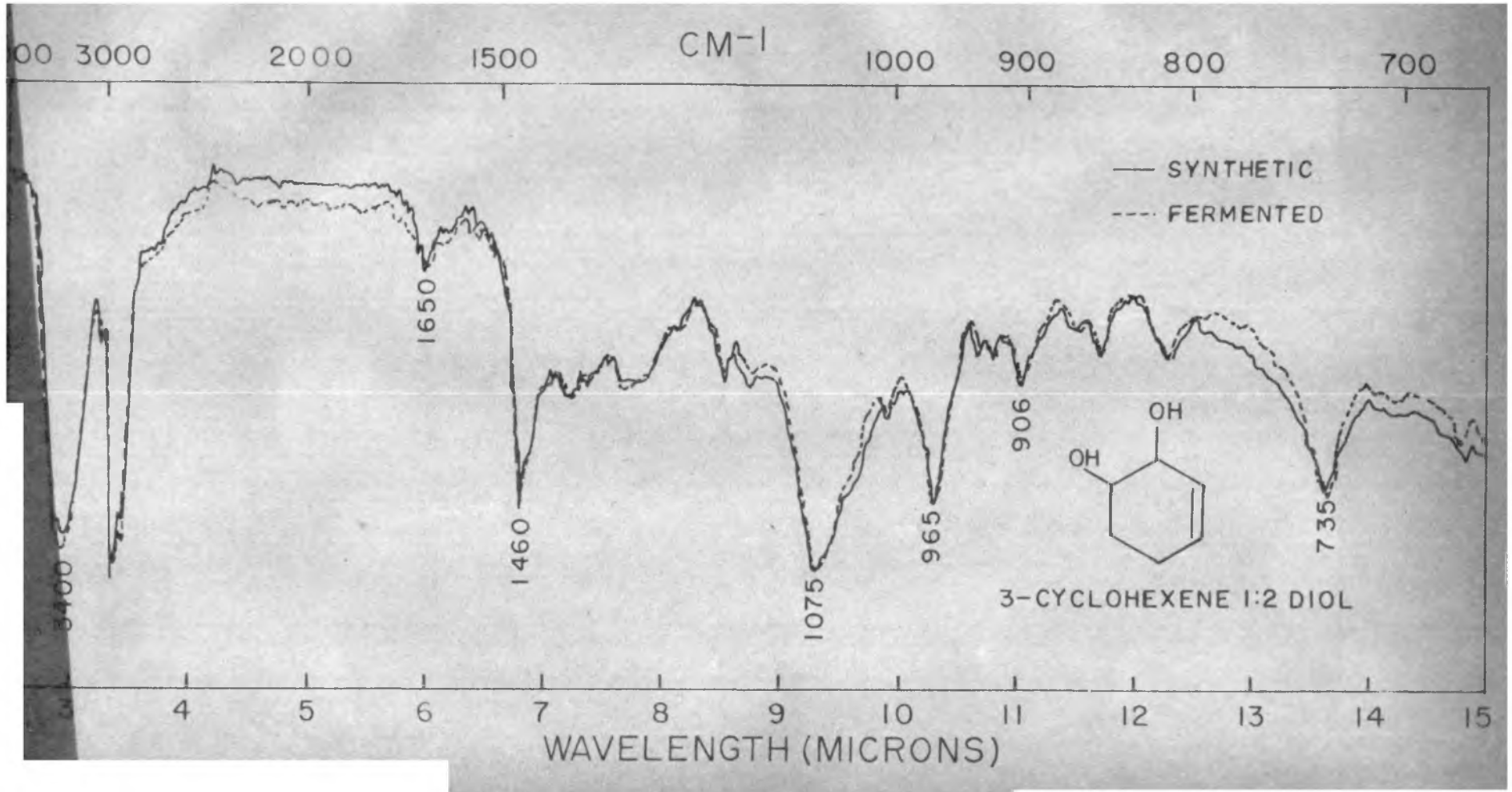

000 3000
2000
1500
CM-1
1000
900
800
700
1740
1350
1245
1045
965
915
735
700
OAC
CYCLOHEXENYL ACETATE
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG VII

in the IR spectrum (Fig.IX, ν_{max} 3390 cm^{-1}) which did not shift on dilution indicating an intramolecular hydrogen bonding. The n-m-r spectrum (Fig.X) indicated a cisoid disubstituted double bond (a finely coupled signal of two-proton intensity at 4.2τ), two cisoid protons on adjacent carbons carrying hydroxyls (a finely-coupled signal of two-proton intensity at 5.9 - 6.3τ), two allylic protons (broad signal at 8.1τ) and two methylenic protons on a ring (broad signal at 8.3τ). Oxidation of compound 4 with manganese dioxide yielded an α-β unsaturated hydroxy ketone (5) (λmax 225 mμ, ϵmax 10,600; IR ν_{max} 3320 cm^{-1} and 1675 cm^{-1}) (Fig.XI) indicating that one of the hydroxyls is allylic to a double bond. Compound 4 consumed one mole of periodate to yield cyclopentadienal (7) identified through its crystalline 2,4-dinitrophenylhydrazone, m.p.141^{0}-142^{0}C (λ max 360mμ ϵ max=24,960), presumably through the unstable dialdehyde intermediate (6). From the physico-chemical data available, compound 4 was characterised as (+) 3-cyclohexene cis-1,2-diol (4). The cis configuration accounts for the physical properties such as high volatility, low polarity (due to hydrogen bonding) and the cisoid coupling at 5.9 - 6.3τ in the n-m-r spectrum (Fig.X). Further, on catalytic reduction of (4) the optically inactive meso-cyclohexane-1,2-diol (9) was obtained, thus providing a chemical proof for the cis-configuration of the adjacent hydroxyl groups in compound 4. The only literature report on the synthesis of the diol (4) is by Bodes and Huyer (1936) who obtained both the cis as well as the trans cyclohexene diols by bromination of 1:3 cyclohexadiene followed by solvolysis. This method did not work satisfactorily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2000
1500
CM-1
1000
900
800
700
SYNTHETIC
FERMENTED
3400
1650
1460
1075
965
906
735
OH
OH
3-CYCLOHEXENE 1:2 DIOL
3
4
5
6
7
8
9
10
11
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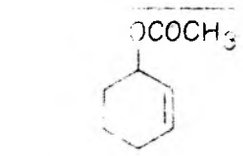
13
14
15
WAVELENGTH (MICRONS)

FIG. IX



CYCLOHEXENYL ACETATE

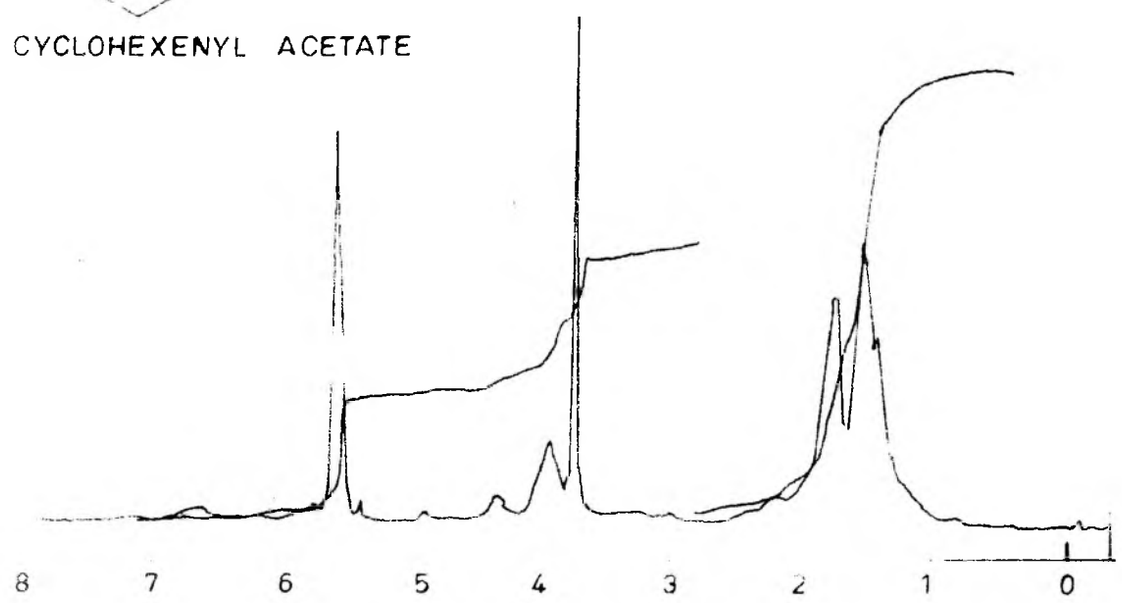
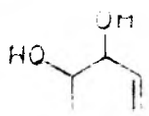
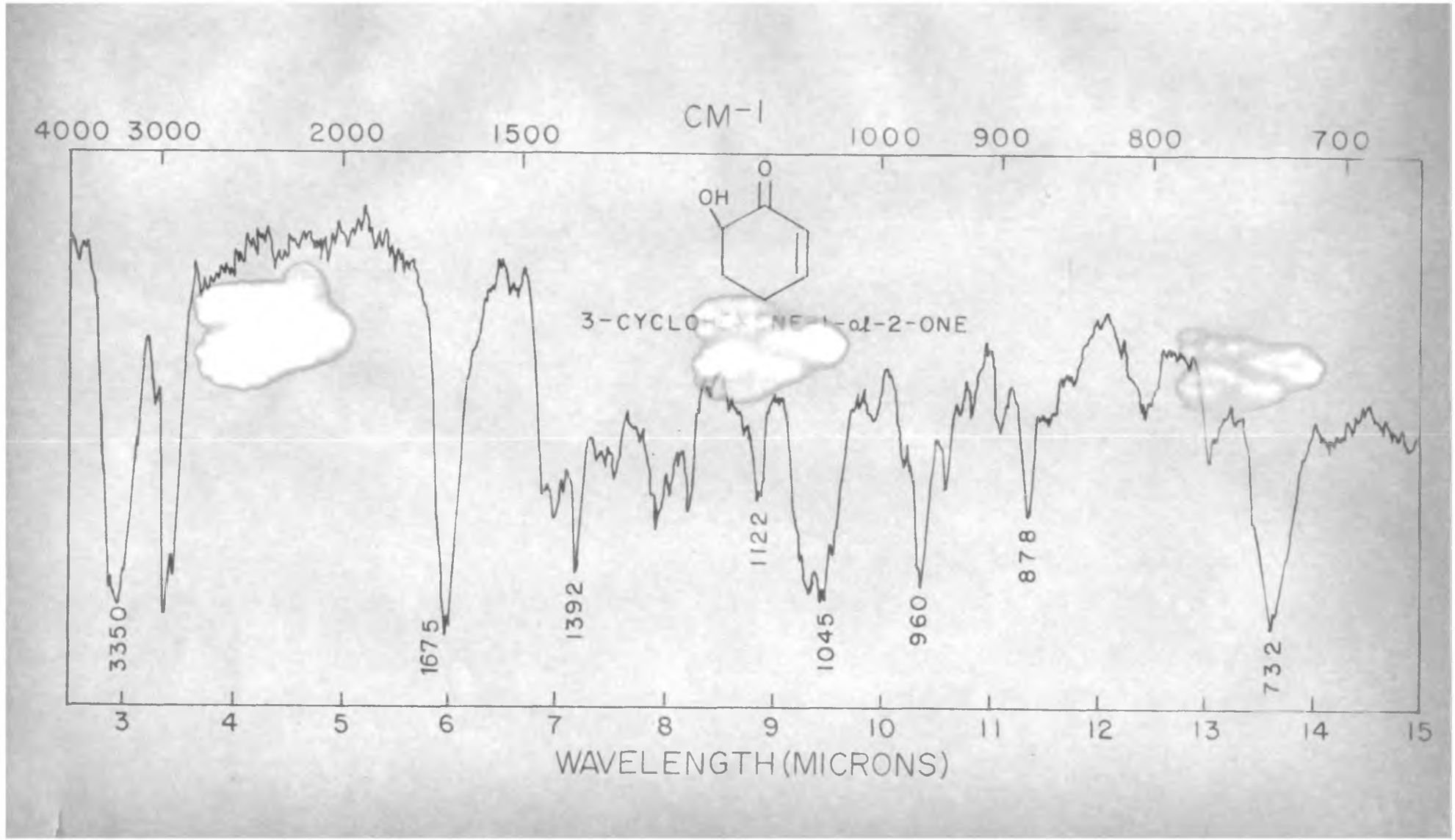


FIG. VIII



3 CYCLOHEXENE 1:2 DIOL

FIG. X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2000
1500
1000
900
800
700
O
OH
3-CYCLO
NE-1-ol-2-ONE
3350
1675
1392
1122
1045
960
878
732
3
4
5
6
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9
10
11
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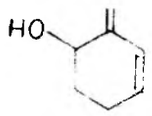
14
15
WAVELENGTH (MICRONS)



3: CYCLOHEXENE
-2- ONE --1- ol

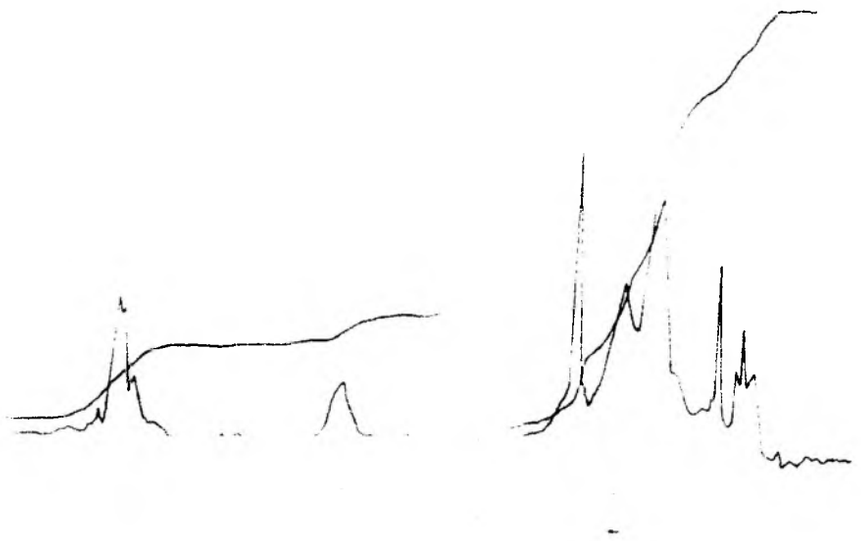


FIG. XII

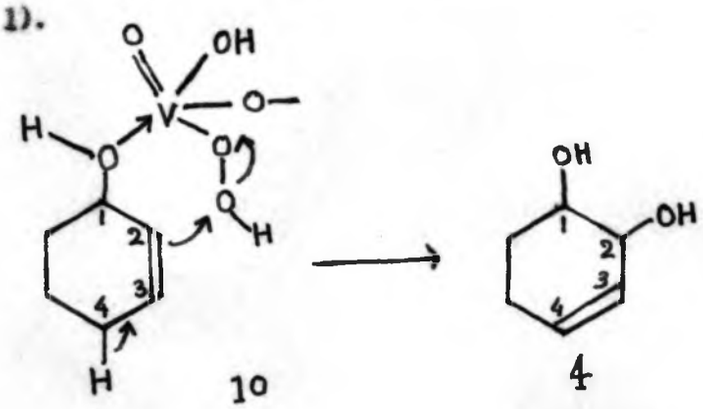
in the investigations undertaken. A method was finally worked out for its synthesis involving a controlled potassium permanganate oxidation of 1-bromo-3-cyclohexene (3) with a simultaneous dehydrobromination to yield the racemic form of the diol (4) in a single step. In spite of strict adherence to the reaction conditions, the yields, however, were extremely variable, ranging from 15 to 45 per cent.

A second method of synthesis of the diol (4) was developed as a result of a chance discovery. While preparing 2-cyclohexene-1-one by the method of Eisenbraun (1963) by vanadium pentoxide and hydrogen peroxide, an examination of the reaction products by thin layer chromatography indicated that along with the ketone (2), appreciable amounts of the mono-hydroxy compound (3), as well as the diol (4) were produced. Following this lead it was possible to synthesise the diol (4) by the oxidation of cyclohexenol (3) by the same reagents. This synthesis, besides providing a convenient method for obtaining the diol (4), provides a clue to the probable mechanism involved in the formation of this compound in the biological system.

An examination of the fermentation products by thin layer chromatography at intervals of 30 min upto a period of 2 hr revealed that the monohydroxy compound (3) is produced at the beginning. Later compounds (2) and (4) accumulate at the expense of compound (2) (Fig. I). Therefore, the oxidation of cyclohexene in the V^{+5} and hydrogen peroxide system follows a

parallel pathway to that in the biological system.

In the compound (3) the alcoholic oxygen may provide one of the ligands which coordinate with the pentavalent vanadium. Oxidation with hydrogen peroxide probably converts this complex to the highly idealised peroxidic intermediate (10). In fact, the vanadium pentoxide-hydrogen peroxide system may be a complex one, involving the polymeric hexavanadate ($H_6V_{10}O_{28}$) (Martinez & Trujillo, 1951). The justification of the peroxide structure shown in the simplified and idealised expression, 10, may be provided from the polarographic behaviour of V^{+5} - hydrogen peroxide system (Kohltoff & Parry, 1951). In the expression, 10, the electrophilic peroxide hydroxyl withdraws electrons from the double bond. The resulting electron deficiency at position 3 may be compensated by the withdrawl of electrons from the C-H bond at position 4. The net outcome of these probable events is the introduction of the second hydroxyl at position 2 along with the migration of the double bond from position 2,3 to position 3,4. The six-membered transition state, 10, is consistent with the observed *cis* disposition of the second hydroxyl group with respect to that at position 1. It also provides a reasonable explanation for the appearance of a hydroxyl function in a seemingly inert homoallylic position in compound *IV* (i.e., position 1).



10 4

It is unlikely, however, that vanadium or free hydrogen peroxide may be involved in the biological system under study, since no detectable amount of vanadium could be demonstrated in this system (J. R. Vakil & P. K. Bhattacharyya - unpublished data). Experience of other workers in analogous hydroxylation reaction with steroids would indicate that free hydrogen peroxide may not be involved (Bergstrom, 1953). It is quite probable, however, that some other metal such as iron, may be implicated in the fungal hydroxylation and oxygen from the atmosphere is the source of the hydroxyl group. From the model reaction the only inference that can be extended to the A. niger system is that it is very likely that in the second hydroxylation with the mould some cyclic five- or six- membered transition states are also involved.

A more detailed discussion on these points will be presented in the concluding chapter of this thesis.

The extracts from blank experiments run with cyclohexene as a substrate but without the mould, exhibited the formation of small amount of cyclohexenone (2) and traces of cyclohexenol (3) on TLC analysis, the former predominating. The formation of the diol (4) was not observed.

The data, however, do not differentiate between two probable modes of formation of the monohydroxylated product (3): (a) by a true allylic oxygenation and (b) oxygenation on a double bond followed by an allylic shift.

CYCLOHEXENE

EXPERIMENTAL

Cyclohexene was prepared by dehydration of cyclohexanol. The physical properties were identical with those reported in literature, b.p.80°-82°C/715 mm. density 0.8102, n^{25} = 1.4435. IR) max 2960, 1640, 1433, 1136, 1034, 918, 878, 718 cm^{-1} (Fig.II) NMR (Fig.III).

Fermentation:

Determination of optimum concentration of cyclohexene in the medium for obtaining maximum transformation products:

Different sets of experiments were carried out in six litre stainless steel fermentors (with varying concentration of cyclohexene in the medium at 28°±1°C (both temp.) with graded levels of cyclohexene 0.2%, 0.4%, 0.6%, 0.8%, 1.0% (v/v). The time allowed for incubation was maintained constant at 2 hr after which the fermentations were stopped and the culture extracted (Chapter II). The extracts were separated into unreacted cyclohexene and the oxygenated derivatives by partitioning between pet.ether and 90% methanol as described earlier. The methanol layer was evaporated to small volume and extracted with chloroform (50 ml) thrice. The chloroform extracts were pooled, and dried (sodium sulphate), and evaporated through a fractionating column and the residue weighed as oxygenated product.

TABLE 1

Effect of concentration of cyclohexane on the formation of oxygenated products

Capacity of fermentor (6 l)	Concn. of cyclohexane vol./vol.	Oxygenated products
4 lit	0.2%	0.200 gm
4 lit	0.4%	0.950 gm
4 lit	0.6%	1.478 gm
4 lit	0.8%	0.700 gm
4 lit	1.0%	0.500 gm

The results (Table 1) indicate that the amount of oxygenated product showed an increase upto an initial concentration of cyclohexane in the medium upto 0.6%. Higher concentrations of cyclohexane seemed to retard the formation of oxygenated products, and at a very high concentration very little conversion was observed.

The formation of oxygenated products as a function of time:

Five sets of experiments were carried out in the fermentors with 0.5% levels of cyclohexane using different periods of incubation. The results are tabulated in Table 2.

TABLE 2

Formation of oxygenated products in the fermentation of cyclohexane with time

Capacity of fermentor	Time (hr)	Concentration (%)	Oxygenated products
4 lit	½	0.5	0.300 gm
4 lit	1	0.5	0.500 gm
4 lit	2	0.5	1.378 gm
4 lit	4	0.5	0.590 gm
4 lit	8	0.5	0.400 gm

The results (Table 2) indicate that with concentrations of 0.5% the maximum conversion to oxygenated products was obtained in 2 hr. After 8 hr there was very little of oxygenated product left in the medium. Control runs without the mould showed that the loss due to evaporation was of the order of 50% during the 8 hr period.

Optimum transformation temperature

Two sets of experiments were carried out at different temperatures 28° and 31°C. The formation of the oxygenated products are tabulated in Table 3.

TABLE 3

Fermentation of cyclohexene at different temperatures

Capacity fermentor	Time (hr)	Conc. %	Temperature	Oxygenated product
4 lit	2	0.5	28°C	1.370 gm
4 lit	2	0.5	31°C	0.300 gm

The results (Table 3) indicate that the amount of oxygenated product showed a sharp decline with increase in temperature from 28° to 31°C. At lower temperature the growth of the mould was slow. The subsequent experiments were run at 27°-28°C.

Isolation of transformation products:

The fermentation was carried out in stainless steel fermentors as described earlier. Cyclohexene (0.5%) was added to 36 hr growth of the fungus (A. niger, NCIM 612) and the fermentation continued for 2 hr with intermittent aeration at ½ vol/vol/min

for 5 min after every 30 min. The contents of the fermentors were filtered through a cheese cloth. The mycelium and the filtrate were processed separately as described earlier.

A preliminary TLC examination of the ether extract revealed the existence of at least three metabolic products. The dried ether extract was evaporated to yield (11.0 gm) of total extract which was separated into the non-polar and polar neutral fractions by partitioning between pet.ether (40^{0}-60^{0}) and 90% aqueous methanol (as described earlier - Chapter II). The pet.ether layers were pooled together and distilled through a Vigreaux fractionating column in order to prevent volatility losses as far as possible. The pet.ether extract yielded 8.0 gm of the non-polar fraction. On fractional distillation this portion yielded a low boiling hydrocarbon b.p.30^{0}-31^{0}C. The volatile fraction was identified as cyclohexene by comparative infrared spectroscopy. The residue after distillation (0.7 gm) was passed through grade I alumina (70 gm) and eluted with the usual solvent mixture. Petroleum ether eluted mostly mould fat. A crystalline alcohol m.p.134^{0}C was eluted in the pet.ether-5% ether fraction and characterised as ergosterol by a comparison of its infrared spectrum.

The 90% methanol layer containing the more polar transformation products was also carefully evaporated through a fractionating column. The turbid aqueous residue was repeatedly extracted (thrice) with 50 ml of chloroform. The chloroform extracts were combined, dried (sodium sulphate) and

again carefully concentrated to a small volume. A TLC examination of this residue revealed the existence of three main spots which were shown to be present in the earlier ether extract.

Separation of the metabolites

A column chromatography of the residue (1.2 gm) over alumina (grade II, 120 gm) was attempted in order to resolve the mixture. Petroleum ether and 1% ether eluted the fastest moving TLC component (2) which was recovered after evaporation of the solvent through fractionating column and purified by distillation yielded (0.1 gm) b.p. 61°-63°C/14 mm. Found C 74.4%, H 8.72%. Calc. for C_6H_8O, C 74.97%, H 8.39%, UV λ max 224.5mμ $\epsilon_{m.}$=10,300, IR ν max 1685, 1400, 1214, 1125, 945, 832, 770, 735 cm^{-1} (Fig.4). The compound gave an orange-red crystalline 2:4 dinitrophenylhydrazone, m.p. 161°-163°C (reported for 2-cyclohexene-1-one 2:4 dinitrophenylhydrazone m.p. 161°-163°C) 2-cyclohexene-1-one (2) was prepared according to the method of (Eisenbraun, 1963) by the action of vanadium pentoxide and hydrogen peroxide on cyclohexene (1). The IR spectra of the mould metabolite and synthetic cyclohexenone were identical. The mixed m.p. of the 2:4 dinitrophenylhydrazones (m.p. 161°-163°C).

Further fractions from the alumina column appeared to be mixtures of the slower moving TLC components and could not be resolved by fractional distillation.

Preparative thin layer chromatography:

The use of thin layer chromatography on a preparative scale helped in the separation of all the compounds in pure form.

Glass plates (25 cm x 25 cm) impregnated with a mixture of silicic acid (E.Merck) 85% and plaster of Paris (15%) were used (34.0 gm). The method of preparation and development of the plates has been described (Chapter II). The amount of sample used for spotting was not more than 0.1 to 0.15 gm per plate. Seven plates were used in each run. The zones obtained after the development of plates were then marked out and the desired areas were scraped off and eluted with ether. The ether eluates were evaporated through a Vigreaux column in each case.

Ketone (2):

The fastest moving component (Zone I) was identified as cyclohexenone (2) (as described earlier).

Alcohol (3):

Zone II: The ether eluates gave an oily residue (0.1 gm) which distilled b.p. 63°-65°C/14 mm $[\alpha]_D$ = +11.65° (conc. 2%). Found C 72.9%, H 10.8%. Calc. for $C_9H_{16}O$, C 73.4%, H 10.2%. IR νmax 3250, 1650, 1290, 1165, 1060, 1010, 965, 901, 860, 855, 815, 735 cm^{-1} (Fig.V) and NMR (Fig.VI). Acetylation with pyridine and acetic anhydride gave a monoacetate indicating that the hydroxyl group was primary or secondary in nature. b.p. 60°-61°/15 mm. Found C 68.67%, H 8.7%. Calc. for $C_{11}H_{18}O_2$, C 68.54%, H 8.63%. IR νmax at 1740, 1350, 1245, 1045, 965, 915, 735, 700 cm^{-1} (Fig.VII). The n-m-r (Fig.VIII).

Manganese dioxide oxidation of the alcohol:

The alcohol (2) (20.0 mg) was taken in chloroform (50 ml) and shaken with active manganese dioxide (500 mg) prepared by the

method described by Turner (1954). The reaction was allowed to proceed for 24 hr at room temperature with continuous shaking on a microid shaker after which the mixture was filtered and the residue washed with hot chloroform (10 ml portions) thrice. The combined filtrates were concentrated to yield (15 mg) of the reaction produc UV spectrum showed λmax 224.5, ϵ_{max}=10,300 indicating the presence of an α,β unsaturated carbonyl function thereby showing that the hydroxyl group is allylic to the double bond.

The compound was identified as cyclohexenol by comparative IR spectrum with an authentic sample of cyclohexenol prepared by the method described below.

Preparation of cyclohexenol (3):

Cyclohexenone (2) (0.5 g) was reduced by stirring with lithium aluminium hydride (2.0 gm) in dry ether (25.0 ml). The reaction mixture was refluxed for 3 hr. Excess lithium aluminium hydride was decomposed by the addition of 2.0 ml ethyl acetate. After addition of 1.7 ml of 10% NaOH the mixture was extracted with petroleum ether (40^0-60^0). The pet.ether layer was dried (sodium sulphate), evaporated through a fractionating column to yield (0.300 gm) of the alcohol. The IR spectrum of the alcohol (cyclohexenol) (3) was superimposable with that of the alcohol obtained from the fermentation.

Diol (4):

Zone III. The ether eluate was carefully fractionated to yield a viscous residue (0.15 gm) purified by distillation b.p.63^0-70^0/14 mm, $[\alpha]_D$ = +16.5° .(conc.2%). Found C 63.6%, H 8.93%.

Calcd. for $C_{15}H_{10}O_2$ C 83.33, H 9.25%. IR ν max 3400, 1650, 1460, 1075, 965, 906, 735 cm^{-1} (Fig.IX) and NMR (Fig.X).

Manganese dioxide oxidation of the diol (4):

The diol (4) (25 mg) was taken in chloroform (50 ml) and shaken with active manganese dioxide (600 mg) (Turner, 1954) for 24 hr at room temperature with continuous shaking on a microid shaker after which the mixture was filtered and the residue washed with portions of hot chloroform (10 ml) three times. The combined chloroform extracts were pooled together and concentrated (fractionating column) to yield (20 mg) of the reaction product. The product obtained showed the presence of an α,β unsaturated hydroxy ketone (5) in its UV and IR spectrum, λ max 225, ϵ =10,600. IR ν max 3350, 1675, 1392, 1122, 1045, 960, 878, 732 cm^{-1} (Fig.XI). NMR gave signals at 5.7τ, 4.05τ, 2.3τ, 1.9τ and 1.68τ (Fig.XII).

Periodate oxidation of diol (4):

The diol (5.0 mg) in a capillary was dropped inside a flask containing 2.0 ml of normal sulphuric acid and 1.0 ml of 0.1 molar periodic acid. A blank experiment without the alcohol was carried out simultaneously under the same conditions. The sample was allowed to stand for 1 hr after which 5 ml of 6.8% Na_2CO_3 solution was added with stirring. To the flask was now added 2.0 ml of 0.11 N sodium arsenite solution and two drops of 50% potassium iodide solution. The reaction mixture was allowed to stand at room temperature for 10 min after which few drops of starch solution was added and the solution titrated against 0.1N iodine solution to a distinct blue colour. A blank experiment in which no diol was added was also run

side by side. The sample took up (0.9 ml of .1N iodine) (titre of the sample - titre of the blank) which corresponded to 1 mol/molecule of (calculated 4.05 mg, sample taken 5.0 mg) alcohol. The reaction mixture was dropped into a warm saturated solution of 2:4 dinitrophenylhydrazine in alcohol (20% ethanolic sulphuric acid, 5.0 ml). The precipitate of the 2:4 dinitrophenylhydrazone was filtered and recrystallised from aqueous ethanol to give m.p. 141°-142°C. Found N 20.8%. calc. for $C_{12}H_{10}N_4O_4$. N 20.43%. λ max 366 mμ, ϵ max 24,900.

Synthesis of the diol (4):

The diol (4) was synthesised by the action of neutral $KMnO_4$ in 2-bromo-1-cyclohexene (prepared by action of N-bromo succinimide in cyclohexene in CCl_4 solution) (Vogel, Pract. Org. Chem. page 926). A dilute solution of 2-bromo-1-cyclohexene (1% solution) 10.0 gm in one litre of acetone was chilled in an ice salt bath to -10°C. A 1% solution of potassium permanganate (1000 ml) was added to the reaction mixture maintaining vigorous stirring. During the addition care was taken so that the temperature did not rise above 5°C. The whole addition was done in exactly fifteen minutes, after which the reaction was stopped and excess of potassium permanganate decomposed immediately by sulphur dioxide. The precipitated manganese dioxide was filtered off and the clear filtrate was concentrated to a small volume through a Vigreaux column. The residue was extracted thrice with 20 ml portions of chloroform. The chloroform layers were pooled, washed with water and dried (sodium sulphate) and evaporated through a fractionating column to give an oily residue (1.5 gm); purified by distillation b.p. 63°-69°C/14 mm. It showed a single

spot in the TLC with identical mobility with the diol obtained from the fermentation of cyclohexene. The diol obtained by synthesis also took up 1 mole of periodate and the 2:4 dinitrophenylhydrazone of the periodate cleavage product did not show any depression in m.p.141°-142° with the corresponding product from the diol obtained by fermentation. IR spectra of the diols were superimposable.

<u>An alternative method for the synthesis of the diol (4) from cyclohexenol (3):</u>

Cyclohexenol (3) (5.0 gm) in 50 ml acetone was cooled with ice salt mixture to -10°C. To the well stirred solution was added the catalyst, prepared by mixing 2 ml of 30% hydrogen peroxide (100 vol), vanadium pentoxide (50 mg) at 5 to 10°C and diluted to 10 ml with precooled (-10°C) acetone. The mixture was filtered as rapidly as possible so that the temperature did not rise above -2°C. The flask was now kept on a water bath and 5 ml of 30% hydrogen peroxide was added dropwise to the stirred solution. Temperature was maintained at 30°C. About 3 ml of hydrogen peroxide was added and the colour changed from orange to green. The mixture was left overnight. The reaction mixture was then refluxed on water bath for 1 hr and excess of acetone distilled off through a fractionating column. The residue (3.0 gm) was dried over anhydrous sodium sulphate, filtered and distilled (b.p.60°-69°C/14 mm) to give the desired compound which on TLC showed identical mobility with that of the fermented sample of the diol obtained from the fermentation and had identical IR and NMR spectra. In some batches contamination

with cyclohexenone was observed. In such cases a chromatography over alumina was resorted to.

Hydrogenation of the diol (4):

The diol (50 mg) in alcohol was hydrogenated over platinum oxide (10 mg) catalyst. It was found that it took up 1 mole of hydrogen indicating presence of one double bond. The catalyst was filtered off and the alcohol on evaporation gave meso cyclohexane 1:2 diol m.p. 96-98°c ($[\alpha]_D = 0$. (Dictionary of organic compounds Ed. Heilbron + Bunbury, Eyre + Spottiswoods. London 1946 Vol. 2 p. 168.)

CHAPTER IVa

DEHYDROGENATION OF 1-METHYL-1-CYCLOHEXENE

CHAPTER IVa

1-METHYL-1-CYCLOHEXENE

DISCUSSION

From the preceding chapter three types of chemical activity of the mould on cyclohexene become evident: (a) allylic hydroxylation, (b) oxygenation on a double bond and (c) the probable oxidation of an allylic hydroxyl to a carbonyl group. It was considered necessary to study the effect of a methyl substituent on the cyclohexene ring particularly on the double bond, since all the observed chemical activity of the mould appeared to be centred around the double bond. A second reason influencing the choice of 1-methyl-1-cyclohexene (11) as a model substrate was that this compound can be regarded as a simple analogue of some of the monoterpenes, such as α-pinene and limonene without the three carbon side chain in the p-position

Thus, as in the case of cyclohexene (1) the conditions for the fermentation of 1-methyl-1-cyclohexene (11) were established by preliminary trials. It was found that the optimum level of methyl-cyclohexene for obtaining the maximum amount of oxygenated products was around 0.5% but at this level of the substrate the incubation period had to be increased to 4 hr at 28°.

Transformation products (Fig.XIII):

The products after fermentation were extracted in the usual manner and separated into acidic and neutral fractions (as described

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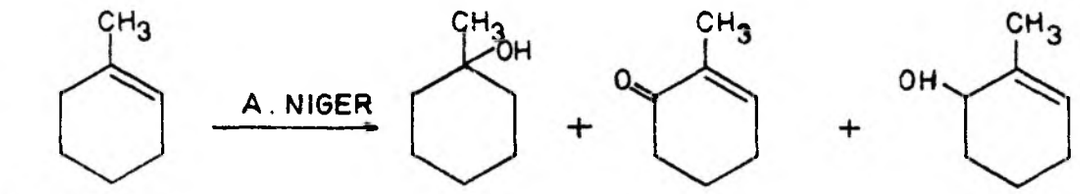


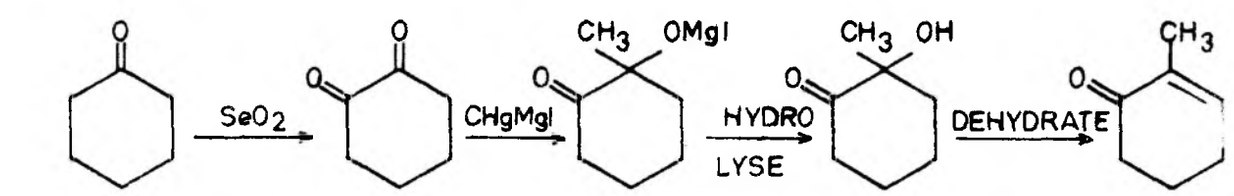
1-METHYL-1-CYCLOHEXENE (11)

1-METHYL-1-CYCLOHEXANOL (13)

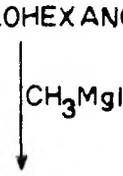
1-METHYL-1-CYCLOHEXENE-6-ONE (12)

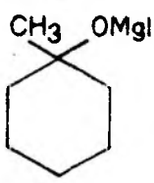
1-METHYL-1-CYCLOHEXENE-6-OL (14)

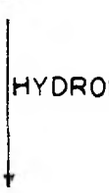
CHEMICAL SYNTHESIS OF 1-METHYL-1-CYCLOHEXANOL, 1-METHYL-1-CYCLOHEXENE-6-ONE, 1-METHYL-1-CYCLOHEXENE-6-OL

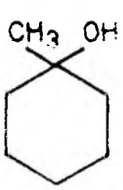


CYCLOHEXANONE



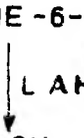


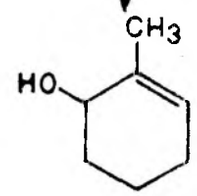




1-METHYL-1-CYCLOHEXANOL

1-METHYL-1-C[illegible]
HEXENE-6-[illegible]





1-METHYL-1-CYCLO-
HEXENE-6-[illegible]
(14)

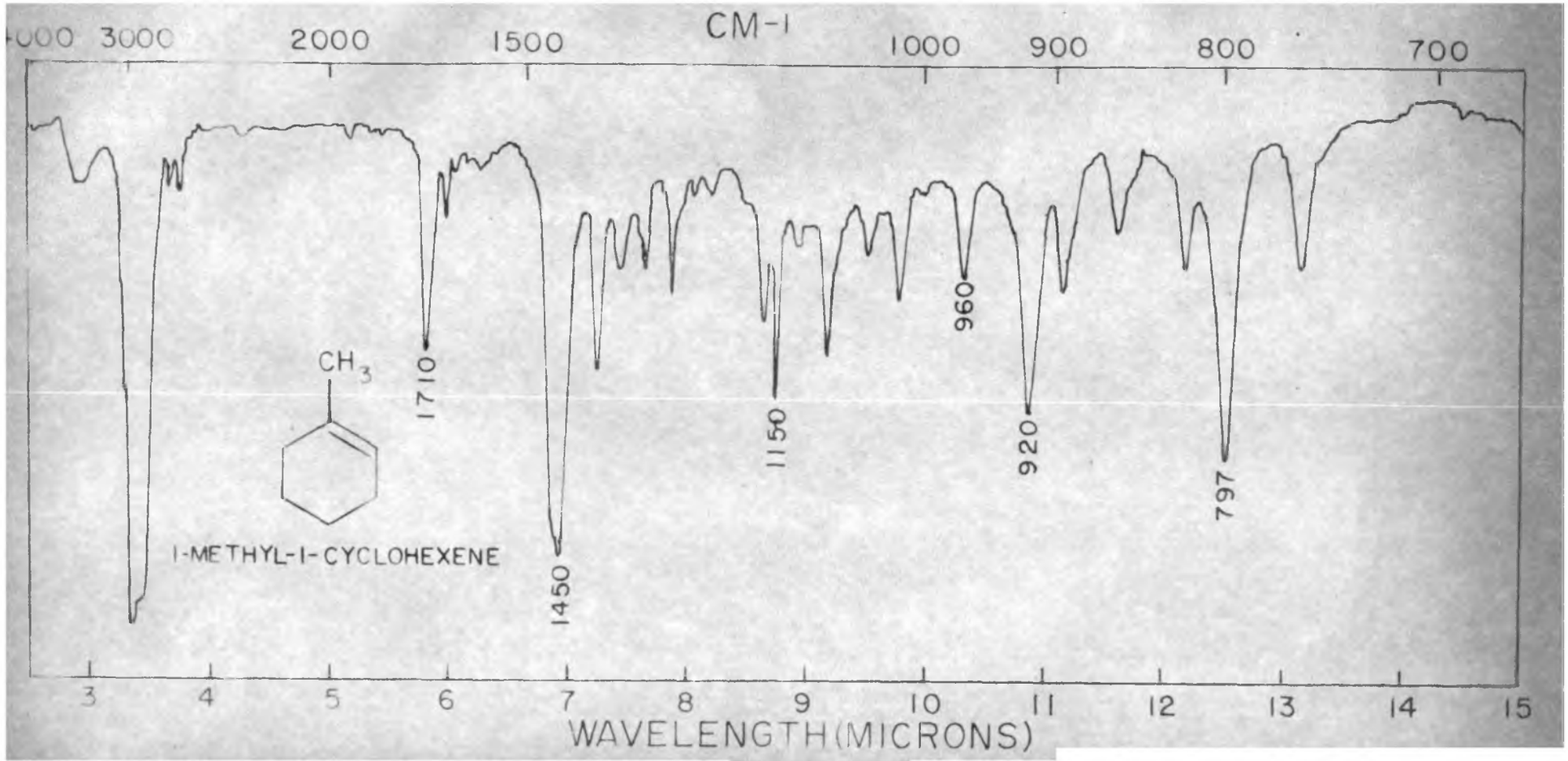
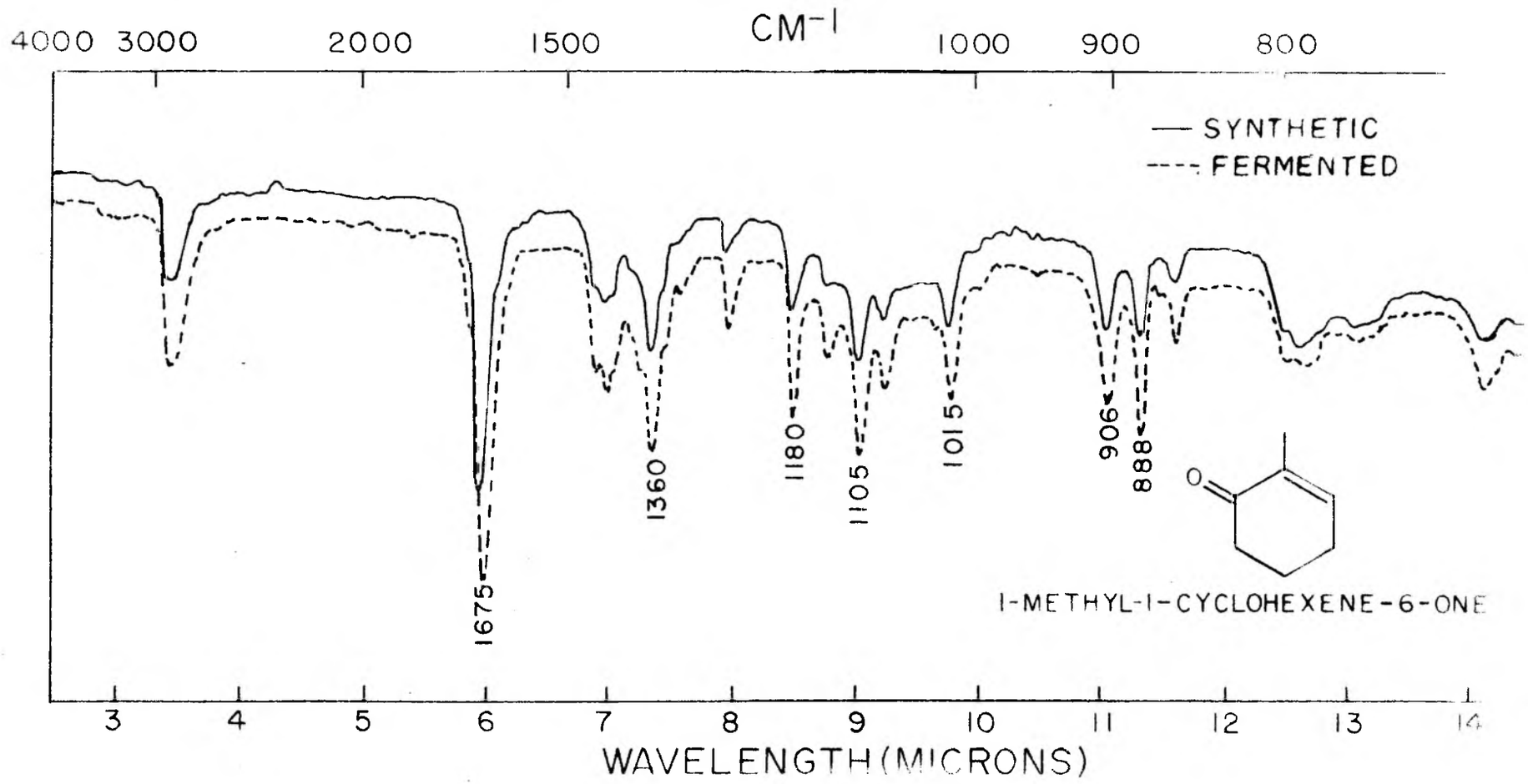

CM-1
4000
3000
2000
1500
1000
900
800
700
1710
1450
1150
960
920
797
CH3
1-METHYL-1-CYCLOHEXENE
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

FIG XX.

in Chapter II). The acidic fraction again did not yield appreciable amount of any metabolite. The neutral fraction was partitioned in the manner described earlier into non-polar and polar sub-fractions by distributing between light petroleum ether and 90% aqueous methanol. The nonpolar fraction after distillation yielded unreacted 1-methyl-1-cyclohexene (11) and wound lipid as a residue. Three main components were detected in the polar sub-fraction by TLC. These components could be easily separated by chromatography over grade II alumina.

Ketone (12):

The fastest moving component which was eluted from the column with petroleum ether + 1% ether showed a single spot on TLC. It analysed for $C_7H_{10}O$. The IR spectrum (Fig.XIV) of this compound showed a strong carbonyl absorption at 1675 cm^{-1} indicating the existence of α,β unsaturated carbonyl function in the molecule. The existence of such a chromophore was also corroborated by the UV spectrum (λmax 235$m\mu$ ϵ = 9400). Compound (12) gave a 2:4 dinitrophenylhydrazone (orange crystals) m.p. 210°C (UV absorption λmax 378$m\mu$, ϵ = 27,100) characteristic of an α,β unsaturated 2,4-dinitrophenyl hydrazone. The n-m-r spectrum showed (Fig.XV) a sharp signal of 3 proton intensity at 8.3τ indicating the presence of methyl group on a double bond. Two allylic methylene proton and two more protons α- to the carbonyl system appeared as a broad signal around 7.7 - 8.7τ. The band for one vinyl proton in the β-position of an α,β unsaturated carbonyl system showed up at 3.35τ. Besides these, there were two ring methylene protons


4000 3000 2000 1500 CM⁻¹ 1000 900 800
SYNTHETIC
FERMENTED
1675
1360
1180
1105
1015
906
888
O
1-METHYL-1-CYCLOHEXENE-6-ONE
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

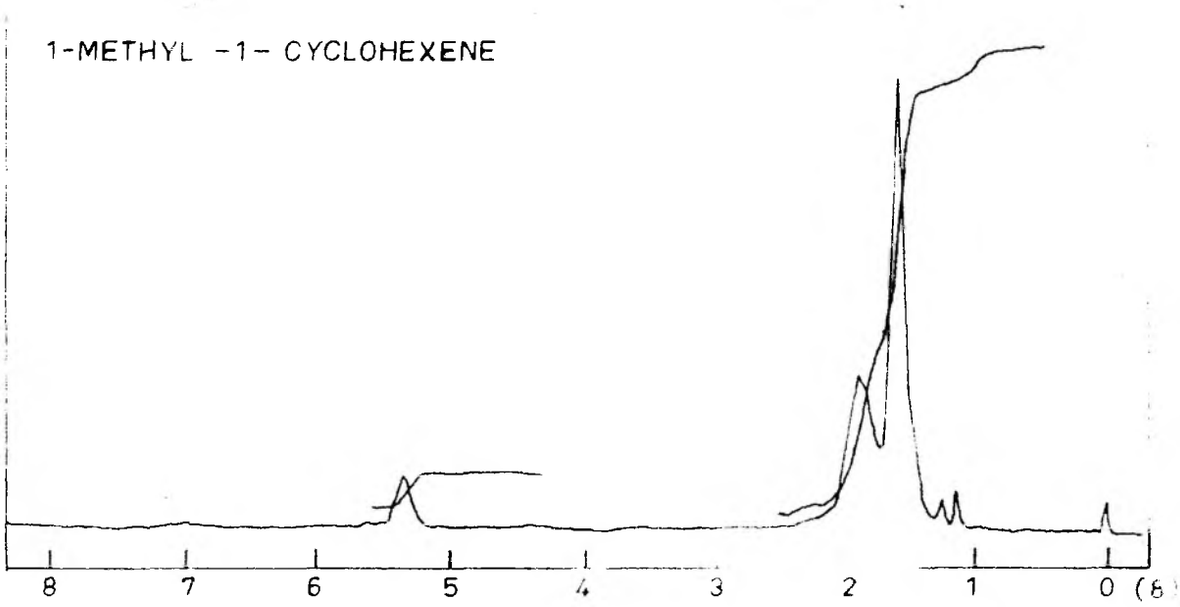


FIG. XXI.

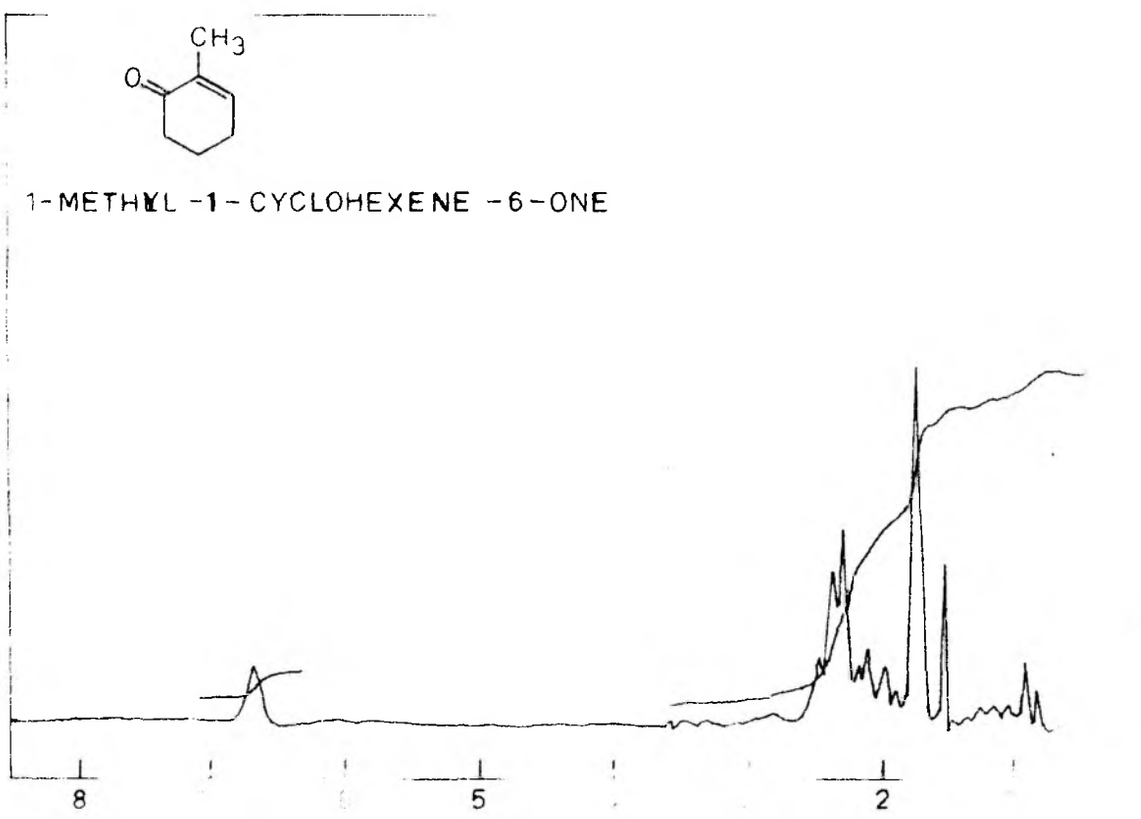


FIG. XV

appearing as a complex signal around 8.4 - 8.6 τ region. From the above spectral properties the only structure admissible for the compound is 1-methyl-1-cyclohexene-6-one (12).

The mould metabolite (12) and an authentic sample of 1-methyl-1-cyclohexene-6-one prepared according to Lewis et al(1947) had superimposable IR spectra and the mixed m.p. of the corresponding 2:4 dinitrophenylhydrazones showed no depression.

Alcohol (13):

The next fraction from the column was eluted with petroleum ether + 5% ether. The elementary analysis corresponded to an empirical formula $C_7H_{14}O$. It was optically inactive. The IR spectrum (Fig.XVI) indicated the presence of a tertiary hydroxyl group in the molecule (3320 cm^{-1}). The NMR spectrum (Fig.XVII) gave a signal of 3 proton intensity at 8.8 τ indicating the presence of a methyl group on a tertiary carbon carrying an oxygen function. The ring methylene protons appeared as a broad signal at 8. τ . Attempts to acetylate the sample with acetic anhydride,-pyridine under the usual conditions failed indicating that the hydroxy group was tertiary in nature. The compound was identified as 1-methyl-1-cyclohexanol (13). The IR spectrum of this alcohol was identical with that reported in literature for 1-methyl-cyclohexene-ol-1. (Smith P.A.S + Baer R.D. J. Am. Chem. Soc. 74 6136.

Alcohol (14):

The most polar component (14) was eluted from the column with 100% ether. It analysed for $C_7H_{12}O$ and was optically active $[\alpha]_D$ + 20.5.

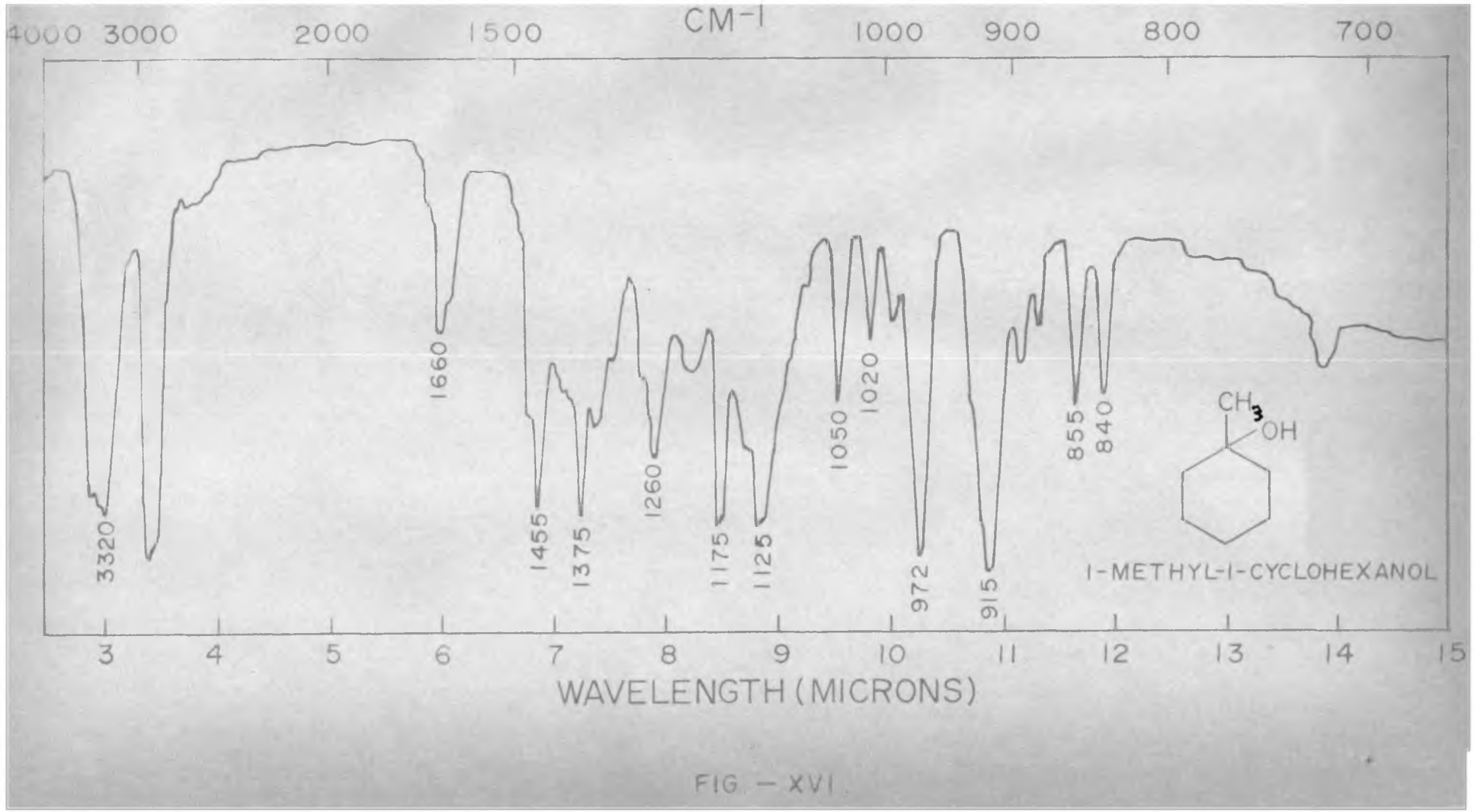
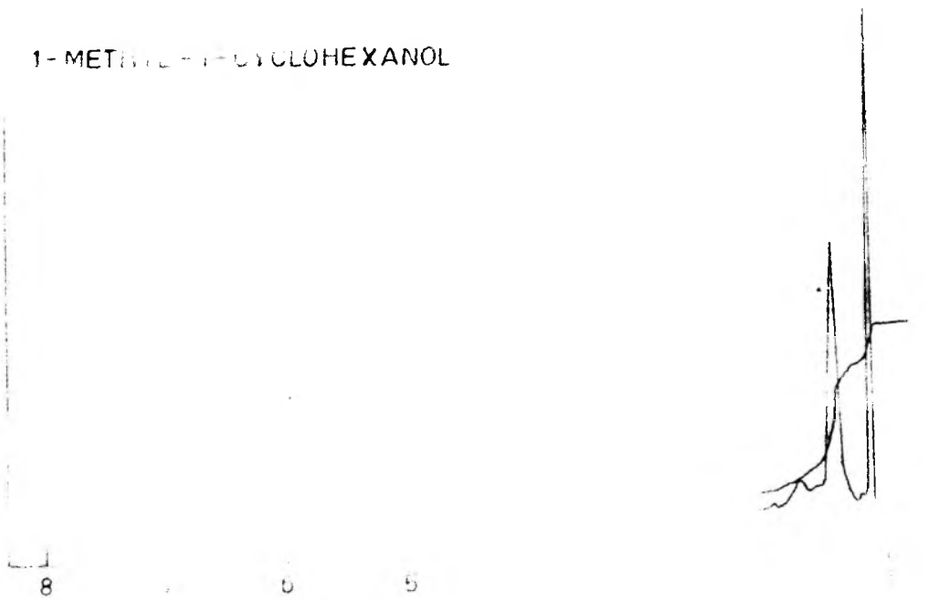

4000 3000 2000 1500 CM-1 1000 900 800 700
3320
1660
1455
1375
1260
1175
1125
1050
1020
972
915
855
840
CH3
OH
1-METHYL-1-CYCLOHEXANOL
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

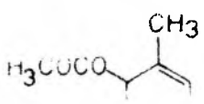
FIG — XVI

1- METHYL - 1- CYCLOHEXANOL



8 6 5

FIG XVII

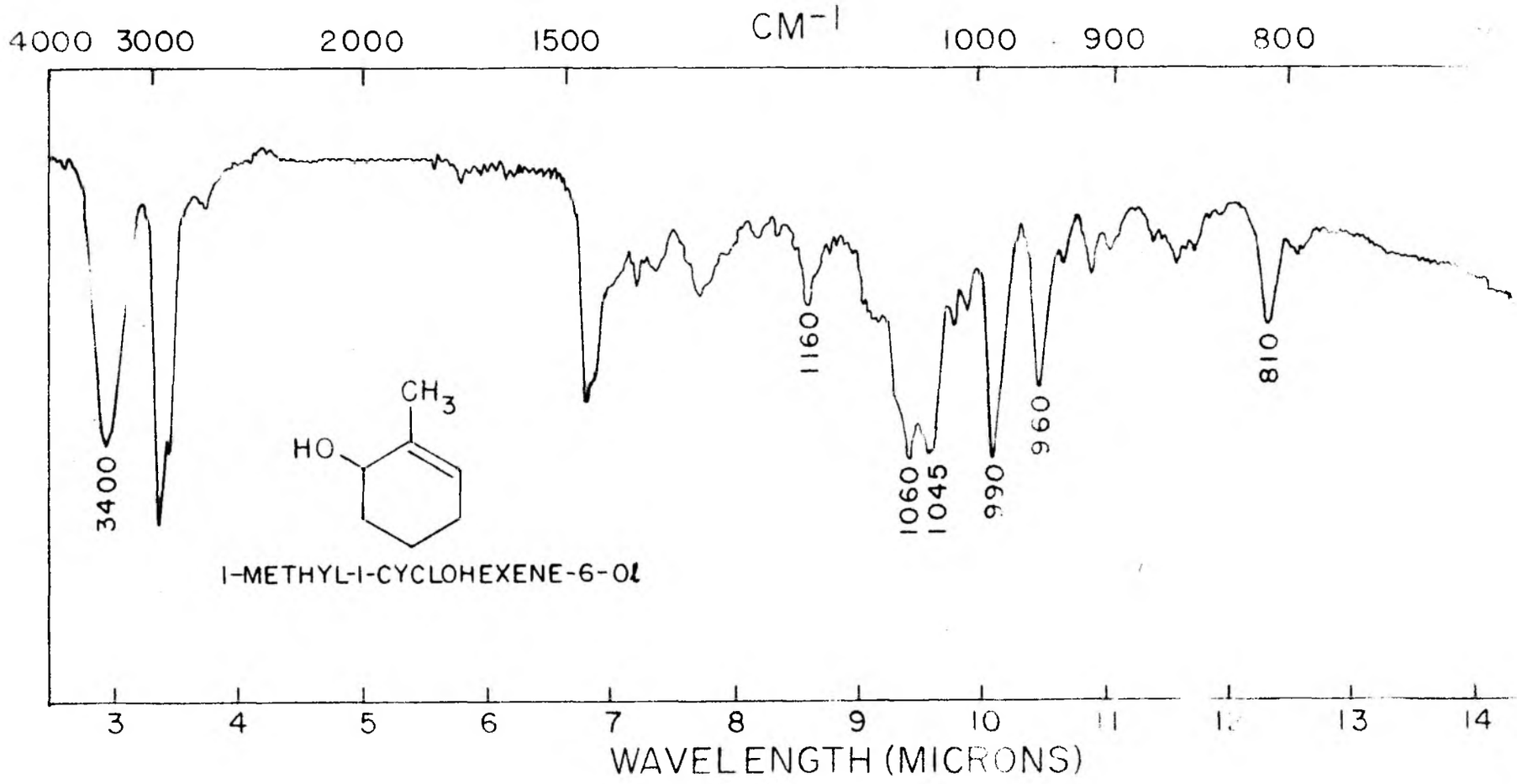


1- METHYL - 1- CYCLOHEXENE - 6- ACETATE

It showed a typical hydroxyl absorption in the IR spectrum (3400 cm^{-1}) (Fig.XVIII) and a trisubstituted double bond (~~1665~~ 810 cm^{-1}). The compound could be easily acetylated with acetic anhydride and pyridine indicating that the hydroxyl group was in a non-hindered position. The mono-acetate of this alcohol analysed for $C_9H_{14}O_2$. The IR spectrum of this derivative showed no hydroxyl band at 3350 cm^{-1} but a characteristic ester band (1740, 1253 cm^{-1}). The n-m-r spectrum (Fig.XIX) of the acetate showed a sharp band at 8.1 τ of three proton intensity indicating the presence of methyl on a trisubstituted double bond and another at 8.0 τ ascribed to the acetate methyl. The ring methylene protons appeared as a complex signal at 8.33 τ. A single vinyl proton showing spin coupling with adjacent methylene protons appeared at 4.55 τ and the proton on the carbon carrying the esterified hydroxyl appeared at 4.83 τ. From the spectral data the tentative structure 14 could be assigned to this compound.

A chemical confirmation of this structure was obtained by oxidation of the free alcohol to the ketone (12) by Jone's reagent.

It is of interest to note that one of the basic reactions observed in the introduction of a hydroxyl function into substrate molecule previously encountered, viz. allylic oxygenation is also observed in the transformation of 1-methyl-1-cyclohexene. This alcohol (14) was again associated with the corresponding α,β unsaturated ketone (12). Like the allylic hydroxylation observed on the cyclohexene nucleus, hydroxylation at position 6 in 1-methyl-1-cyclohexene was stereospecific leading to the opti-


4000 3000 2000 1500 CM⁻¹ 1000 900 800
3400
1160
1060
1045
990
960
810
CH3
HO
1-METHYL-1-CYCLOHEXENE-6-OL
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

cally active alcohol (14). Along with the allylic oxygenation, a hydration reaction to yield the saturated 1-methyl-cyclohexene-1-ol (13) was also evident.

It is interesting to find that the dihydroxylation reaction leading to cyclohexene, 1,2-cis diol (4) from cyclohexene (1) could not be demonstrated with 1-methyl-1-cyclohexene (11) presumably due to the steric hindrance introduced by the methyl group. However, it is difficult to judge as to how far the inductive effect from the methyl group influences these reactions. That such an effect is involved may be construed from the fact that the position 6 rather than the alternate allylic position 3 in the ring was involved in the hydroxylation process.

The substrate blanks run with the experiment produced the ketone (12) and the alcohol (14) in trace quantities as judged by comparative TLC. The formation of the hydration production was not detectable. Here also the amount of ketone obtained was far in excess of that of the alcohol.

It is, therefore, evident that just as with cyclohexene, the primary site of fungal oxygenation in 1-methyl-1-cyclohexene is also identical with the most reactive i.e., the autoxidation site.

CHAPTER IVa

EXPERIMENTAL

1-METHYL-1-CYCLOHEXENE

1-Methyl-1-cyclohexene was prepared by the method described in Chapter II. It was further purified by refluxing over metallic sodium and redistillation, b.p. 110°-112°/715 mm. n_D = 1.4503 D = 0.8145. The physical properties of the preparation were identical with those reported in literature. IR ν_{max} 3450, 2990, 1720, 1676, 1441, 1375, 1339, 1303, 1263, 1155, 1141, 1088, 1052, 1027, 967, 916, 894, 858, 820, 797, 760 cm^{-1} (Fig.XX). NMR (Fig.XXI).

Fermentation:

The conditions for the fermentation and the extraction procedure have already been discussed (Chapter II). In case of 1-methyl-1-cyclohexene it was found that optimum concentration was 0.5% and the optimum time of incubation 4 hr with this concentration.

Separation:

The neutral ether layer after extraction (Chapter II) was dried and evaporated through a fractionating column to yield (14 gm) of total extract which was separated into the polar and nonpolar neutral fractions by partitioning between petroleum ether and 90% methanol (Chapter II). The nonpolar fraction (pet.ether) on evaporation gave a residue (12.2 gm) which distilled at 110°-112°C/714 mm. The volatile component was identifiedd as

1-methyl-1-cyclohexene from its IR spectrum (Fig.XX). The residue (3.66 gm) was chromatographed over alumina (66 gm) and eluted with the usual solvents (described earlier in Chapter II) to yield mostly mould lipid and fatty material. The polar fraction obtained after evaporation of the methanol layer through a Vigreaux column to a small volume was diluted with water and extracted with (3 x 50 ml) chloroform. The chloroform extracts were pooled together, dried and evaporated to yield an oily residue (2.01 gm) which showed the presence of at least 3 components in TLC.

Identification of Metabolites (Fig.XIII):

Ketone (12): The viscous oily residue (2 gm) was chromatographed over grade II alumina (200 gm) and eluted with a mixture of solvents as detailed in Chapter II. Petroleum ether + 1% ether eluate from the column was evaporated to get a residue (200 mg) which showed a single spot in TLC. The compound was further purified by distillation b.p.60°-70°/11 mm. Anal. C 76.64% H 9.16%. Calc. for $C_7H_{10}O$, C 76.32%, H 9.15%. IR νmax 1673, 1360, 1190, 1105, 1015, 966, 838 cm^{-1} (Fig.XIV). UV λ max 235 mμ ϵ = 9400. NMR spectrum (Fig.XV). It gave a 2:4 dinitrophenyl hydrazone, m.p.208°-209°C (reported for 1-methyl-1-cyclohexene-6-one 2:4 dinitrophenylhydrazone, m.p. 210°C). (λ max 378 mμ ϵ = 27,150). The 2:4 dinitrophenylhydrazone obtained from the fermentation product did not show any depression in the m.p. when mixed with an authentic sample (mmp 208°-210°C).

For identification the ketone was synthesised according to the method described in literature (Lewis, 1947).

Alcohol (13):

The petroleum ether + 5% ether eluate was fractionally distilled to yield a residue (500 mg) which showed a single spot in TLC. The residue was further purified by distillation, b.p.60°-61°/10 mm. Anal. Found: C 76.64%, H 9.16%. Calc. for $C_7H_{14}O$, C 76.32%, H 9.15%. IR ν max 3320, 1660, 1455, 1375, 1260, 1175, 1125, 1050, 1020, 972, 915, 855, 840, 725 cm^{-1}. (Fig.XVI). NMR bands 8.8 τ (Fig.XVII). IR spectra identical with that reported for 1-methyl-1-cyclohexanol.

An attempt to acetylate the product with acetic anhydride and pyridine was unsuccessful and gave the unreacted alcohol.

Alcohol (14):

The 100% ether eluate from the column on evaporation gave an oily residue (0.7 gm) which showed a single spot in TLC. The compound was distilled. b.p.66°-67°/14 mm. $[\alpha]_D$ +20.8. Anal. C 74.05%, H 10.05%. Calc. for $C_7H_{12}O$, C 74.95%, H 10.78%. IR νmax 3350, 1160, 1060, 1045, 990, 960, 912, 885, 810 cm^{-1}(Fig.XVIII). The compound (100 mg) was easily acetylated with acetic anhydride (1 drop) and pyridine (2 ml) to give the monoacetate (80 mg) b.p.69°-70°/14 mm. Anal. C 69.7%, H 9.05%. Calc. for $C_9H_{14}O_2$, C 70.1%, H 9.15%. IR νmax 1740, 1450, 1375, 1245, 1030, 990, 960, 941, 920 cm^{-1}. NMR (Fig.XIX).

Oxidation of alcohol (14) to 1-methyl-1-cyclohexene-6-one (12):

The alcohol (150 mg) in 2 ml of acetone (ice cold) was titrated with Jone's reagent to a red colour. The mixture was decomposed with water, extracted with ether (3 x 25 ml) and the

ether extract was dried (sodium sulphate) and evaporated to give a liquid b.p.66°-67°C/14 mm. It was identified as 1-methyl-1-cyclohexene-6-one by spectral comparison with the authentic compound.

CHAPTER IIIa

FERMENTATION OF 1-METHYL-2-CYCLOHEXANE

CHAPTER IVb

1-METHYL-3-CYCLOHEXENE

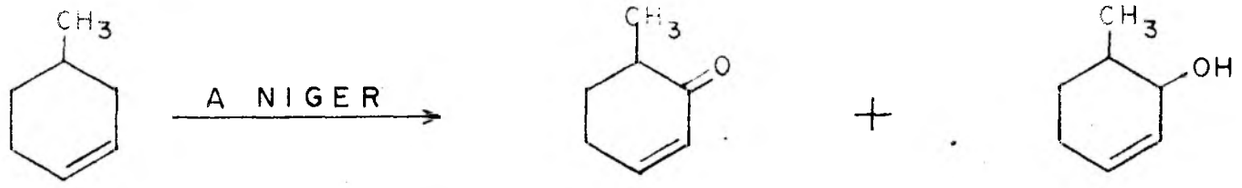
DISCUSSION

The results with cyclohexene (1) and 1-methyl-1-cyclohexene (11) indicated that the first sites of hydroxylation by the mould were allylic to double bond. However, in case of cyclohexene, 2-cyclohexene-1-ol (3) was further oxidised to the (+)-cyclohexene-1,2-cis diol (4). The presence of a methyl group on the double bond probably interfered with the second hydroxylation process with 1-methyl-1-cyclohexene (11) which yielded only the mono oxygenated derivatives such as 1-methyl-1-cyclohexene-6-ol (14) and 1-methyl-1-cyclohexene-6-one (12) besides a hydration product (13).

It was, therefore, considered necessary to study the behaviour in the fungal system of 1-methyl-3-cyclohexene (15)where the double bond was widely separated for the methyl group and the steric hindrance to the dihydroxylation process was less pronounced. Just as with the isomeric 1-methyl-1-cyclohexene (11) the optimum level of this hydrocarbon (15) was 0.5% and the optimum incubation period 4 hr at this substrate level at a temperature of 27°-28°C. The conditions for the fermentation were essentially the same as those described for 1-methyl-1-cyclohexene.

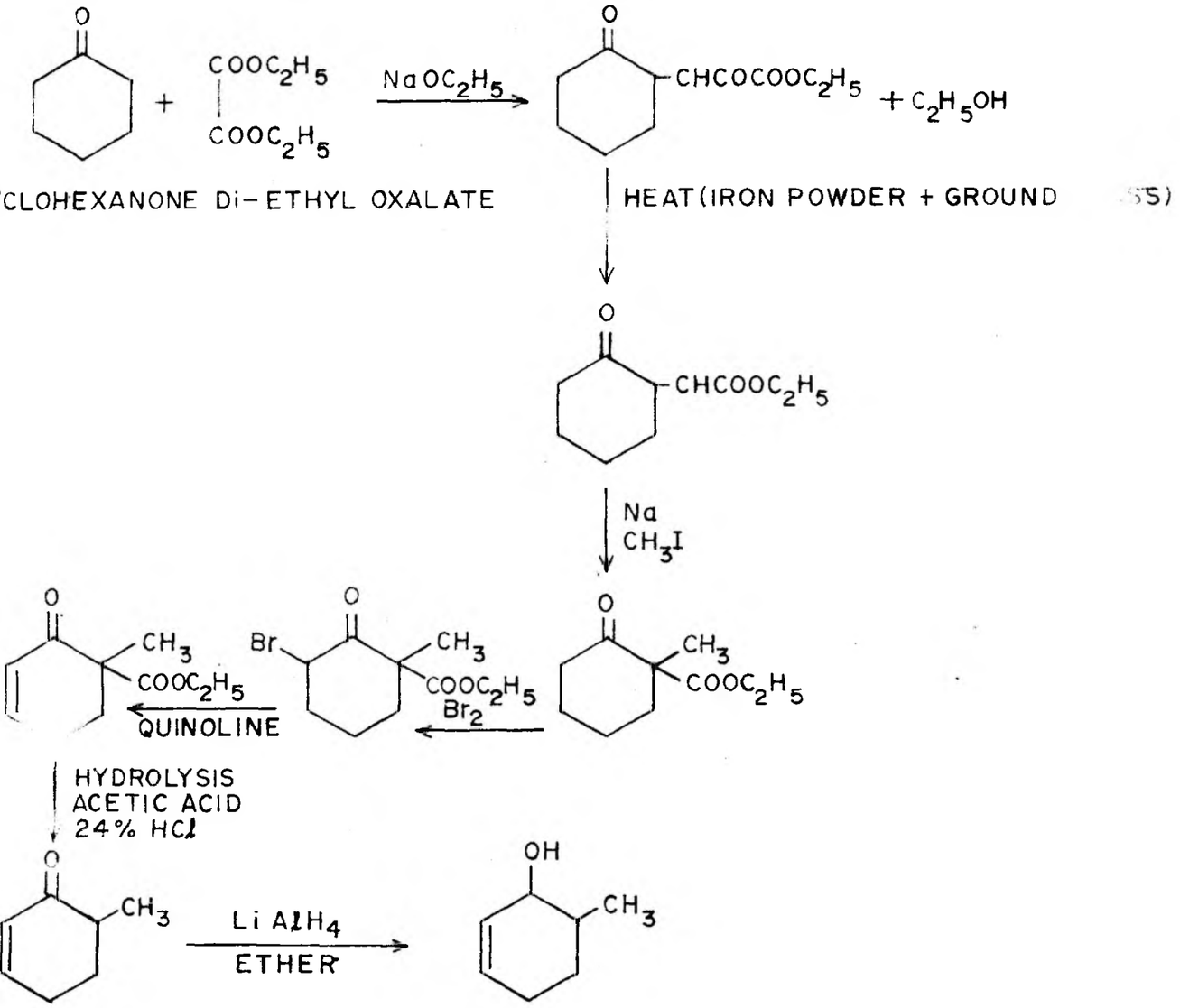
Transformation products (Fig.XXIV):

The products were extracted in the usual manner as described (Chapter II) and separated into acidic and neutral layers. The acidic layer on evaporation did not yield any metabolite. The



1-METHYL-3-CYCLOHEXENE. 1-METHYL-3-CYCLOHEXENE-2-ONE. 1-METHYL-3-CYCLOHEXENE-2-OL
(15) (16) (17)

CHEMICAL SYNTHESIS OF 1-METHYL-3-CYCLOHEXENE-2-ONE + 1-METHYL-3-CYCLOHEXENE-2-OL



1-METHYL-3-CYCLOHEXENE-2-ONE. 1-METHYL-3-CYCLOHEXENE-2-OL
(16) (17)

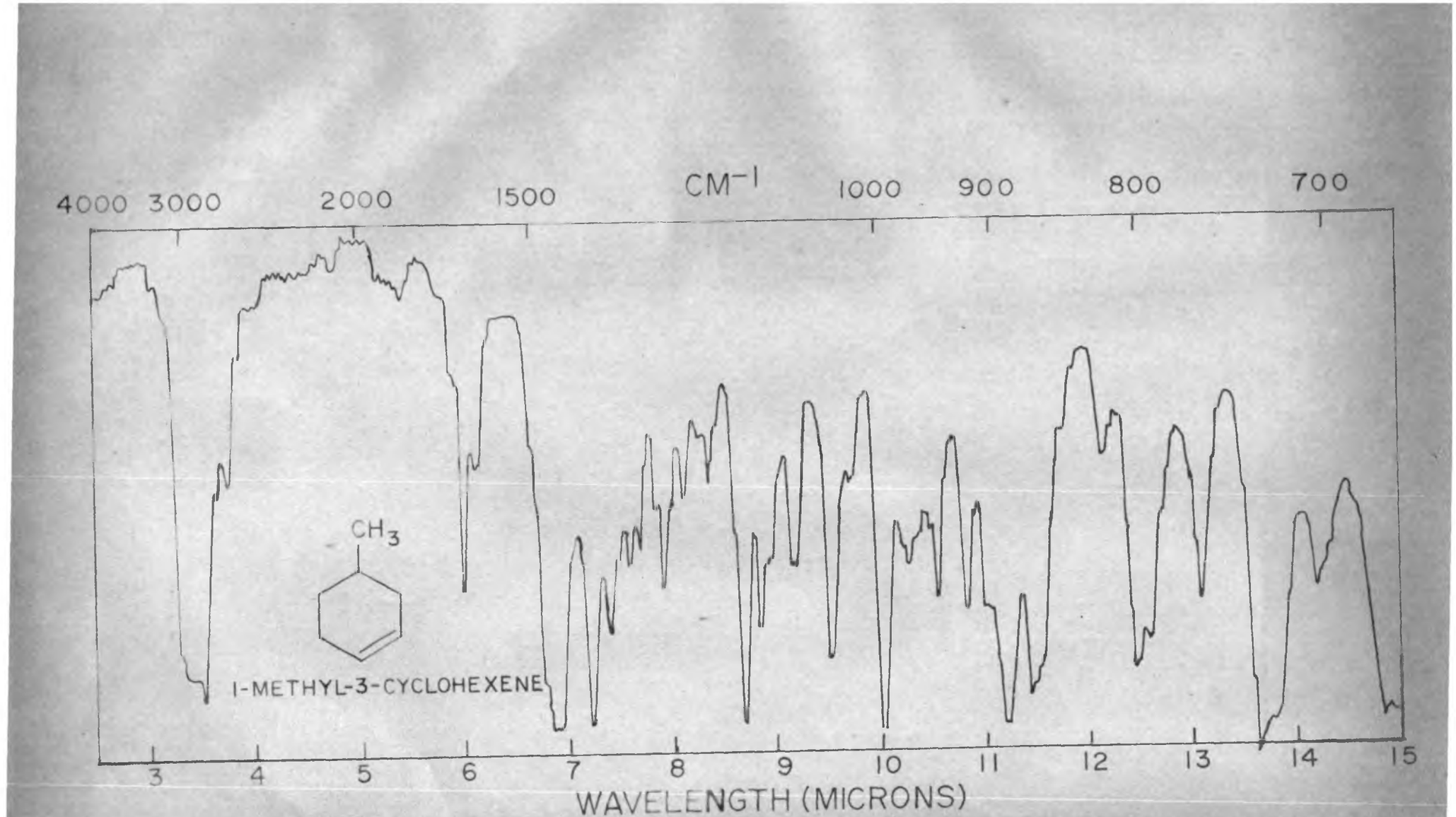
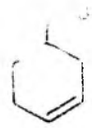

4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
CH3
1-METHYL-3-CYCLOHEXENE
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XXII



1-METHYL - 3- CYCLOHEXENE

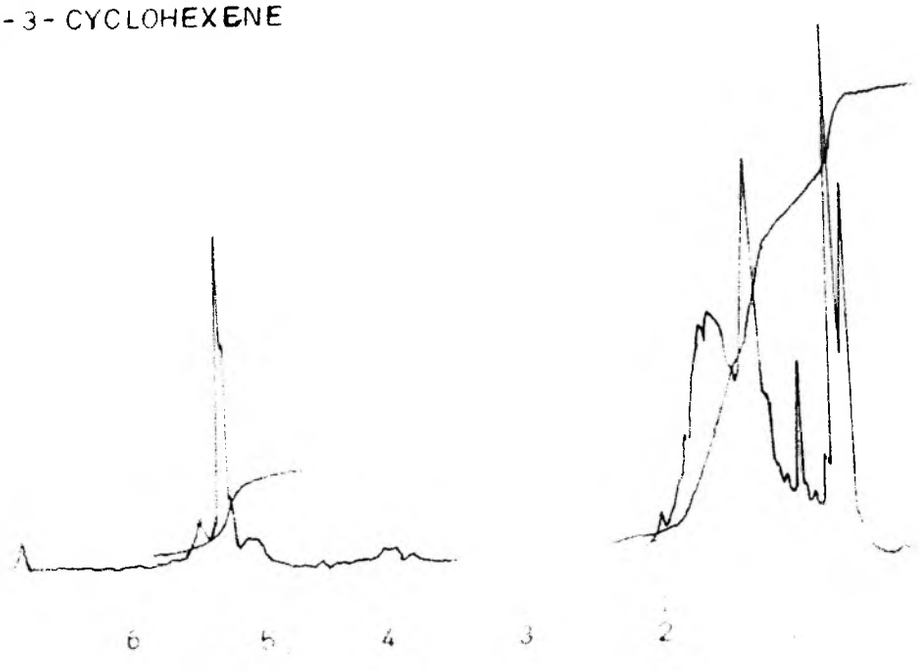


FIG XXIII

neutral layer yielded polar and non-polar fractions. The non-polar fraction yielded mainly unreacted 1-methyl-3-cyclohexene (15) and mould fat. The polar fraction obtained from the methanol layer on preliminary investigation by TLC also showed the presence of two major and one minor components. The components were separated by chromatography over alumina grade II.

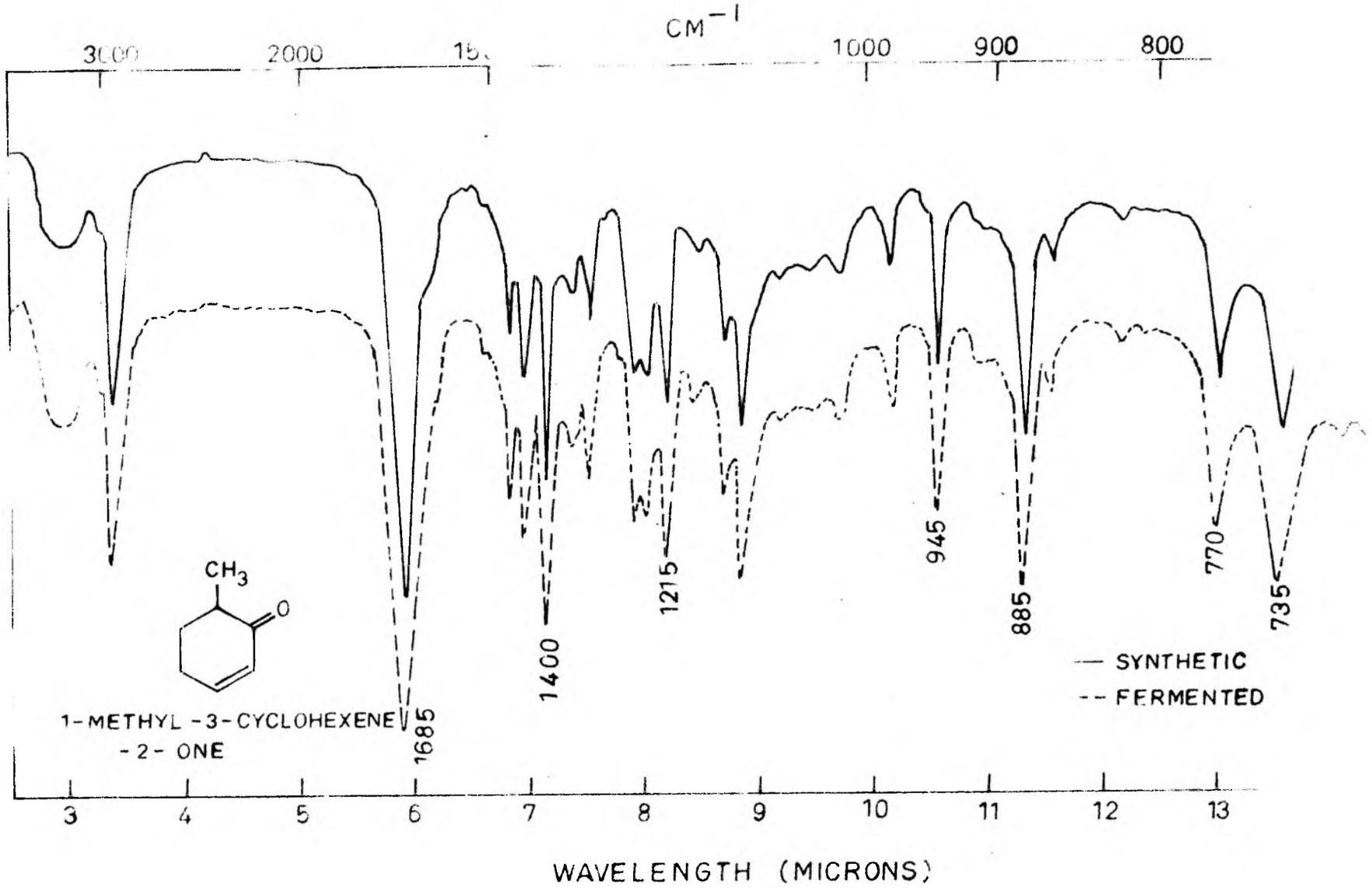
Ketone (16):

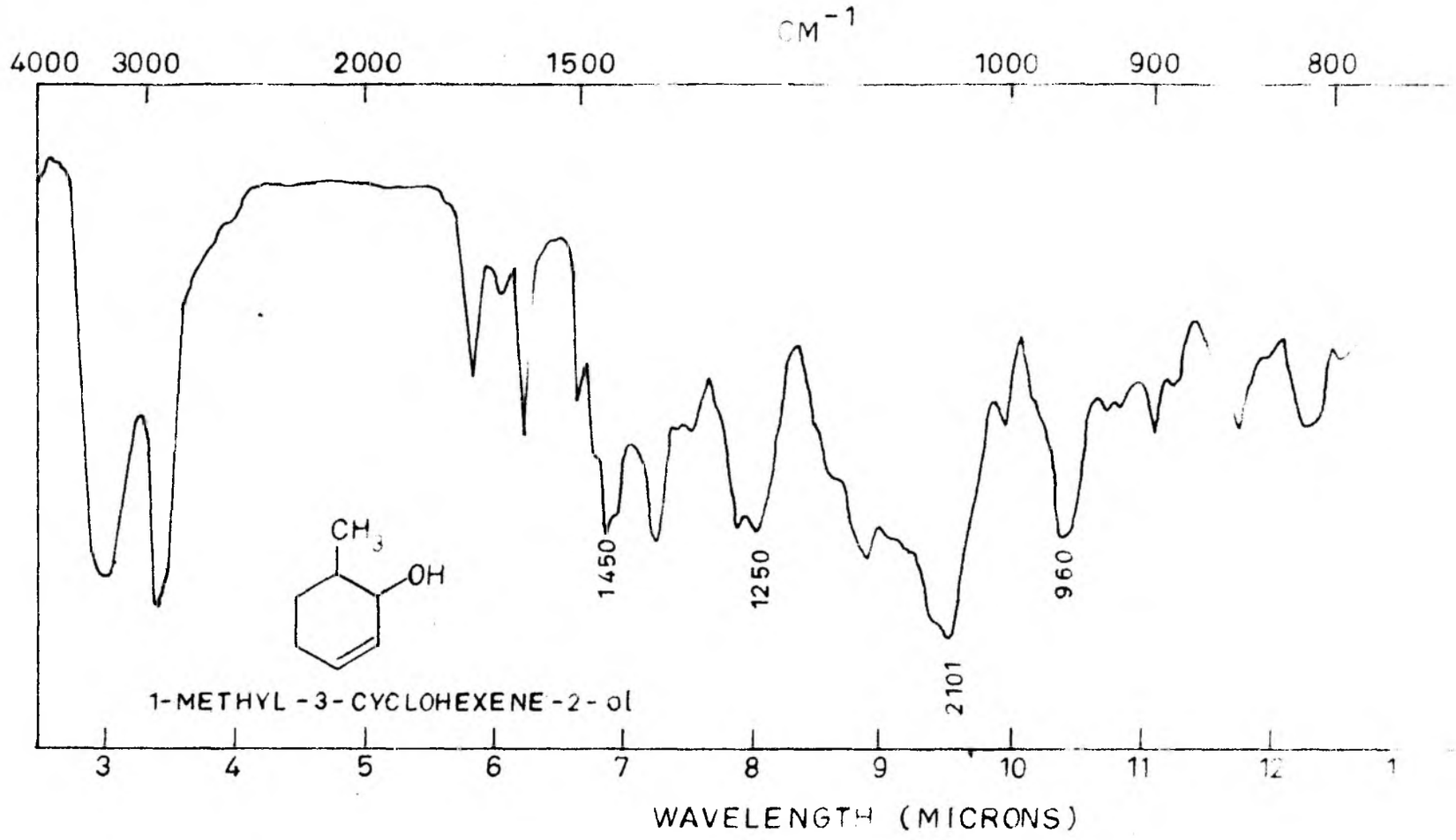
The petroleum ether + 1% ether eluate from the column gave a product which analysed for $C_7H_{10}O$. The infrared spectra of this compound indicated presence of α,β unsaturated carbonyl function (νmax 1695 cm^{-1}, Fig.XXV) a finding which was corroborated by UV λmax at 225 mμ, ϵmax = 10,400. It gave 2:4 dinitrophenylhydrazone, m.p. 156°-157° (λmax 362 mμ, ϵ max = 27,120.) From the above physico-chemical properties it was identified as 1-methyl-3-cyclohexene-2-one and this identification was confirmed by its spectral comparison with an authentic sample. The melting point of 2:4 dinitrophenylhydrazone of the synthetic sample as well as that from the mould metabolite did not show any depression.

Alcohol (17):

The ether eluates from column on evaporation gave a compound which analysed for $C_7H_{12}O$. The IR spectrum (Fig.XXVI) of this compound showed hydroxyl absorptions at 3320 cm^{-1}. On oxidation with Jone's reagent it was converted to the above ketone (16).

It was, therefore, established that the alcohol was 1-methyl-3-cyclohexene-2-ol (17). One of the surprising findings about its physical property was that it was optically inactive.


CM⁻¹
3000
2000
1500
1000
900
800
CH3
O
1-METHYL -3-CYCLOHEXENE
-2- ONE
1685
1400
1215
945
885
770
735
— SYNTHETIC
-- FERMENTED
3
4
5
6
7
8
9
10
11
12
13
WAVELENGTH (MICRONS)


CM⁻¹
4000
3000
2000
1500
1000
900
800
CH3
OH
1450
1250
2101
960
1-METHYL-3-CYCLOHEXENE-2-ol
3
4
5
6
7
8
9
10
11
12
WAVELENGTH (MICRONS)

The third component could not be isolated in amounts sufficient for further chemical studies. However, its mobility in TLC corresponded to that of a monooxygenated compound since it moved faster than compound (17).

It is also noteworthy that in the case of1-methyl-3-cyclohexene the oxygen function entered at the most reactive position between double bond and the methyl group. This position is also the site for autoxidation as the extract from substrate blanks also gave two TLC spots with mobilities identical with those of the ketone (16) and the alcohol (17). However, the amounts of these compounds formed in the experiment with the mould appeared to be several hundred times greater. Further, in the autoxidation experiments the ketone was produced in overwhelmingly larger amounts but in the fungal system the alcohol was the major product. This type of difference in product pattern from autoxidation and fungal transformation has also been observed in case of verbenol and verbenone from α-pinene as well as in the cases of cyclohexene and 1-methyl-1-cyclohexene. There was always a preponderance of the alcohol over ketone in the fungal metabolites.

The reason for lack of optical activity in the hydroxylated product is not clear. Perhaps in this case a racemisation process may account for the lack of apparent stereoselectivity in the hydroxylation process. This will be discussed in more detail in the concluding chapter.

Another feature in the transformation of this hydrocarbon is that in spite of conditions favourable for the dihydroxylation reaction as in cyclohexene, no dioxygenated product could be detected in the medium. The mobility of the unidentified compound in TLC was much faster than what could be expected for a dihydroxylated compound. Perhaps the methyl group, although away from the double bond, may still provide for enough steric hindrance to prevent the formation of the cyclic transition state.

CHAPTER IVb

1-METHYL-3-CYCLOHEXENE

EXPERIMENTAL

The preparation of 1-methyl-3-cyclohexene has been described in Chapter II. The hydrocarbon was purified by refluxing over sodium metal and redistillation (b.p.104°-105°C, n_D = 1.4943, D = 0.8091). The IR spectrum of the compound was identical with that reported in literature for 1-methyl-3-cyclohexene (Fig.XXII).

1-Methyl-4-cyclohexanol was prepared according to Wicker (1956) by hydrogenation of p-cresol with Raney's Nickel as catalyst.

Fermentation:

The conditions for the fermentation and the extraction procedure have already been described (Chapter II). Preliminary trial experiments with graded levels of methyl cyclohexene and different incubation periods in the manner described for cyclohexene established that 0.5% (v/v) of this hydrocarbon was optimal and the best period of incubation 4 hrs at 27°-28°C to get the maximum yields of oxygenated products at this level of substrate.

Separation of metabolites(Fig.XXIII):

The neutral ether layer obtained after extraction was dried and evaporated to get an oily residue (13.8 gm) which contained both the polar and non-polar fractions. These were separated by a Craig-type distribution between 90% aqueous methanol and petroleum ether. The petroleum ether layer on eva-

poration gave an oily residue (10.8 gm) most of which distilled at 104°-106°C indicating that it was the aireated hydrocarbon which was identified by its spectral property. The residue (0.63 gm) was chromatographed over grade I alumina and eluted with solvents as described earlier to yield mainly mould lipids and fatty materials. The polar fraction obtained after evaporation of methanol layer gave an oily liquid having a pungent odour (1.6 gm). On preliminary investigation with TLC it showed two main components and one minor one.

Identification:

The oily residue (1.6 gm) was chromatographed over grade II alumina (160 gm) and eluted with the solvent system as described earlier.

Ketone (16):

The petroleum ether + 1% ether eluate from the column on evaporation gave an oily residue (0.43 gm) which was further purified by distillation b.p. 69°-71°/18 mm. Anal. C 75.9%, H 9.35%. Calc. for $C_7H_{10}O$. C 76.39, H 9.15%. IR νmax 1685, 1650, 1470, 1445, 1400, 1335, 1260, 1215, 1150, 1125, 1080, 985, 885, 770 and 735 cm^{-1} (Fig. XXV). UV spectrum λmax 225 mμ ϵ max = 10,400. The compound gave an orange red 2:4 dinitrophenylhydrazone m.p. 154°-156°C. λ max 362 mμ, ϵ = 27,120 (reported for 1-methyl-3-cyclohexene-2-one 2:4 dinitrophenylhydrazone (m.p. 156°-157°C). It did not show any depression in m.p. when mixed with an authentic sample. The identity was established by the synthesis

of an authentic sample of this ketone as described in literature (Naseiti et al 1956). The IR spectra (Fig.XXV) of the mould metabolite and the synthetic samples were superimposable.

Alcohol (17):

The 100% ether eluates obtained from the column on evaporation gave a residue (0.73 gm) which showed a single spot in T.L.C. It was further purified by distillation, b.p.68°-70°C/15 mm $[\alpha]_D$ = 0.0. Anal. C 74.2%, H 10.5%. Calc. for $C_7H_{12}O$, C 74.95%, H 10.78%. IR ν_{max} 3320, 1610, 1450, 1250, 1050, 960, 900, 812, 738, 735 cm^{-1} (Fig.XXVI).

The oxidation of the alcohol (17) to 1-methyl-3-cyclohexene-2-one(16):

The alcohol (100 mg) in 5 ml of acetone (ice bath) was oxidised by Jone's reagent to a definite red colour. The product was worked up, after decomposing the reaction mixture with water by extraction with ether. The dried (sodium sulphate) ether extract on evaporation gave an oily residue (75 mg) which was identified as 1-methyl-3-cyclohexene-2-one from its spectral properties.

CHAPTER V

FERMENTATION OF LIQUORS

CHAPTER V

LIMONENE

DISCUSSION

Limonene, $C_{10}H_{16}$ (18):

This monoterpene occurs very widely distributed in nature and has played a fundamental part in the development of terpene chemistry. Limonene forms the main constituent of terpene fraction of a number of oils such as the oils of lemon, orange, etc. (Simonsen, 1953).

The choice of limonene as a substrate was obvious, since besides being the simplest terpene it also can be looked upon as a model compound in which an additional isopropenyl group has been substituted at the p-position in 1-methyl-1-cyclohexene (11).

It was considered desirable to investigate whether the fungal reactions observed with 1-methyl-1-cyclohexene do also take place in the p-menthene nucleus. It may be recapitulated that 1-methyl-1-cyclohexene gave one hydration product and two allylic oxidation products.

Fermentation:

The conditions for the fermentation were identical with those reported for cyclohexene (1). The hydrocarbon was added at 0.6% level and incubated for a period of 4 hr at 27° to 28°C. The procedure for extraction and separation has already been described in the earlier chapter.

Transformation products (Fig.XXXII):

The products after fermentation were extracted in the usual manner as described (Chapter II) into acidic and neutral layers. The acid fraction did not yield any metabolite. The neutral fraction was partitioned between 90% aqueous methanol and light petroleum ether to get the non-polar and the polar fractions (procedure described in Chapter II).

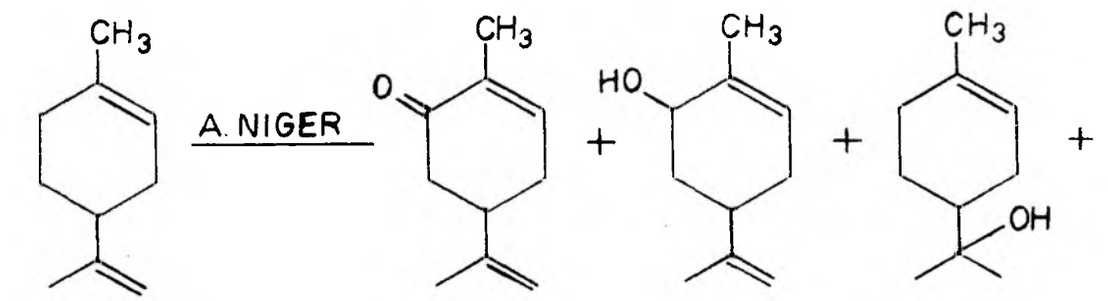
The non-polar fraction after distillation yielded unreacted limonene (13) and mould lipids. The examination of the polar fraction obtained from the methanol layer showed three major and a few minor components by TLC. The sweet smelling oil was now chromatographed over alumina grade II.

Ketone (19):

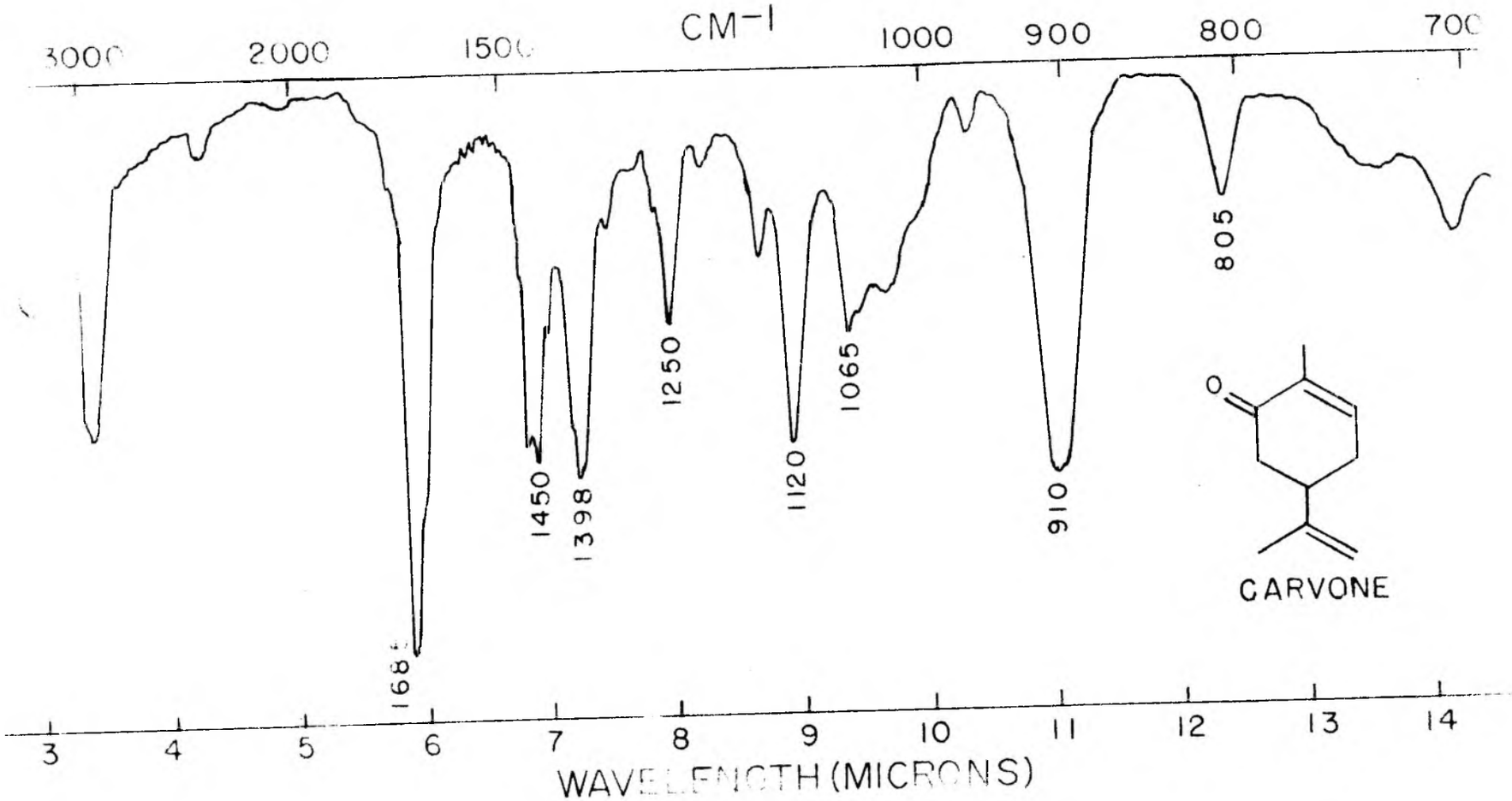
The petroleum ether + 1% ether from the column eluted a single component (TLC). The compound analysed for $C_{10}H_{14}O$ and was optically active, α_D = +69.1. The IR spectrum showed a strong absorption at 1685 cm^{-1} indicating the presence of α-β unsaturated carbonyl group (Fig.XXVII). The ultraviolet absorption also corroborated the existence of such a function λmax 235mμ, ϵ= 18,340. It gave a crystalline oxime m.p.72°C. From these data the compound was identified as (+)carvone. The oxime did not show any depression in the mixed m.p. with an authentic sample of carvone oxime (m.p.72°). The identity of the ketone was established conclusively by running comparative and mixed VPC with an authentic sample supplied by the Essential Oils Division of this Laboratory.

FIG. XXXII

TRANSFORMATION OF (+) LIMONENE BY A. NIGER



IMONENE (18) CARVONE (19) CARVEOL (21) ∝-TERPINEOL (22)


CM-1
3000
2000
1500
1000
900
800
700
1685
1450
1398
1250
1120
1065
910
805
O
CARVONE
3
4
5
6
7
8
9
10
11
12
13
14
WAVELENGTH (MICRONS)

Alcohol (20):

The next fraction eluted from the column with petroleum ether + 25% ether showed a single spot in TLC and a single peak in VPC. The compound analysed for $C_{10}H_{16}O$ $[\alpha]_D$ = +22.4. The IR spectrum showed a hydroxyl group (3350 cm^{-1}) double bond (1670 and 1660 cm^{-1}) and an isobutylenic double bond (890 cm^{-1}). The n-m-r spectrum of this alcohol indicated the presence of a tertiary methyl group on a carbon carrying an oxygen function (sharp signal of 3 proton intensity at 8.74 τ, four methylenic ring protons (diffuse signals around 8.30 τ), a hydroxyl proton (8.33 τ disappears on treatment with D_2O, a tertiary proton allylic to two double bonds (strongly coupled complex signal around 5.0 τ) a cisoid disubstituted double bond (a split signal of two proton intensity at 5.3 τ. The only structure which fits in with the above data is represented by (20), p-menthadiene 2:8-1-ol. However, unfortunately no reference sample was available for comparative spectral studies. An attempt to acetylate the product by acetic anhydride and pyridine gave back the unreacted alcohol indicating that the hydroxyl group was tertiary in nature.

Alcohols (21) and (22):

The next fraction obtained from 100% ether eluate was found to contain two compounds on TLC and VPC examination. From the TLC data the compounds which were suspected to be present were carveol (21), α-terpineol (22) and perillyl alcohol. Comparative VPC of the mixture was obtained after the addition of these compounds along with the fermented product. The data presented

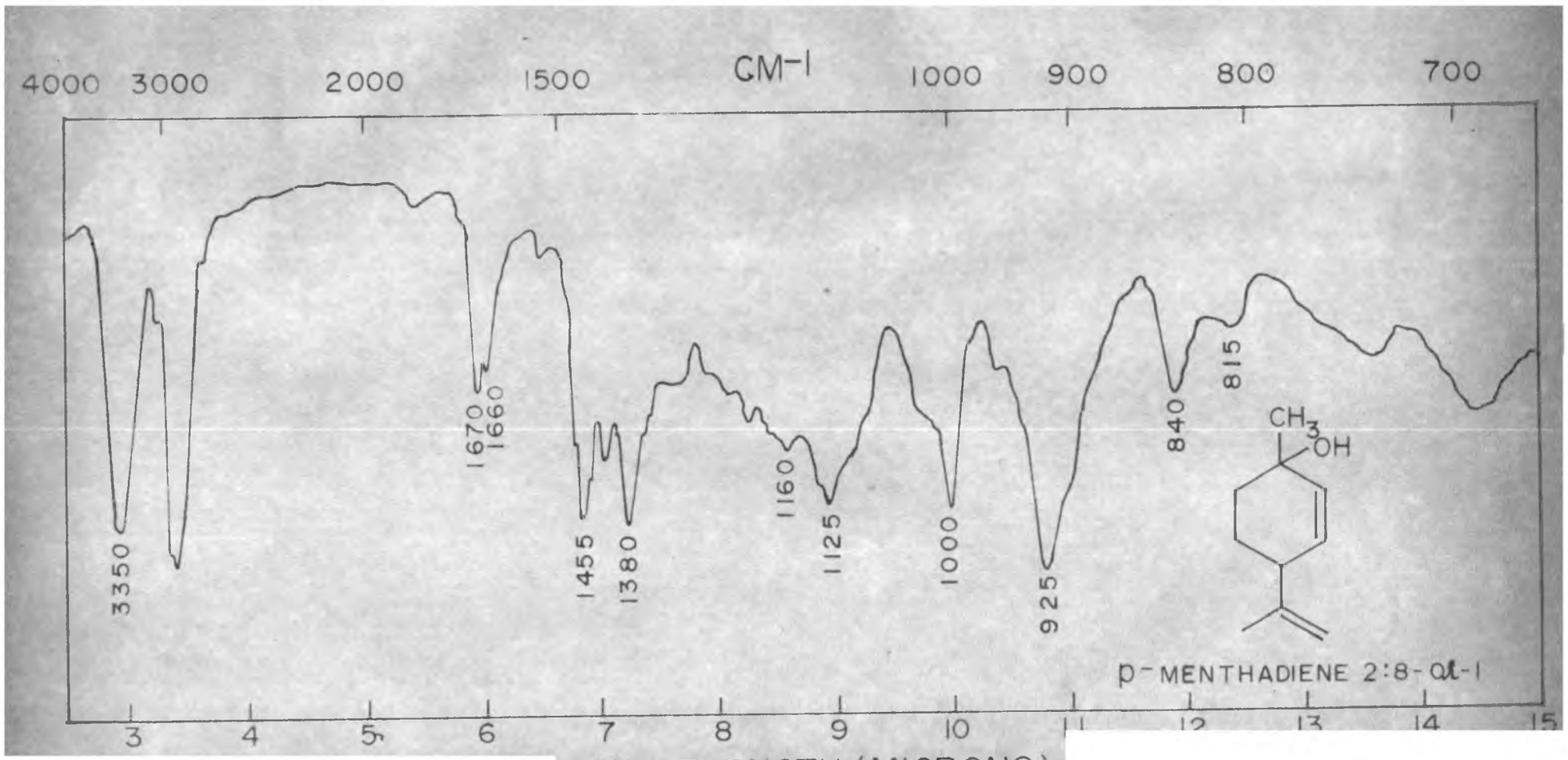

4000 3000 2000 1500 CM-1 1000 900 800 700
3350
1670
1660
1455
1380
1160
1125
1000
925
840
815
CH3
OH
p-MENTHADIENE 2:8-ol-1
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG XXVIII

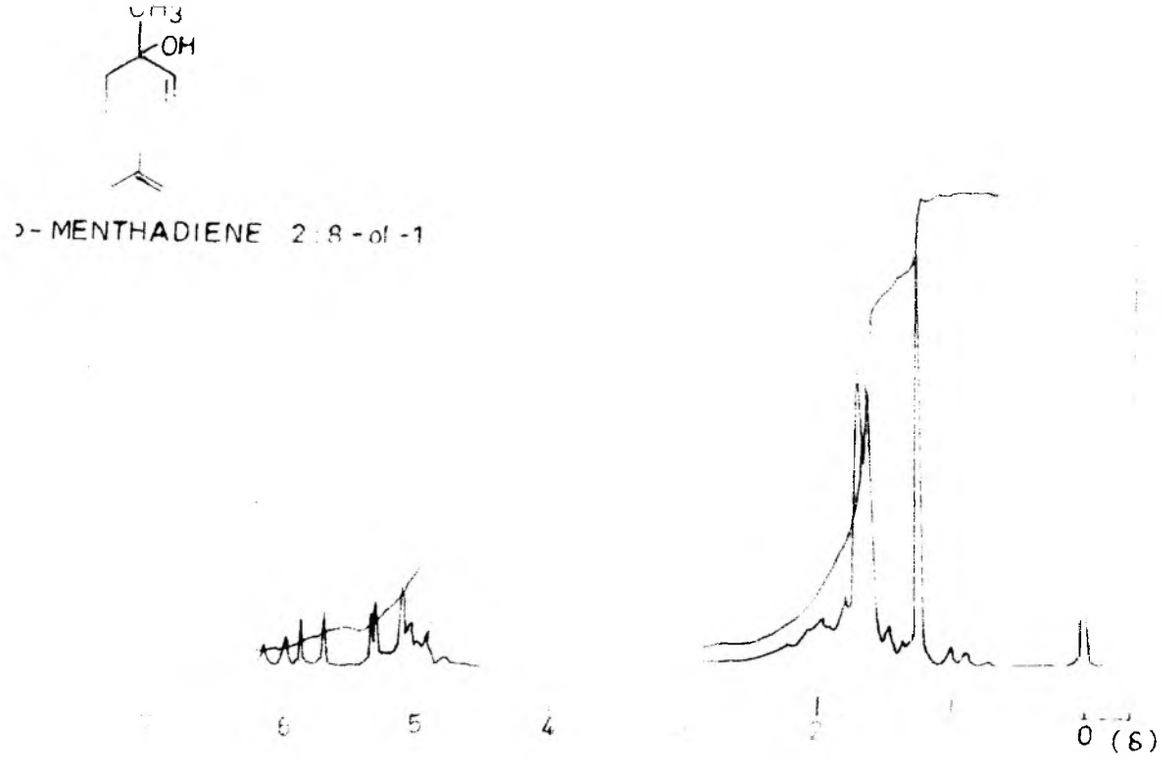


FIG XXXI

indicated that α-terpineol (22) and carveol (21) were present in the mixture. Separation of the two compounds was easily achieved by acetylating the mixture of the alcohols. Carveol being a secondary alcohol was acetylated readily, while the hydroxyl group in α-terpineol could not be acetylated under mild conditions because of its tertiary nature. The acetylated mixture was separated by chromatography over alumina grade II. The petroleum ether fraction yielded an ester which after saponification yielded compound, $C_{10}H_{16}O$. $[\alpha]_D$ + 23.9 which was conclusively identified as (+)carveol (21) by comparison of its IR bands with that reported for carveol (21) (Fig.XXIX). The identity was further established by converting the alcohol (27) to carvone (19) by Jone's oxidation.

The other eluate from the colum following the acetylated derivative yielded the tertiary alcohol $C_{10}H_{18}O$. Spectral comparison and comparative VPC with an authentic sample established its identity as + α-terpineol (22) $[\alpha]_D$ + 100.4. (Fig.XXX).

It is noteworthy that all the basic reactions employed in the formation of the hydroxyl group by the mould are observed in the transformation products of limonene (18). The formation of α-terpineol (22) is mediated by a simple hydration reaction analogous to the formation of 1-methyl-1-cyclohexanol (13) from 1-methyl-1-cyclohexene (11). The formation of carveol (21) and carvone (19) could also be anticipated by allylic oxygenation processes. The stereochemistry of these products will indicate that these are indeed truly allylic oxidation products, as the

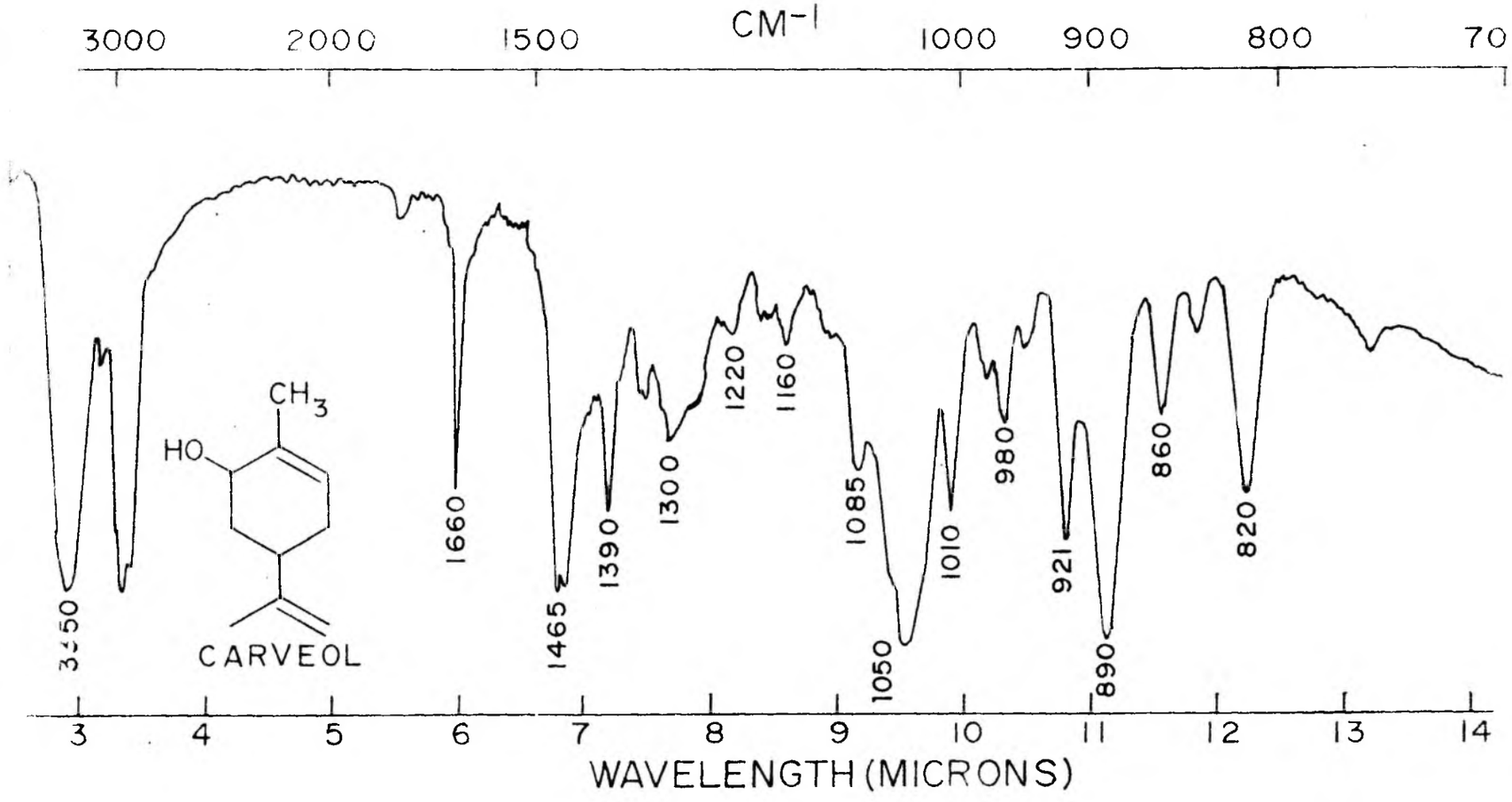

3000
2000
1500
CM-1
1000
900
800
70
3450
1660
1465
1390
1300
1220
1160
1085
1050
1010
980
921
890
860
820
HO
CH3
CARVEOL
3
4
5
6
7
8
9
10
11
12
13
14
WAVELENGTH (MICRONS)

FIG XXIX

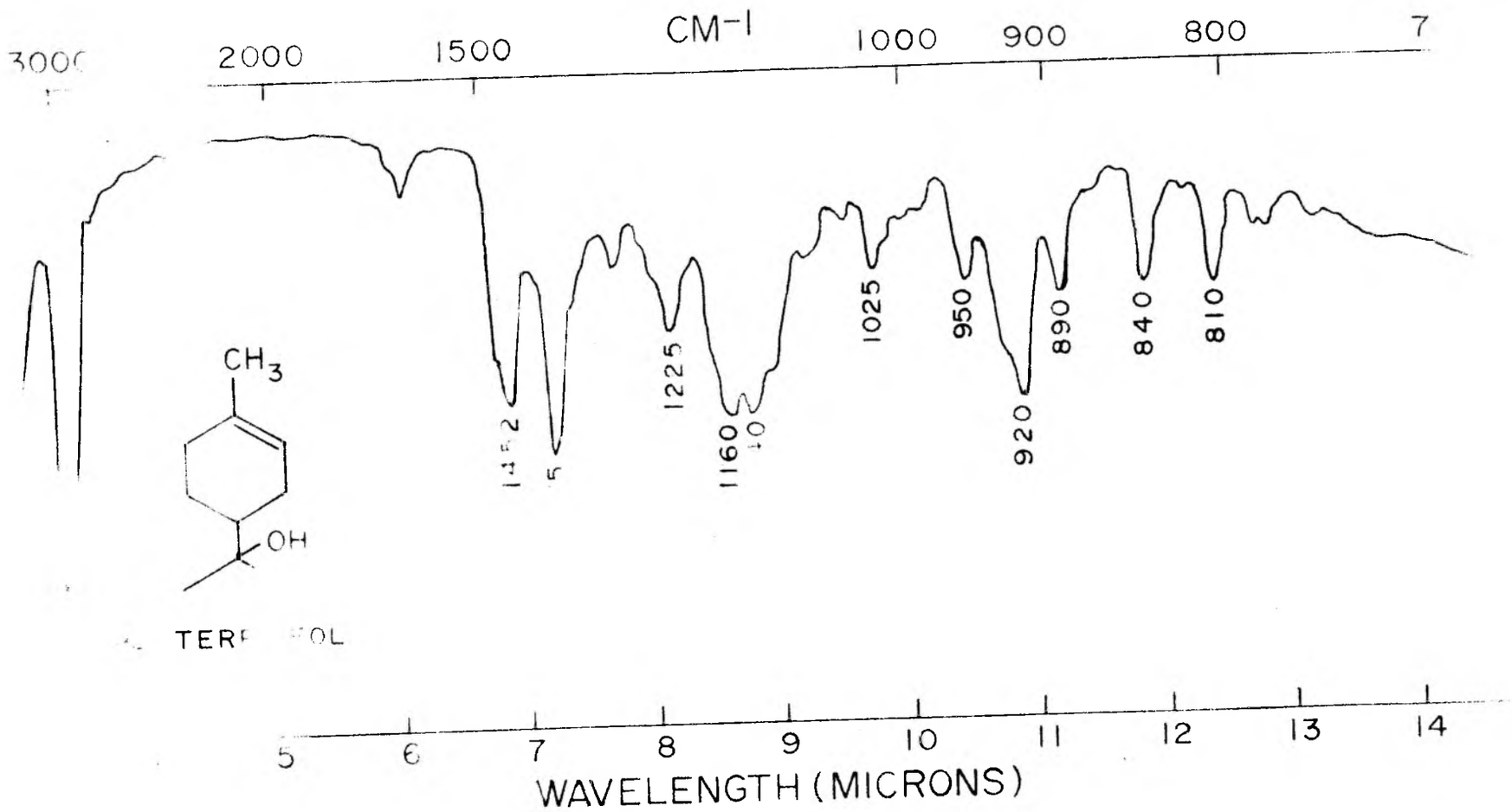
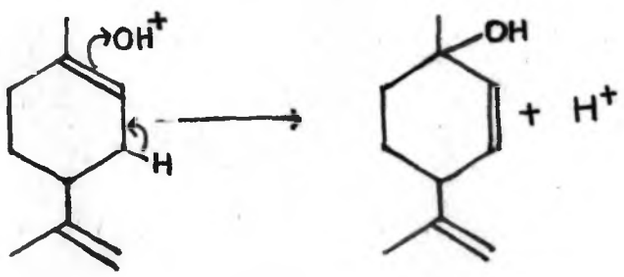

3000
2000
1500
CM-1
1000
900
800
7
1225
1160
1025
950
920
890
840
810
CH3
OH
TERP OL
5
6
7
8
9
10
11
12
13
14
WAVELENGTH (MICRONS)

FIG. XXX

attack on the double bond followed by an allylic shift would have led to the formation of (-)carvone. This type of oxygenation is, however, observed in the formation of p-menthadiene 2-8-ol-1 (20) which is formed presumably by an attack of the electrophilic hydroxyl on the double bond followed by an allylic shift.



Another significant feature of these transformations is that again the oxygenations seem to be oriented at autoxidation sites as both carveol and carvone are obtained by autoxidation of limonene.

The substrate blanks run along with the fermentation side by side formed carvone as evidenced by TLC.

CHAPTER V

LIMONENE

EXPERIMENTAL

Limonene (13) was fractionated from orange oil (locally supplied) by fractional distillation by batch stripping method under total reflux (Chapter II). The fraction boiling between 172°-174°C was collected. This was further purified by redistilling over metallic sodium, VPC of the distillate indicated that it was 99.5% pure. The physical properties of the compound n_D = 1.4743, D = 0.8437, α_D = + 104°.

Fermentation:

The conditions for the fermentation and the extraction procedure were the same as described before (Chapter II). In case of limonene it was found that optimum concentration was 0.6%, time of incubation 4 hr at this substrate level. The temperature was maintained at 27°-28°C.

Separation:

The dried neutral ether extract was evaporated to yield (18 gm) of total extract which was seprarated into the non-polar and polar neutral fractions by partitioning between 90% aqueous methanol and petroleum ether (Chapter II). The non-polar fraction (pet.ether) was evaporated to yield a residue (14 gm) which distilled at 172°/174°C. and was identified as limonene (13) from its spectral properties. There was no decrease in the rotation of the recovered sample α_D = +103.8°. The residue (0.5 gm) was passed over grade I alumina (50 gm) and eluted with

petroleum ether + ether.

The petroleum ether fraction from the column yielded mostly mould lipid and fatty material. The viscous oil (2.6 gm) recovered from the polar fraction showed the presence of at least 5 to 6 components on TLC. VPC of the product indicated the presence of three major components and about 5 minor components.

Separation and identification of components (Fig. XXXII):

The viscous oil (2.6 gm) from the methanolic layer was chromatographed over alumina grade II (260 gm) and eluted with a mixture of solvents as described earlier. Petroleum ether + 1% ether eluate from the column gave a fraction showing a single spot in TLC and in VPC.

Ketone (19):

The solvent was removed and the residue (200 mg) was further purified by distillation b.p. 60°-61°/10 mm $[\alpha]_D$ + 69.1. Found C 79.24%, H 9.11%. Calcd. for $C_{10}H_{14}O$, C 79.95%, H 9.39%. IR ν max 1685, 1450, 1393, 1250, 1120, 1065, 910 cm^{-1} (Fig. XXVII). UV λ max 235 mμ, ϵ= 10,340. It gave an oxime m.p. 72° (reported for carvone oxime). It did not show any depression in the m.p. when mixed with an authentic sample of the oxime prepared from carvone. From the above data it was identified as carvone also by its mixed VPC with an authentic sample. (Retention time 4 min, temp. 123°C, column P, carrier gas H_2, flow rate 9 sec/10 ml).

Compound (20):

The next fraction eluted from the column petroleum ether + 25% ether on evaporation through a fractionating column gave a residue (0.5 gm) which was further purified by distillation b.p. 61°-62°/10 mm α_D = 22.4. Found. C 78.74%, H 10.30%. Calcd. for $C_{10}H_{16}O$. C 78.89%, H 10.59%. IR max 3350, 1670, 1660, 1455, 1380, 1160, 1125, 1000, 926, 890, 840, 815, 740, 690 cm^{-1} (Fig. XXVIII). NMR (Fig. XXX). The compound was recovered unchanged after attempted acetylation with acetic anhydride and pyridine at room temperature.

Separation of compounds (21) and (22):

The fraction obtained from the 100% ether eluate (1.0 gm) of the column was shown to be a mixture of two alcohols by TLC. The compounds suspected to be present were carveol (21), α-terpineol (22), perillyl alcohol. A mixed VPC of the mixture was run with the authentic samples and it was shown that carveol and α-terpineol accounted for both the compounds. (Retention time: carveol 4 min 48 sec; α-terpineol 2 min 24 sec; Temp. 128°C, column P, carrier gas H_2, flow rate 9 sec/10 ml).

The above mixture of alcohol (0.7 gm) was acetylated with 2.0 ml of dry pyridine and 3 to 4 ml of acetic anhydride at room temperature overnight. The reaction mixture was poured into ice cold water, extracted with ether. The ether extract was washed with dilute hydrochloric acid, then with aqueous bicarbonate and water, dried and evaporated to give a viscous liquid (0.8 gm).

Chromatography of the acetylated product:

The product obtained from the above procedure showed presence of two distinct spots in TLC one of which corresponded with α-terpineol. It was chromatographed over grade II alumina (30 gm) and eluted with petroleum ether and ether.

Alcohol (21):

The petroleum ether fraction (A) after evaporation gave the ester (0.5 gm) which had a sweet smell. The ester was saponified by 5% alcoholic KOH (5.0 ml) in the usual manner. The free alcohol was recovered by ether extraction and purified by distillation b.p. 101°-102°/10 mm $[\alpha]_D$ + 23.9. Anal. C 78.70%, H 10.30%. Calc. for $C_{10}H_{16}O$. C 78.99%, H 10.59%. IR ν max 3350, 1660, 1465, 1390, 1300, 1220, 1160, 1085, 1050, 1010, 900, 962, 921, 890, 860, 845, 820 cm^{-1} (Fig. XXIX).

Oxidation of the alcohol (21) to carvone (19):

The alcohol (21) (100 mg) was treated with Jone's reagent in acetone solution in ice bath till the colour of the reagent persisted (red) and kept overnight. The reaction mixture was then poured in water, extracted with ether (50 ml) thrice and dried (sodium sulphate). On evaporation, the ether layer gave a residue (90 mg) which was distilled and identified as (+)carvone, which had identical IR spectrum with that of an authentic sample.

Compound (22):

Fraction (B): The fraction obtained in the 100% ether eluate from the column was purified by distillation b.p. 104°/15 mm.

$[\alpha]_D$ = 103.5. Anal. C 76.93%, H 11.66%. Calc. for $C_{10}H_{18}O$. C 77.96%, H 11.76%. IR max 3350, 1650, 1452, 1375, 1300, 1225, 1160, 1140, 1025, 950, 920, 890, 840, 810 cm^{-1}. (Fig.XXX).

The compound was recovered unchanged after treatment with acetic anhydride and pyridine overnight at room temperature. The identity of the compound with α-terpineol (22) was established by a comparative and mixed VPC and TLC with an authentic sample (Retention time 2 min 24 sec; temp. 129°C, column P, carrier gas H_2 flow rate 9 sec/10 ml).

CHAPTER VI

FERMENTATION OF l-α-PINENE

CHAPTER VI

l-α-PINENE

DISCUSSION

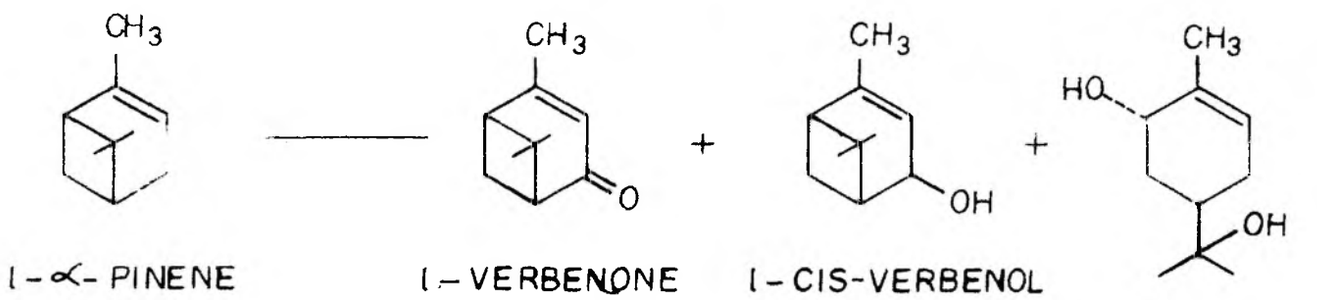
It has been mentioned in the introductory chapters that earlier experience with the fermentation with α-pinene (Prema & Bhattacharyya 1962a) with the same strain of Aspergillus niger indicated that both the d and l-forms in a commercial sample of α-pinene (23) containing 70% d and 30% l isomers were oxidised. The products, however, were optically pure (+)cis verbenol (24), (+)verbenone (25) and (+)trans sobferol (26) and presumably had their origin in the d isomer. The specific rotation of the unreacted pinene recovered after fermentation was practically the same as the starting material indicating that no enrichment of any of the isomers had taken place during the fermentation.

To determine the fate of l-α-pinene (23) in the fungal system fermentations were carried out with this substrate under the conditions developed for α-pinene.

The products after fermentation were separated in the usual manner (Prema & Bhattacharyya, 1962a) into acidic and neutral fractions. The neutral fraction was also separated into polar and nonpolar material. The nonpolar material yielded mainly unreacted l-α-pinene (23). The examination of the polar fraction from l-α-pinene fermentation by comparative TLC with the corresponding fraction from d-α-pinene revealed that the products in both cases

FIG XXXIII

TRANSFORMATION OF l-∝(−) PINENE BY A.NIGER



l-∝- PINENE (23)

l-VERBENONE (25)

l-CIS-VERBENOL (24)

l-TRANS-SOBREROl (26)

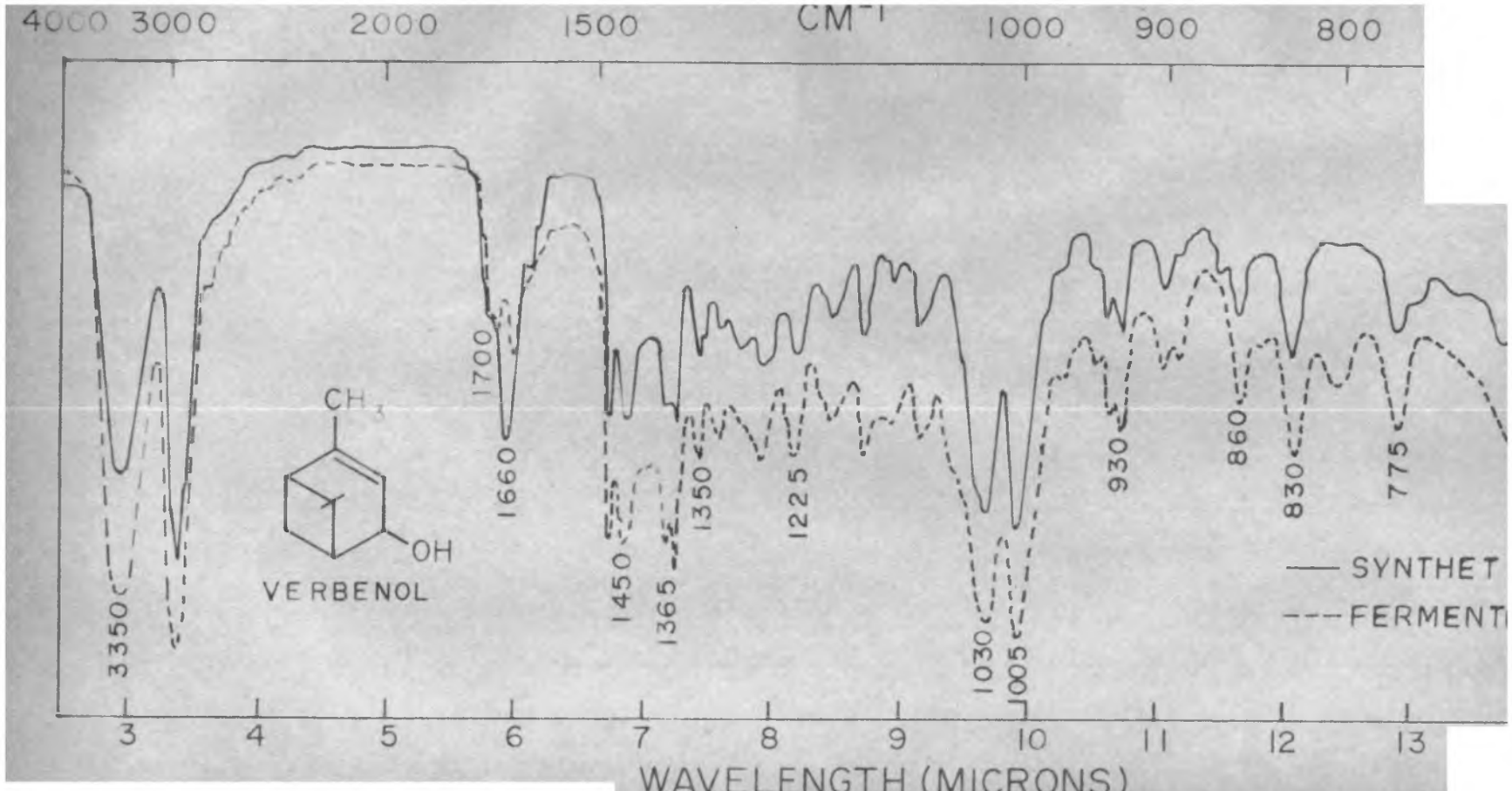

4000 3000 2000 1500 CM⁻¹ 1000 900 800
3350
1700
1660
1450
1365
1350
1225
1030
1005
930
860
830
775
CH3
OH
VERBENOL
— SYNTHET
--- FERMENT
3 4 5 6 7 8 9 10 11 12 13
WAVELENGTH (MICRONS)

FIG XXXV

were identical (Fig.XXXIII). Three of the major spots were identified as verbenone (25), cis-verbenol (24) and trans-sobrerol (26) by a comparative TLC with authentic samples. The polar fraction was subjected to chromatography over alumina. The major fraction eluting with 100% ether was purified by distillation. It analysed for $C_{10}H_{16}O$ and had a specific rotation of $[\alpha]_D$ -64. This compound was identified as (-)cis-verbenol (24) by a spectral comparison with +cis verbenol. (Fig.XXXV).

The minor fractions were not examined. The yields of verbenol from l-α-pinene was, however, lower than that reported by Prema & Bhattacharyya (1962a) for (+) cis verbenol from l α-pinene.

It is, therefore, a baffling phenomenon to find that both d and l α-pinene are metabolised through the same intermediates but with a mixture of d and l α-pinene only the accumulation of metabolites belonging to the (+) series such as +verbenol (24) is observed. Probably the rates of subsequent biotransformation of verbenol by oxidation may be different for the d(+) and (-) isomers. This postulate may account for the lower yields of (-)verbenol from l-α-pinene and the observed optical purity of the transformation products from a mixture of 70% d and 30% l α-pinene.

This explanation, however, remains to be supported by more rigorous studies on the rates of oxidation of the intermediate products on α-pinene fermentation.

CHAPTER VI

l-α-PINENE

EXPERIMENTAL

l-α-Pinene (23) was prepared by isomerisation of l-β-pinene (27) with gum resin for 18 to 24 hr at reflux temperature according to Austerweil (1926). The liquid b.p. 152°-154°/711 mm was collected and purified by fractionation through a spinning band column (1:20 reflux ratio) and the main fraction boiling at 152°-154°C was collected. It showed single peak in VPC (99.5%) $\alpha_D = -39.6^\circ$, $n_D = 1.4729$, $D = 0.8595$, the physical properties were identical with those reported in literature.

Fermentation:

In the case of α-pinene the fermentations were carried out in shake flasks essentially according to Prema & Bhattacharyya (Chapter II) and the extraction procedures were identical to those described earlier. In case of l-α-pinene the optimum concentration was 0.5% , time of incubation 8 hr at this concentration, temperature 27°-28°C. The substrate was added in two lots using 0.5% each time at 4 hr intervals and the fermentation stopped after 8 hr. The method of extraction was as before.

Separation:

The ether extract from forty shake flasks containing the neutral component on evaporation yielded 25 gm of product which was further separated into polar and nonpolar components by partitioning between petroleum ether and 90% aqueous methanol.

The nonpolar fraction (pet.ether) on evaporation gave a residue (21.2 gm) which was distilled (b.p. 152°-154°C) and identified as l-α-pinene by comparison of its spectral properties with an authentic sample. It did not show any appreciable change in the optical rotation $\alpha_D = -39^0$. The residue obtained after removal of pinene from the total nonpolar component on chromatography over grade I alumina (50 gm) and elution of the column with pet. ether yielded mostly mould lipid and fatty material.

The poler fraction (methanol layer) was concentrated to a small volume diluted with water and extracted with chloroform (50 ml x 3 times). The chloroform extracts were pooled together, dried and evaporated to get an oily residue (2 gm) which on preliminary TLC examination exhibited three spots having identical mobilities with authentic samples of (+)verbenone (25), (+)cis verbenol (24) and (+)trans sobrerol (26) (as obtained by fermentation of dl-α-pinene according to Prema & Bhattacharyya, 1962a) (Fig.XXXIII).

Identification of -cis verbenol (24):

The oily residue (2 gm) was chromatographed over grade II alumina (200 gm) and eluted with the solvent system consisting of petroleum ether and ether mixture. The main ether fraction (100%) obtained from the elution of the column on evaporation gave a residue (500 mg) which was purified by distillation b.p. 110°/15 mm. $[\alpha]_D = -64^0$. Anal. C 77.3%. H 10.15%. Calc. for $C_{10}H_{16}O$. C 77.99%. H 10.39%. IR ν_{max} 3350, 1700,

1660, 1460, 1450, 1395, 1330, 1250, 1225, 1145, 1080, 1030, 1005, 930, 900, 860, 830, 775, 730 cm^{-1}. The identity was established by its mixed VPC with an authentic sample of (+)*cis* verbenol (24). (Retention time 4 min 20 sec; temp. 120°C, column P, carrier gas H_2, flow rate 9 sec/10 ml). The IR spectra of both the samples were superimposable (Fig.XXXV).

CHAPTER XII
FERMENTATION OF L-α-PINENE

CHAPTER VII

β-PINENE

DISCUSSION

β-Pinene (27), $C_{10}H_{16}$, also known as nopinene, is found in most essential oils which contain α-pinene but in much smaller proportions. The (+) form is detected only in Ferula galbaniflua and in Cryptomeranthus mittallii. The structure of the compound was deduced from the corresponding saturated α-hydroxy acid which has been named nopinic acid (Rodd, 1953). The synthesis of β-pinene was achieved by Bennet et al (1933). The choice of β-pinene as the substrate was influenced by the fact that in the case of α-pinene the products obtained were mainly oriented in the autooxidation sites and it was, therefore, necessary to investigate if the mould would behave in the same manner towards β-pinene. It may be recalled in this connection that the pinene utilising Pseudomonad as described in the introductory Chapter (I) gave almost identical products from α and β-pinenes.

Fermentation:

In the case of β-pinene the fermentations were carried out in shake flasks. The conditions were essentially the same as those with α-pinene but the second addition of the hydrocarbon was omitted.

Transformation products (Fig.XXXIV):

The products obtained after fermentation were extracted and separated into acidic and neutral layers. The acidic fraction did not yield any major metabolite. The neutral fraction was partitioned between 90% aqueous methanol and light petroleum ether to yield both polar and nonpolar fractions. The nonpolar fraction after distillation yielded unreacted l-β-pinene and mould fat. The examination of the polar fraction from the methanol layer in TLC showed the presence of at least three components.

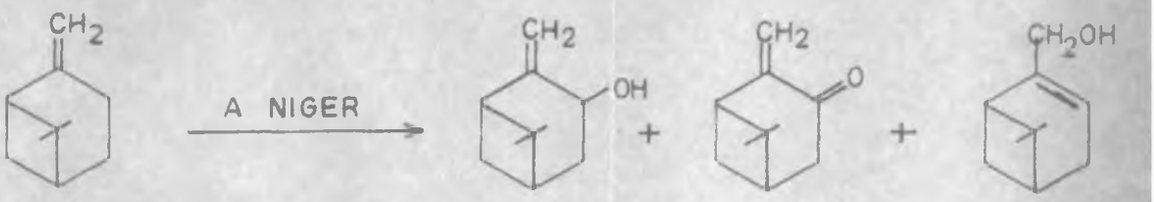
Identification:

Ketone (23): The oily residue was chromatographed over grade II alumina. The petroleum ether + 1% ether eluate from the column gave a sweet smelling compound which analysed for $C_{10}H_{14}O$. The IR spectrum of this compound (Fig.XXXVI) showed a band at 1695 cm^{-1} indicating the presence of an α,β unsaturated carbonyl function, a deduction which was corraborated by the UV spectrum (λ max 242mμ, ϵ =6240). The compound was identified as (-)pinocarvone (23) by a comparative and mixed VPC run with an authentic sample which was obtained by synthesis according to the method of Stallcup (1941).

Alcohol (29):

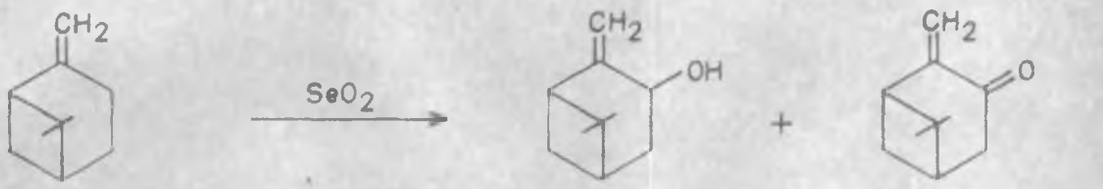
The ether eluate from the column gave a mixture of two alcohols which were suspected to be myrtenol (29) and pinocarveol (30) from their behaviour in the VPC when run along with synthtic samples. The alcohols were separated by preparation of acid

TRANSFORMATION OF l·β (−) PINENE BY A NIGER

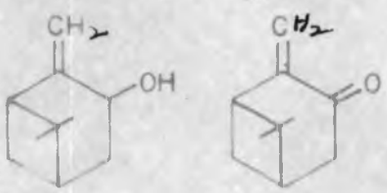


β - PINENE (27) | PINO-CARVEOL (30) | PINO-CARVONE (28) | MYRTENOL (29)

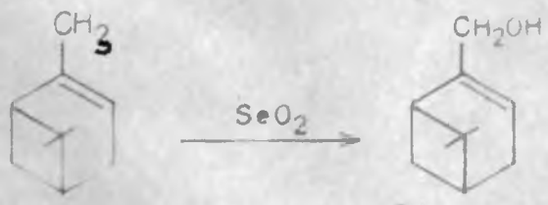
CHEMICAL SYNTHESIS OF PINO-CARVEOL, PINO-CARVONE, MYRTENOL



β - PINENE (27) | PINO-CARVEOL (30) | PINO-CARVONE (28)



PINO-CARVEOL | PINO-CARVONE



α - PINENE | MYRTENOL

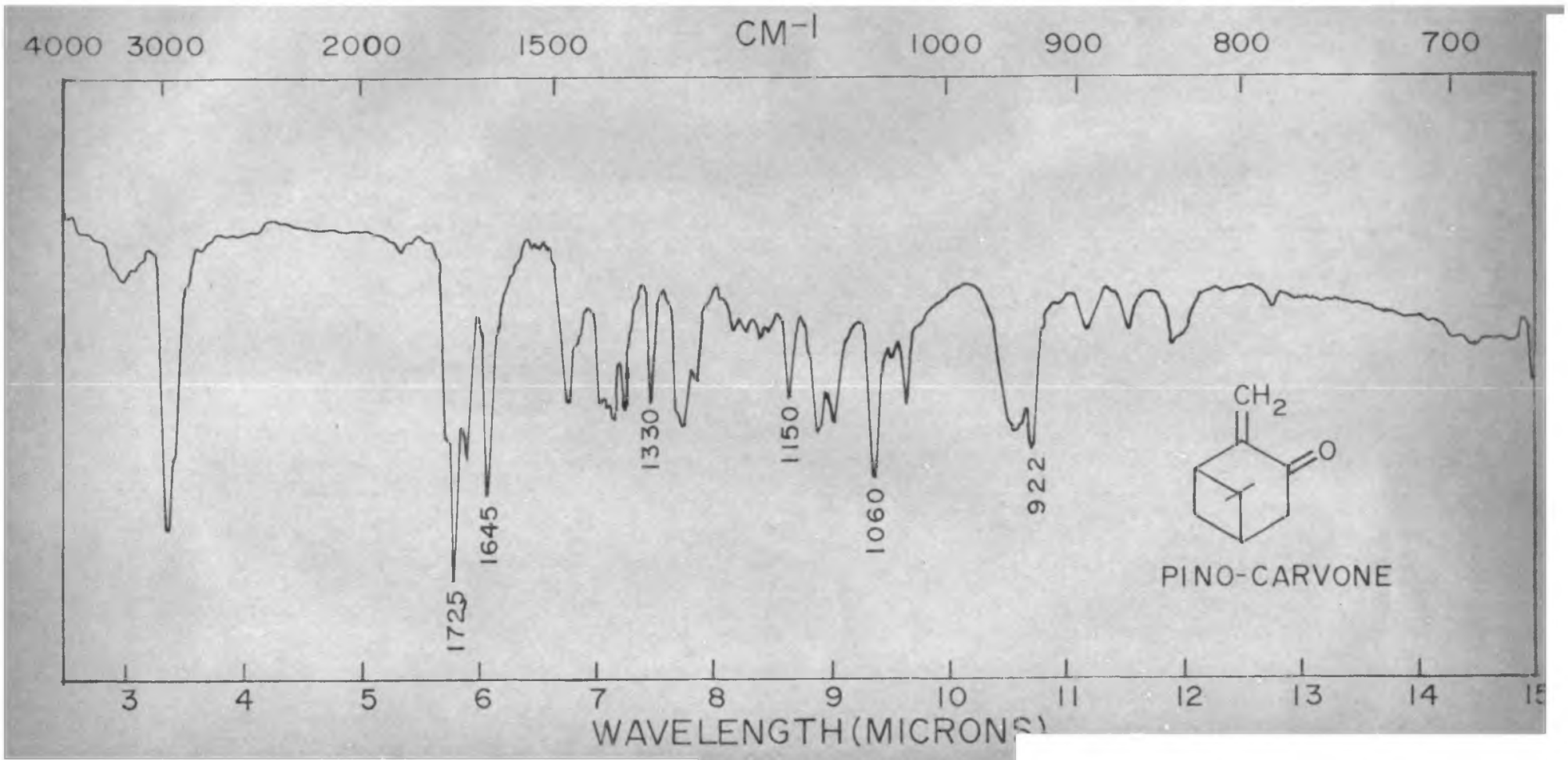

4000 3000 2000 1500 CM-1 1000 900 800 700
1725
1645
1330
1150
1060
922
CH2
O
PINO-CARVONE
3 4 5 6 7 8 9 10 11 12 13 14
WAVELENGTH (MICRONS)

FIG. – XXXVI

phthalate. Myrtenol (29) readily gave a crystalline acid phthalate m.p.118°-119°C (reported for myrtenol acid phthalate 120°). The acid phthalate was saponified to get the free alcohol which analysed for $C_{10}H_{16}O$, $[\alpha]_D$ = -46.45. The free alcohol showed a hydroxyl absorption in the IR spectrum (3340 cm^{-1}). The compound was identified as (-)myrtenol (29) by spectral comparison with an authentic sample prepared according to DuPont (1934) (Fig.XXXVIII).

Alcohol (30):

The residual fraction which was extracted out of the acid phthalate mixture by ether was recovered by evaporation of the solvent. The elementary analysis of this compound indicated a molecular formula of $C_{10}H_{16}O$. The IR spectrum showed a band (ν max 3450 cm^{-1}) characteristic of a hydroxyl group. The alcohol was identified as (-)pinocarveol (30) by a spectral comparison with an authentic sample obtained by synthesis according to Stallcup (1941) (Fig.XXXVII).

The formation of myrtenol, pinocarveol and pinocarvone once again exhibits the same two basic types of oxygenation, an allylic oxidation and oxygenation on a double bond. It is clear from the products formed that the mould differs from the Pseudomonas described earlier, since it metabolises α & β-pinenes by different pathways. Even in the case of β-pinene (27)the observed modes of attack are oriented towards the autooxidation sites. Myrtenol, pinocarveol and pinocarvone are known to be autooxidation products of β-pinene. In the substrate blanks the formation of pinocarvone could be detected by TLC. In this case the formation of sobrerol could not be demonstrated. But that the attack of the electrophilic oxygen is oriented towards the double bond is evidenced by the formation of myrtenol (29).

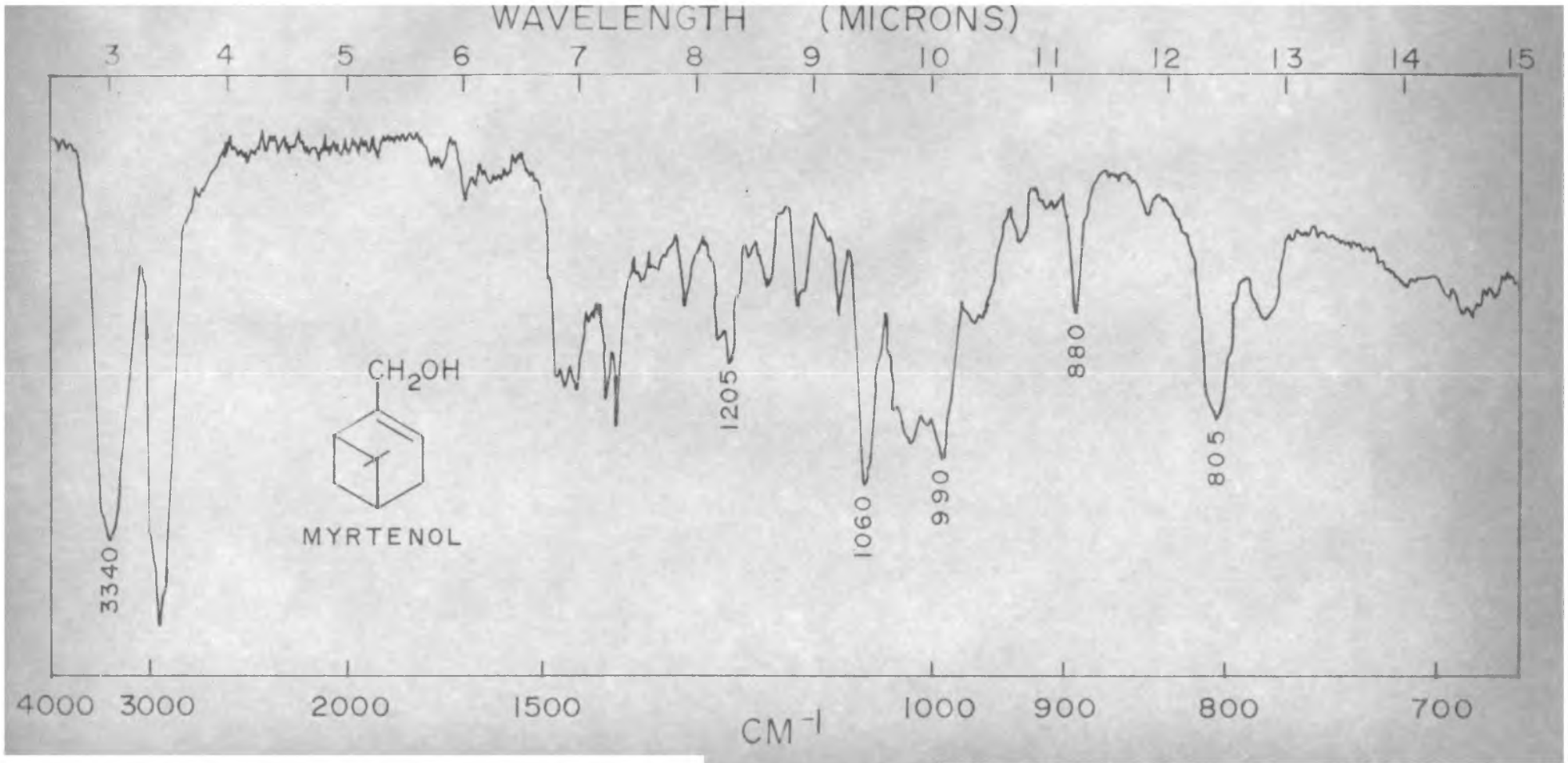

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
11
12
13
14
15
3340
CH2OH
MYRTENOL
1205
1060
990
880
805
4000
3000
2000
1500
1000
900
800
700
CM-1

FIG. XXXVIII

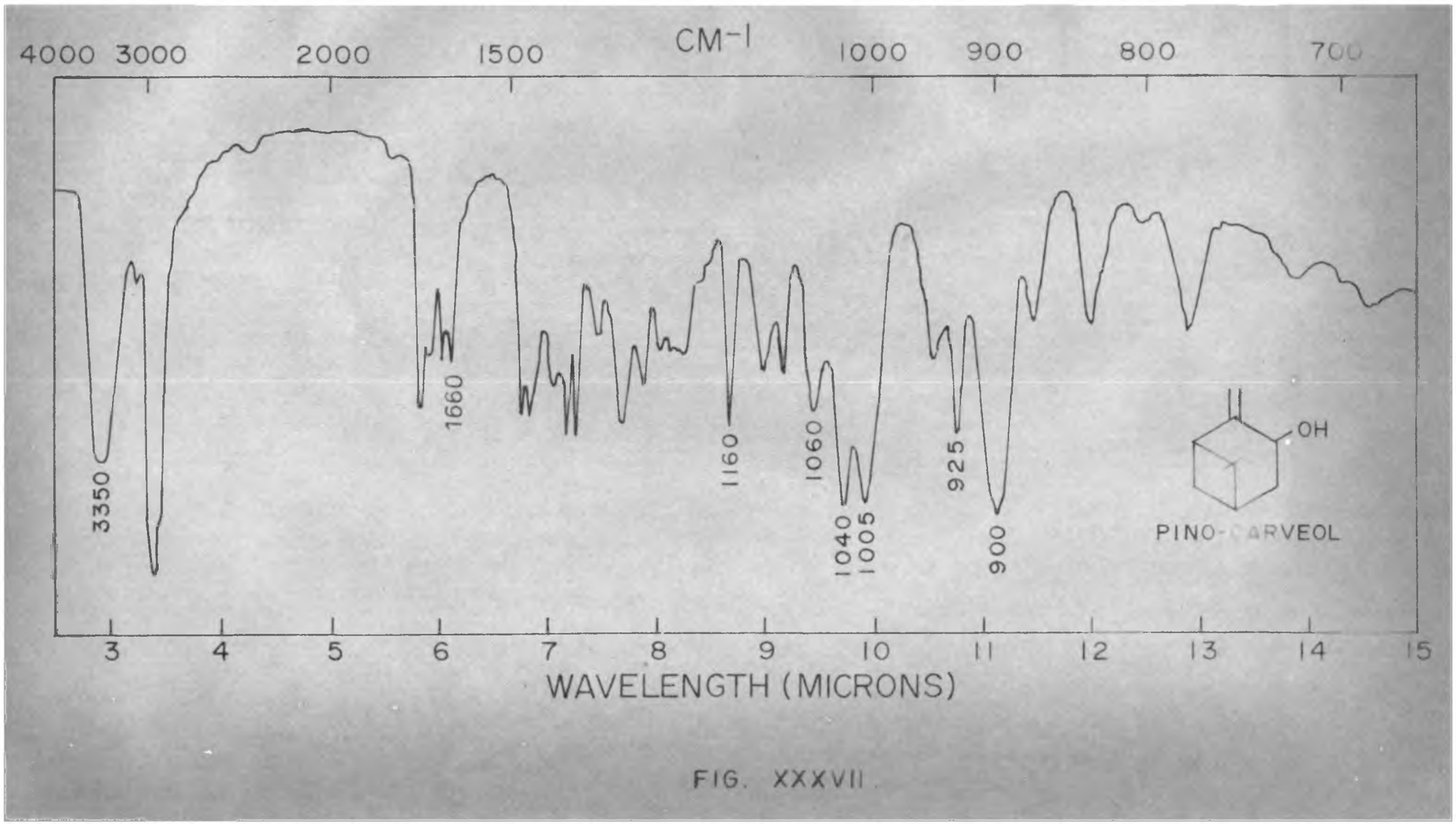

4000 3000 2000 1500 CM-1 1000 900 800 700
3350
1660
1160
1060
1040
1005
925
900
OH
PINO-CARVEOL
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XXXVII

hydrocarbon b.p. 164°-166° identified as l-β-pinene (27) from its spectral data. There was no significant change in the rotation $\alpha_D = -13.2°$. The residue (0.8 gm) was passed over grade I alumina (30 gm) and eluted with the solvent consisting of petroleum ether and ether mixtures. The products mainly obtained were either mould fat or lipid material.

The polar fraction obtained after evaporation of methanol layer was concentrated to a small volume and extracted with 50 ml of chloroform (3x25 ml). The chloroform layers were pooled together and evaporated to give an oily residue (1.8 gm) which was chromatographed over grade II alumina (180 gm) and eluted with the solvent system as described.

Identification of metabolite (Fig.XXXIV):

Entana (27): The petroleum ether + 1% ether eluates obtained from the column on evaporation gave an oily residue (200 mg) which showed a single spot on TLC. It was further purified by distillation b.p. 67°-69°/14 mm $[\alpha]_D = -68.5°$. Anal. C 79.65%, H 9.06%. Calcd. for $C_{10}H_{14}O$. C 79.95%, H 9.39%. IR ν max 1725, 1695, 1645, 1457, 1400, 1330, 1295, 1150, 1120, 1105, 1060, 1025, 945, 922, 885, 835 cm^{-1}. The spectrum (Fig.XXXVI) was identical with that of an authentic sample of pinocarvone obtained according to Stallcup (1941). UV λ max 242, ϵ = 5240. The identity of the compound was further confirmed by running comparative and mixed VPC with an authentic sample. (retention time 2 min 15 sec; temp. 120°, column P, carrier gas H_2, flow rate 9 sec/10 ml).

Alcohol (29):

The 100% ether eluate from the column gave on evaporation gave a residue (1 gm) which showed two distinct spots in TLC having very close R_f values. The compounds suspected to be present were (-)cis-pinocarveol (30) and myrtenol (29) according to TLC and VPC data. The presence of these compounds was confirmed by running the VPC with added myrtenol and pinocarveol with the metabolite. (myrtenol - retention time 4 min 35 sec; pinocarveol - retention time 2 min 30 sec; temp. 120°C, column P, carrier gas H_2, flow rate 9 sec/10 ml). The two components were separated by preparing the acid phthalate essentially according to Vogel (Practical Org. Chemistry, p.649). Myrtenol (29) formed acid phthalate while pinocarveol did not. Myrtenol acid phthalate was recrystallised from hexane m.p.118°C (reported m.p. of myrtenol acid phthalate 120°C) and did not show any depression in m.p. when mixed with an authentic sample of myrtenol acid phthalate (118°-119°C). The acid phthalate was hydrolysed with 5N alcoholic KOH and extracted in the usual manner with ether, to get the free alcohol (29) b.p. 103°-104°C/11 mm $[\alpha]_D$ = -46.45°. Anal. C 78.09%, H 10.49%. Calcd. for $C_{10}H_{16}O$. C 78.89%, H 10.59%. IR ν max 3340, 1660, 1460, 1380, 1372, 1261, 1205, 1160, 1130, 1090, 1060, 1020, 990, 960, 920, 890, 805, 775 cm^{-1}. The spectrum (Fig.XXXVIII) was identical with that of an authentic sample of myrtenol synthesised according to DuPont (1934) by the action of selenium dioxide on α-pinene.

Alcohol (30):

The residual mother liquor from the preparation of acid phthalate was now extracted with ether. The ether which on

evaporation gave a residue (0.3 gm) which showed a single spot in TLC. It was farther purified by distillation b.p. 70°-72°/14 mm. $[\alpha]_D$ = -72°. Anal. C 79.71%, H 10.32%. Calcd. for $C_{10}H_{16}O$. C 78.89%, H 10.59%. IR ν max 3350, 1670, 1650, 1200, 1160, 1110, 1095, 1060, 1040, 1005, 950, 925, 900, 835, 775 cm^{-1} (identical with that of an authentic sample of pinocarveol (Fig.XXXVII) prepared by the method of Stallcup (1941, 1942) by the action of selenium dioxide on β-pinene.

CHAPTER VIII

GENERAL DISCUSSION

CHAPTER VIII

GENERAL DISCUSSION

Even a cursory examination of the fungal transformations of model compounds and terpenes described in the earlier chapters will reveal that the primary and most predominant step in these biotransformations is the introduction of oxygen function in these hydrocarbon substrates. These oxygenation processes will be discussed here in the light of some previously proposed mechanisms of oxygen transfer reactions.

Unfortunately, even though a large amount of data is available on oxygenating enzymes today (Mason 1957, Hoyer et al 1963a, 1963B, Hayaishi 1964), very little is really known about the nature of active oxygen and the exact mechanism of the oxygenation reactions. In the present *A.niger* system the transformations could not be studied at enzymatic levels, since in preliminary trials cell-free extracts from the fungal pellets showed very little or no hydroxylating activity with these compounds. Even manometric studies with intact cells indicated that most of the hydrocarbon substrates tend to inhibit the rather persistent and large endogenous respiration of the mould and a long time is needed to overcome this inhibition. Two reasons can be put forward to explain this phenomenon. (A) - the endogenous energy source in the fungal cells because of their growth on energy-rich media prior to the addition of the hydrocarbons stored in the form of carbohydrates (starch or glycogen) is large enough to support

vigorous respiration of fungal cells and (B) - the hydroxylation reaction under the experimental conditions may be of minor significance in mould metabolism, yielding little useful energy to the organism (Hayaishi, 1963).

In the absence of any information on the exact nature of the enzymes involved in the fungal hydroxylation reaction, speculations will be centered on three broad topics:

A) The comparison of those hydroxylation reaction with other better-known hydroxylating systems.

B) The activation of oxygen and the nature of active oxygen.

C) The probable chemical events in fungal oxygenation processes.

The oxygenation enzymes:

Mason (1957) classified the enzymes directly or indirectly dealing with molecular oxygen into three main classes: (1) the oxygen transferases, (2) mixed function oxidases and (3) electron transferases. Of these, the first two are directly concerned with the introduction of oxygen in substrate molecule. Hayaishi (1962, 1963) has suggested that the names "dioxygenase" and "monooxygenase" for the first and second class of enzymes more effectively categorise their functions.

The "oxygen transferases" or "dioxygenases" catalyse the consumption of one mole of oxygen per mole of the substrate in such a manner that both the atoms of the consumed oxygen molecule appear in the product.

$$A + O_2 \xrightarrow{\text{Enzyme}} AO_2$$

alternatively, $2A + O_2 \longrightarrow 2AO$ (not observed)

Usually the dioxygenases are involved in the oxidative cleavage of aromatic rings, common examples being pyrocatechase, metapyrocatechase, 3-hydroxyanthranilic acid-oxidase, protocatechic acid oxidase, tryptophan pyrrolase, anthranilate hydroxyase, etc. (Boyer et al, 1963). The only enzyme of this class which is concerned with a direct oxygenation of aliphatic compounds, is lipoxidase. This enzyme is involved in the oxidative degradation of unsaturated fatty acids containing methylene interrupted cis-double bonds (Tappel 1963).

The "mixed-function" oxidases or "monooxygenases" consist of two different but interdependent catalytic activities, viz. (a) reduction of one atom of oxygen in the oxygen molecule coupled with (b) a specific oxygenation of the substrate with the other atom of oxygen. The overall transformations have been formulated by Mason (1957) in two alternative sets of equations depending on which one of the above activities precede the other.

Scheme I (Type II of Mason)

$$\text{Enzyme} + O_2 \longrightarrow \text{Enzyme}\sim O_2$$

$$\text{Enzyme}\sim O_2 + AH \longrightarrow AOH + \text{Enzyme}\sim O$$

$$\text{Enzyme}\sim O + BH_2 \longrightarrow \text{Enzyme} + B + H_2O$$

where AH is the substrate and BH_2 is the electron acceptor.

Scheme II (Type III, Mason)

$$\text{Enzyme} + O_2 \longrightarrow \text{Enzyme}\sim O_2$$

$$\text{Enzyme}\sim O_2 + BH_2 \longrightarrow \text{Enzyme}\sim O + H_2O + B$$

$$\text{Enzyme}\sim O + AH \longrightarrow \text{Enzyme} + AOH$$

A large number of enzymes such as the aromatic ring hydroxylating enzymes, squalene oxidocyclase, the phenolase complex, luciferase and probably the steroid hydroxylases belong to this class. The lactonising enzyme responsible for Bayer-Villager type ring lactonisation in camphor (as discussed in the introductory chapter of this thesis) is another interesting example of mixed function oxidation.

The characteristic features of the mixed function oxidation are: (a) the oxygen atom entering the substrate molecule is almost entirely derived from atmospheric oxygen, (b) the hydroxide ions from the medium do not take part to any significant extent in the reaction and (c) the presence of other electron donors such as reduced pyridine nucleotide reduced flavins, tetrahydrofolate and its analogues, ascorbic acid, etc. besides the substrates is an obligate requirement for functioning (Mason, 1957).

Hayano and coworkers (Hayano et al, 1955) were able to show by studies with H_2O^{18} and O_2^{18} that the hydroxyl group introduced by the adrenal steroid 11β-hydroxylase into steroid substrates is derived almost entirely from O_2^{18} and is not even exchangeable with the hydroxyl in the medium to any significant extent. Identical conclusions have also been drawn in studies with different aromatic hydroxylases (Mason et al, 1955) squalene oxidicyclase (Cohen & Block, 1956) and steroid 7α, 11α and 11β hydroxylases in moulds and rats (Hayano et al, 1956, 1953; Bergstrom et al 1958, Corey and Gregoriou, 1959).

The absolute stereospecificity of the oxygenation process in steroids has been established by the demonstration that the epimeric 7β and 11β hydrogens remain more or less intact during the hydroxylations at 7α and 11α positions (Bergstrom et al 1958).

The oxygenation processes observed in the current investigation bear certain obvious similarities with the steroid-hydroxylation by the mould: (a) the experimental conditions necessary are essentially identical excepting for substrate concentration and incubation period, (b) the oxygenations, excepting in the case of 1-methyl-3-cyclohexene-1-ol, are characterised by a high degree of stereospecificity and furthermore, (c) the experimental strain of *A. niger* (NCIM 612) has been shown to hydroxylate steroidal substrates such as progesterone (Prema & Bhattacharyya, 1962a) and 18-hydroxyprogesterone (Godse, 1962) almost exclusively at the 11α position. On the other hand, there is one difference observed between the transformation of terpenes and steroids regarding the sites of oxygenation in the substrate molecule. In terpenoids and the model compounds there is an overwhelming tendency for the oxygen molecule to appear at the autooxidation sites, which are obviously the most reactive ones. In contrast, the 11α position of the steroids is by no means a chemically reactive position. This point will be discussed further later on.

No information is available as yet as to whether the hydroxylating enzymes involved in the transformation of model compounds are adaptive or constitutive. However, in the case of steroids, indirect but convincing evidence is available about the adaptive

nature of the hydroxylating enzyme (Perlmann, 1956). The enzyme in Pseudomonad capable of oxidising camphor or the pinenes (Conrad et al, 1963) are also substrate-induced.

Types of oxygenation:

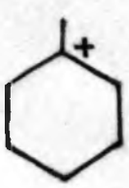
It has been mentioned earlier that mechanistically, the types of oxygenations observed in the present studies can be classified into three categories.

1) Hydration of double bond: examples, formation of 1-methyl-1-cyclohexanol from 1-methyl-1-cyclohexene and α-terpineol from limonene.

2) Oxygenation at allylic positions - which is observed with all the substrates.

3) Oxygenation on a double bond with rearrangement. In the present studies the type of reaction was observed in the formation of 3-cyclohexene-cis-1,2-diol (4), 2,8 p-menthadiene-ol-1 (20), sobrerol (26) and myrtenol (29).

Regarding the first type of hydroxylation, the hydration reaction, no elaborate comments need be made excepting for the fact that these were observed on double bonds which are heavily substituted and are, therefore, more susceptible to protonation. The hydroxyl group was very probably derived from the hydroxyl of the medium as a result of neutralisation of carbonium ions (31) and (32).



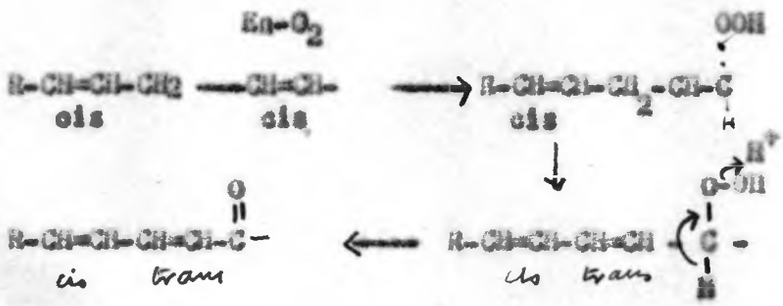
31

32

The other two types of reactions are true oxygenation processes having some of the characteristics of mixed function oxidation.

The nature of active oxygen:

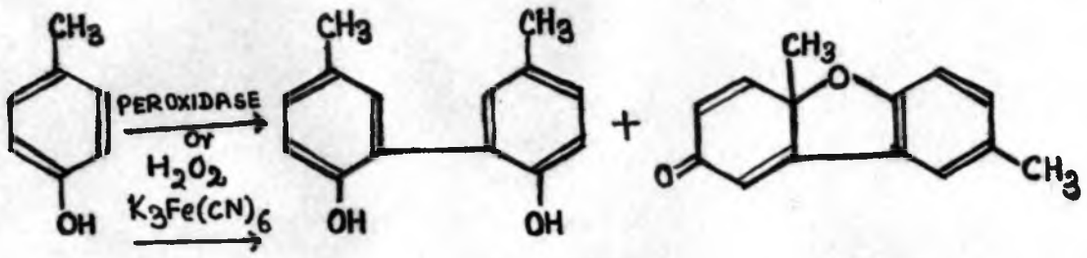
It is still not certain whether the reactive species of oxygen in most of the dioxygenases as well as the monooxygenases is an ion or a free radical. In some cases, however, from the nature of the reaction products a diagnosis is possible. The enzyme, lipoxidase, which utilises molecular oxygen to break down unsaturated fatty acids seem to operate through free radical intermediates (Fridovich and Handler, 1960). One of the most plausible mechanisms proposed for lipoxidase action (Holman, 1947) is that the primary step consists in a single electron reduction of molecular oxygen abstracting an electron from one of the double bonds. The new radical (33) can eject a hydrogen to form the conjugated hydroperoxide (34) which undergoes hydrolysis to the ketone.

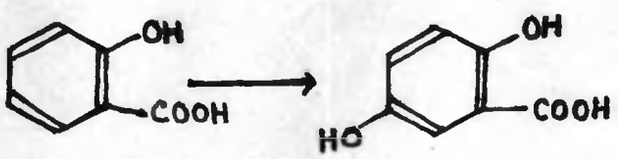


Lipoxidase also represents one of the few cases where no metal appears to be involved in the process of activation of

oxygen. In this case the carbonyl compound is obtained as an intermediate rather than the alcohol - a fact consistent with the involvement of a hydroperoxidic intermediate.

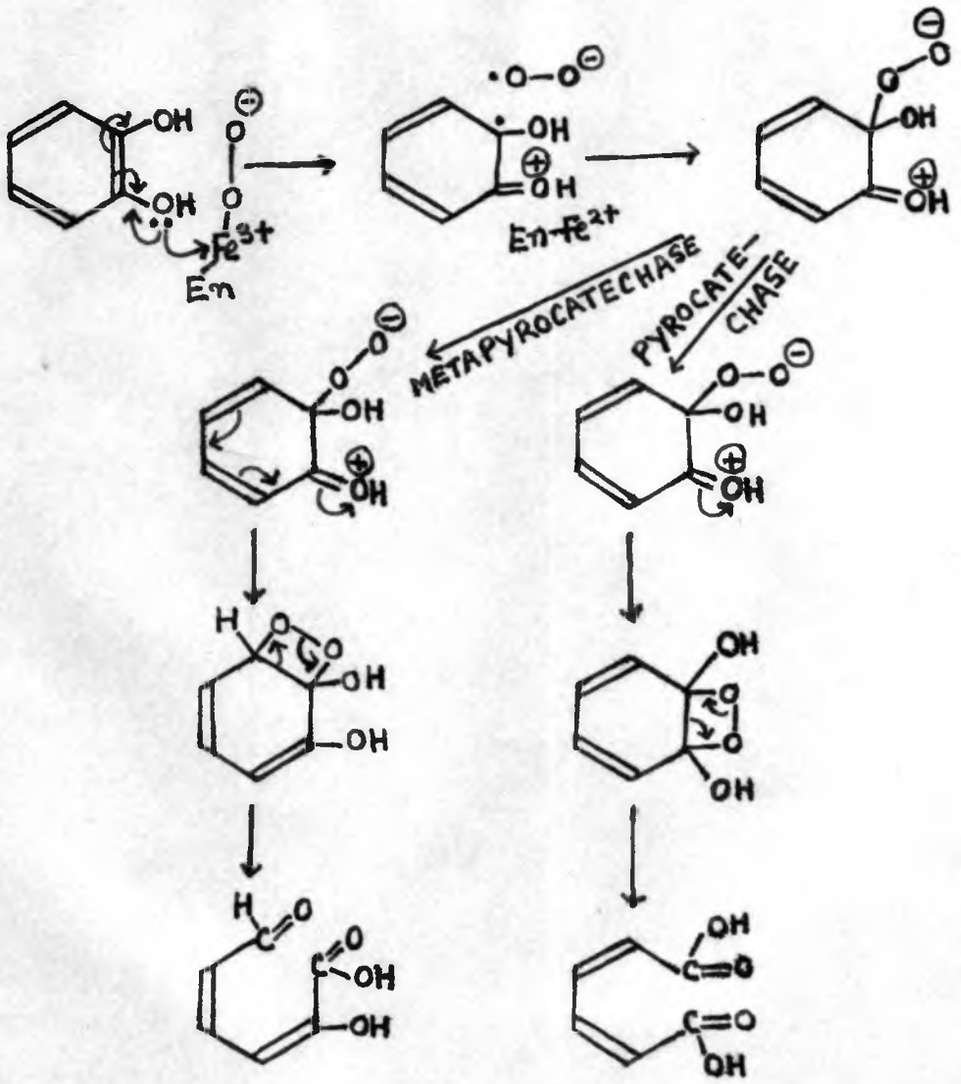
Some enzymes closely related to the monooxidases - peroxidase oxidases - which hydroxylate and oxidise aromatic compounds using hydrogen peroxide as the electron accepter also involve single electron transfers giving rise to free radicals as intermediates (Chance, 1954). The products formed in these enzymic actions are often identical with those obtained by ferricyanide oxidation (Thyagarajan, 1958) a process known to involve single electron transfer.





Hayaishi (1964) developed a mechanism for dioxygenation in pyrocatechase and metapyrocatechase which catalyse the oxidative cleavage (35) to cis-cis muconic acid (36) and to respective hydroxy-cis-cis muconic semialdehyde (37) by incorporating both the atoms of molecular oxygen into the products. These enzymes contain iron as prosthetic groups, presumably bound with the sulphydryl groups of the proteins. In the sequence proposed, the iron in the ferrous state combines with oxygen to form

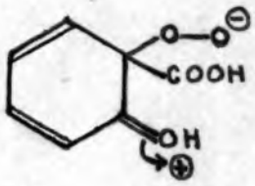
En-Fe^{++} — O_2 complex which can exist in mesomeric form such as En-Fe^{+++}— O_2^-. The iron in the ferric state in this species complexes with the phenolic hydroxyl group to form a ternary complex. An electron from the lone pair of the phenolic oxygen is transferred to reduce the ferric iron. The anionic oxygen radical is simultaneously released from the complex attacking the α-carbon of catechol to form a peroxidic intermediate. The enzyme protein now directs the flow of electrons to result in an 1,6 cyclic intermediate in metapyrocatechase and 1,2 cyclic peroxide in catechase which cleave to hydroxy maconic semialdehyde and muconic acid respectively.



In support of this mechanism the formation of the ferric state immediately after the addition of substrate has been demonstrated in the ESR spectra which showed a signal for Fe^{3+} at a g value of 4.2.

Hayaishi has used this mechanism to explain other enzymes bringing about dioxygenation of aromatic ring such as D-tryptophan pyrrolase degrading D-tryptophan to D-formyl kynurenine and anthranilate hydroxylase (from a Pseudomonad) which converts anthranilic acid to catechol.

The mechanism also can explain certain monooxygenations of the mixed function type. In the case of salicylate hydroxylase which converts salicylic acid (38) to catechol (35) in presence of $FADH_2$. Hayaishi postulates the reduction of the hydroperoxide intermediate with the suitable electron donor such as $FADH_2$. A general mechanism was also proposed by



Hayaishi in which the initial step of activation is brought about first by divalent iron which forms a ternary complex of oxygen and the substrates.

In all these schemes the active form of oxygen is essentially the anion radical, $\cdot O\text{-}O^{-}$.

Hayaishi's mechanism cannot, unfortunately, be extended to explain all the mixed function hydroxylations without

extensive alteration or amendment. In a whole host of monooxygenase reactions the products indicate that the reactive oxygen is highly electrophilic in nature and is directed towards the electronegative sites of the molecule. The nonspecific hydroxylation brought about about by the enzymes from liver microsomes (Mitoma et al, 1956) and the squalene oxidocyclase reaction (Tchen & Block 1956) which cyclises squalene to lanosterol in a concerted manner are examples of this type. Model experiments on free radical addition to squalene by Breslow et al (1962) showed that very little cyclisation of squalene was observed. It is, therefore, pertinent to examine some of the forms of electrophilic oxygen postulated by other investigators.

Cationic hydroxyl, OH^+:

The cationic hydroxyl has been implicated in many organic reactions. Although OH^+ is known to exist in the mass spectrograph, Coulson has poisted out from theoretical grounds that even if formed, OH^+ would be extremely unstable in aqueous systems(Coulson, 1956). However, now it is recognised that under certain conditions highly electrophilic OH radical may also be produced and can function as a cationic species (Norman and Radda, 1962).

Periferryl and ferryl cations: Enzyme Fe^{++}-O_2 & Enzyme-Fe^{++}-O.

The obligatory involvement of heavy metals such as iron in all the enzymatic monooxygenation reactions is still a subject of controversy. In some cases such as lactive oxidative decarboxylase (Sutton, 1957) and salicylate hydroxylase (Hayaishi, 1964)

the evidence regarding the presence of heavy metals is not clearcut. But a vast majority of these enzymes contain iron and in some cases copper as integral components. Based on the behaviour of iron in autooxidation Mason (1957) proposed the hypothesis that electrophilic species such as En-Fe-O_2^{++} and Enzyme Fe^{++} O may function as the electrophilic hydroxylating species. Evidence for such higher valence states for iron has been provided by George (1953). The primary product of combination of enzyme-bound iron with molecular oxygen is the perferryl cation, En Fe^{++}-O_2 which has also been implicated in Hayaishi's scheme discussed earlier. The ferryl-cation, the tetravalent oxygenated iron species, En-Fe^{++}-O, is derived by the following sequence of reactions:

$$En - Fe^{++} \; O_2 \longrightarrow En - Fe^{++} - O_2$$

$$En\text{-}Fe^{++} \; O_2 + DH_2 \longrightarrow Enzyme - Fe^{++} - O$$

According to Mason both these cations can mediate the oxygenation reaction at electronegative centres - depending on which of the activities reduction or hydroxylation precede the other (Type II and Type III mechanism).

<u>The probable mechanisms for the hydroxylation of hydroaromatic models and terpenes:</u>

It will be now worthwhile to speculate on what type of activated oxygen among the types described above, may possibly be involved in the hydroxylations observed in the present work. In the absence of any relevant data let it be assumed, although rather arbitrarily, that the same or very similar activation

processes of molecular oxygen precede both the types of oxygenation reactions viz. allylic oxidation and oxidation on a double bond.

Autooxidation vs fungal transformations:

It was observed all throughout this work as well as in some previous results (Prema & Bhattacharyya, 1962) that as far as the simple substrate molecules are concerned there is an overwhelming tendency for the oxygen molecule to enter the sites for autooxidation. In fact the control experiments run with aeration and agitation with the substrate but without the moulds gave some products which were identical with the fungal metabolites. Cyclohexene in these experiments gave traces of 2-cyclohexene-1-one and 2-cyclohexene-ol-1 but not the diol. Both the methyl cyclohexenes gave the corresponding alcohol and ketone (the formation of hydration product was not observed). Limonene, α-pinene and β-pinene behaved in a similar manner yielding carvone and carveol, verbenone, myrtenol, pinocarveol respectively. The main differences between the fungal transformation and autooxidation are three: (a) the quantities of fungal transformations product were much higher, ranging from fifty to thousand-fold of those obtained by autooxidation under identical conditions, (b) the proportions of alcohols to ketones are much higher in the fungal experiments and (c) the fungal oxygenations were stereospecific (excepting in the case of 1-methyl-3-cyclohexane-ol-2).

Of all the known oxygenases the enzyme, lipoxidase, oxygenates unsaturated fatty acids in a manner closely reminiscent

of that of autooxidation. It is possible to postulate a lipoxidase type of mechanism for these reactions involving the hydroperoxide radical.

$$R\text{-}\overset{|}{C}H_2 + O - O \longrightarrow R\text{-}\overset{|}{C}H\cdot + {}^{\cdot}OOH$$

$$R\text{-}\overset{|}{C}H_2 + {}^{\cdot}OOH \longrightarrow R\text{-}\overset{|}{C}H\cdot + HO\text{-}OH$$

$$HO\text{-}OH \rightleftharpoons 2OH\cdot$$

$$R\overset{|}{C}H\cdot + {}^{\cdot}OH \longrightarrow R\text{-}\overset{|}{C}H\text{-}OH$$

$$R\overset{|}{C}H + {}^{\cdot}OOH \longrightarrow R\text{-}CH\text{-}OOH$$

$$R\overset{|}{C}H\cdot OOH + H^+ \longrightarrow R\text{-}\overset{|}{C}{=}O + H_3^+O$$

It is possible also to implicate a mixed mechanism related to the above incorporating a metal which can withdraw from or donate an electron to a free radical to convert it to a cation or anion:

$$RH + O_2 \rightarrow R^{\cdot} + {}^{\cdot}OOH$$

$${}^{\cdot}OOH + e \rightarrow OOH^-$$

$$R^{\cdot} \longrightarrow R^+ + e$$

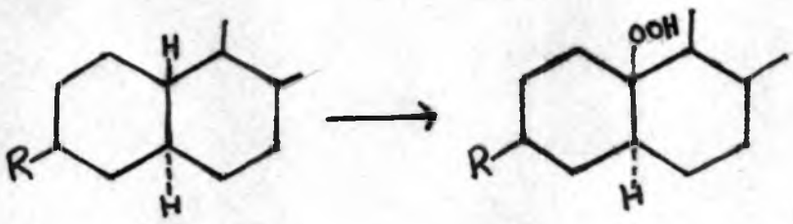
$$R^+ + H_2O \rightarrow ROH + H^+$$

$$OOH^- + H^+ \rightarrow H_2O_2$$

$$R^+ + H_2O_2 \longrightarrow ROOH + H^+$$

To account for the stereospecificity one has only to involve the three-dimensional structure of the protein which orients the substrate molecule in such a way that the oxidation site of the substrate can receive the active oxygen in a fixed geometry. As

a model system of such stereospecific oxygen transfer has been observed in the autooxidation of 19-nor-steroids where exclusively the 10β-hydroperoxide was obtained (Shapiro et al, 1964).

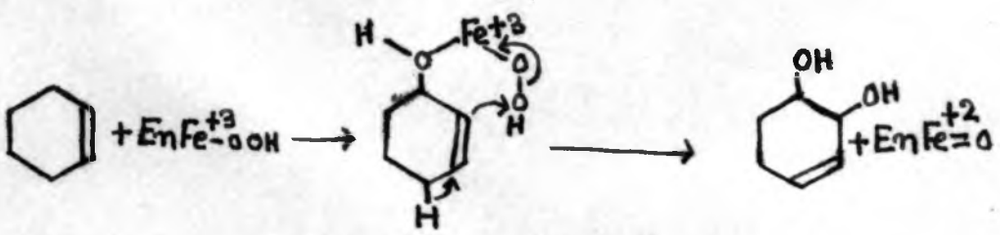


There are however two objections to this mechanism. (1) At least in the case of steroid hydroxylations analogous to terpene oxidation it is known that free hydrogen peroxide does not take part (Grant, 1956; Grant & Brownie 1958) and (2) with the lipoxidase type of mechanism one would expect the preponderance of carbonyl compounds over alcohols in the products. It is very probable though that the autooxidations observed in controls were caused by a mechanism involving hydroperoxides.

Ionic mechanisms:

The anion radical mechanism advanced by Hayaishi is not in itself compatible with the observed hydroxylations, since from the product pattern the direct involvement of an electrophilic oxygen is indicated. However, with some modification the Hayaishi scheme can be implicated in the second hydroxylation of cyclohexenol in the following manner.

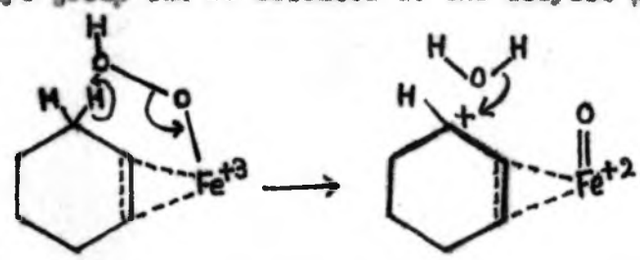
$$En\text{-}Fe^{+3}\text{-}O\text{-}O^{-} + H^{+} \longrightarrow EnFe^{+3} - O\text{-}OH$$



$$En\text{-}Fe^{+2}{=}O + AH_2 \longrightarrow En\text{-}Fe^{+2} + A + H_2O$$

It should be noted that just like the vanadium pentoxide hydroxylations this mechanism also involves a cyclic six-membered transition state. The driving force for the reaction is the withdrawl of the outer valence electrons inside the shell in these transition metals in the highly oxidised state.

To explain allylic hydroxylation it may be visualised that a ligand (though weak in aqueous systems) to hold complexed iron may be provided by the electrons from the double bond and then the hydroxyl group can be oriented at the allylic position.



The incipient electrophilic hydroxyl can abstract a hydride from the allylic position to leave a carbonium ion which may pick up a hydroxyl.

However, with the above formulation two problems arise. (1) The free carbonium ion is expected to have a great tendency to racemise and hence it is difficult to explain the observed stereospecificity. (2) It is difficult to see why the hydroxide ions in the medium would not take part in the neutralisation process (assuming that the analogy with steroid hydroxylation holds true in this case).

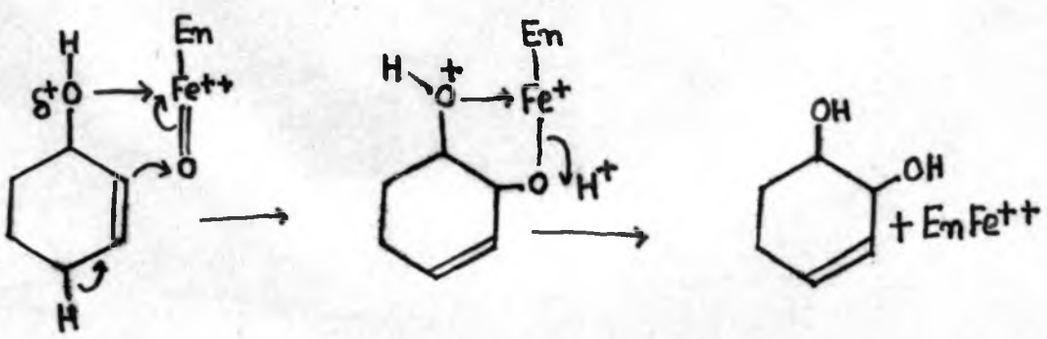
These difficulties are not insurmountable and a probable solution which may also be applicable to this system will be discussed in the next section.

It is to be noted that the oxygenation system En $Fe^{3+}-O^{-}$ proposed by Hayaishi is a mesomeric form of the peroxyl cation postulated by Mason and the above formulations are essentially based on the Type II mechanism. It now remains to be seen if the Type III mechanism involving a partially reduced ferryl cation En $Fe^{++}-O$ can fit in any better with the data obtained in the present work. In this case due to the tendency of the higher oxidised iron to withdraw electrons into the inner shell it is possible to postulate the following mesomeric structures.

$$En - Fe^{++} = O \leftrightarrow En-Fe^{+} -O^{+}$$

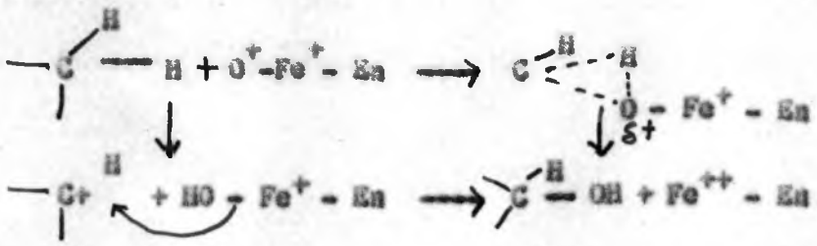
It may be noted that this formulation is somewhat contradictory to the observed polarisation of carbonyl compounds where oxygen has a greater capacity of stabilising a negative charge. It may be argued that the tendency of electrons to drift towards the oxidised iron may be favoured than the enolisation process in this ferryl cation.

The species $En-Fe^{+}-O^{+}$ has the advantage of having a highly electron deficient oxygen and is expected to react with electronegative sites. Furthermore, one can formulate a mechanism for the second hydroxylation of cyclohexenol involving one or the other mesomeric forms in a five-membered transition state in the following manner.



As the first step in the coordinated intermediate there is an electron drag towards the inner shell of iron, the driving force for the reaction, and the deficiency is satisfied by the withdrawal of electrons from the double bond eventually resulting in the allylic shift. In the complex (40) the iron is divalent and the Fe^{+}-O bond can ionise with the mediation of a proton.

In the hydroxylation of a saturated carbon the species $En-Fe^{+}-O^{+}$ can participate according to either one of the two possible mechanisms. In the first mechanism the electrophilic oxygen can form single electron bonds simultaneously with both the carbon and hydrogen to form the transition state.



The C-O bond may strengthen at the expense of C-H bond resulting in the insertion of the oxygen atom between carbon and hydrogen. In the second mechanism the electrophilic oxygen extracts a hydride from the substrate and instantaneously ionizes to yield the free $En-Fe^{++}$ and hydroxide ion. The resulting carbonium ion reacts preferentially and

selectively with the same hydroxide ion released from the complex before it has a chance to collapse. Because of the proximity, a situation which is dictated by the enzyme protein, the probability of collision of this hydroxide ion with the carbonium-ion site is expected to be overwhelmingly greater than that of any other hydroxyl from the medium. This argument can be justified from the observed trends in intramolecular catalysis. For instance, the hydrolysis of aspirin is dependent of hydrogen ion concentration over a wide range of pH (4-8) and is predominantly due to an intramolecular attack by the vicinal carboxylate ion (Bender 1962).

The participation of such "hot" carbonium ions has been postulated to explain the observed stereospecificity in the action of nitrous acid on primary amines (Corey et al 1963). Further, the data obtained by Ryano et al (1956) in the steroid hydroxylation indicate that although the hydroxyl oxygen in these cases may be largely derived from atmosphere, the incorporation is not quite quantitative. There might have been some small incorporation of the hydroxide from the medium and this finding is not inconsistent with the carbonium ion mechanism proposed above. Further, the racemisation observed in the present work in 1-methyl-3-cyclohexene-ol-2 may be ascribed to the rapid stabilization of the "hot" carbonium ion into a cold planar (41) one, by an extension of resonance due to the inductive effect (42) from the 1-methyl group before the combination with the hydroxyl

ation (Corey et al 1963). It is also pertinent in this connection to mention that tetralin gives rise to racemic α-tetralol in the same *A.niger* system (Ramachandani & Bhattacharyya, unpublished data).

41 42

The above considerations would, therefore, indicate that out of all the species of active forms of oxygen described, Mason's ferryl cation, $En\text{-}Fe^{++}O$ seems to fit in better with the observed hydroxylation processes.

It should also be mentioned in this connection that Ingraham (1961) proposed another type of mesomeric structure for the perferryl cation species involving a polarisation of electrons as in $En\text{-}Fe^{4+}\text{-}O_2^{=}$. Objections can be raised to this structure since it does not take into account the electron drag from the valence shells towards the inner shells, a process which provides energy for driving the hydroxylation reaction. However, the formulation $En\text{-}Fe^{3+}\text{-}O\text{-}O^{\cdot}$ by Hayaishi is justifiable because of the resonance of the following type,

$$E\text{-}Fe^{++}=O^{\cdot}\text{-}O^{-} \longleftrightarrow E\text{-}Fe^{+++}\text{-}O\text{-}O^{\cdot}$$

Caution must, however, be exercised before accepting any conclusions made about the nature of the reactive oxygen in the hydroxylation of terpenes and model compounds since in spite

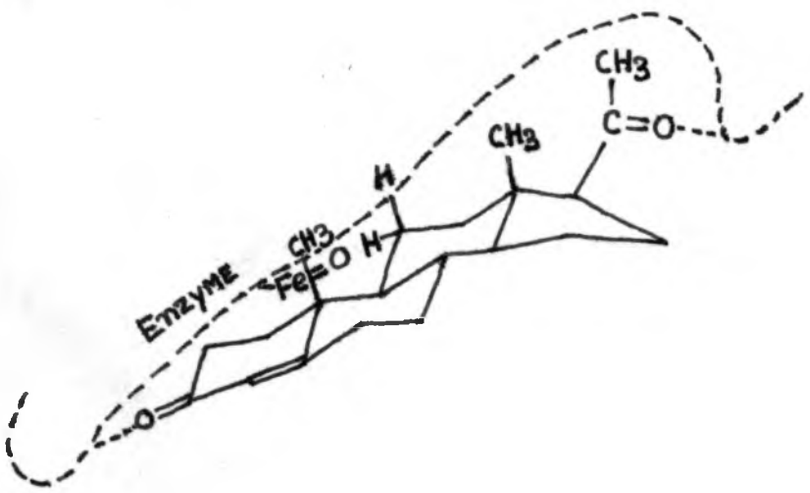
of a large amount of experimental data available of the types of hydroxylation, no rigorous proof can be given for any of the formulations. One really does not know whether the terpene hydroxylation and steroid hydroxylations are mediated by the same or different mechanism.

These investigations were undertaken with the hope that they might yield a better understanding of the fungal hydroxylations in general. Although some definitive trends have been observed, the development of a general theory to explain the biological hydroxylations of the mixed function type remains beyond the investigator's reach. Speculations can be justified only upto the point where after making some assumptions one does not need a fresh set of assumptions to support the previous ones.

Further the present studies raise more problems than they solve. For instance some disturbing question may be raised. How many enzymes are involved in these transformations? Does the mould synthesize a new enzyme for each substrate? Do the same enzymes bring about allylic hydroxylation and oxygenation on a double bond?

From the standpoint of economy of the cells, it is difficult to visualise the formation of large number of enzymes, even though adaptive in nature, in this organism. On the other hand, it is difficult to explain stereospecific hydroxylations of a whole host of substrates ranging from cyclohexane on

one hand and steroids on the other by a single enzyme. As a rather awkward teleological explanation based on a single-enzyme hypothesis, it may be assumed that the smaller substrates such as cyclohexene are only directed towards the catalytic site without a firm binding on the protein surface in a certain geometry so that the most reactive site can receive the oxygen. The tertiary and quaternary structure of the protein prevents any other approach of the substrate molecule but one. A certain degree of freedom is still possible in these molecules to orient themselves in order to present the autoxidation site to the active oxygen. In the case of steroids on the other hand, the substrate is firmly bound to the protein, as idealised in the expression below (43) with the 11α position in close proximity to the active oxygen site:



CHAPTER IX

SUMMARY AND CONCLUSIONS

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The present studies were undertaken in order to investigate the mode of degradation of model compounds such as cyclohexene, 1-methyl-1-cyclohexene, 1-methyl-3-cyclohexene and some terpenoid hydrocarbons such as (+) limonene, l-α-pinene and β-pinene by a strain of *Aspergillus niger* (NCIM 612). This strain was used as the test organism since earlier work indicated that it could metabolise various terpenoid substrates with widely different structures.

Bench scale fermentations using both Chain type fermentors and shake flasks were undertaken in order to isolate and characterise the transformation products. The ether extracts of the fermented broth and mycelial pellets were separated into acidic and neutral components. The acidic fraction did not yield appreciable amounts of metabolites in any of the fermentations. The product formation was monitored by thin layer chromatography and vapour phase chromatography.

Cyclohexene:

The neutral polar metabolites from cyclohexene on chromatography yielded a faster moving component identified as cyclohexene by its physico-chemical properties and

comparative IR spectroscopy with an authentic sample. The slower moving fractions were separated by preparative thin layer chromatography and identified as (+) cyclohexene-ol-1 and (+) 3-cyclohexene cis 1:2 diol by its physico-chemical characterisation and comparative IR spectroscopy with an authentic sample. It was found that the action of vanadium pentoxide and hydrogen peroxide on 2-cyclohexene-ol-1 in a model reaction also yielded the 3-cyclohexene-1,2 cis-diol.

1-Methyl-1-cyclohexene:

The neutral ether layer from 1-methyl-1-cyclohexene fermentation yielded on chromatography three components which were identified as 1-methyl-1-cyclohexene-6-one, (+) 1-methyl-1-cyclohexene-6-ol and 1-methyl-1-cyclohexanol from their physico-chemical characteristics and comparative IR spectroscopy with authentic samples.

1-Methyl-3-cyclohexene:

From the fermentation of 1-methyl-3-cyclohexene two compounds could be identified after chromatography of the neutral ether layer, a ketone, 1-methyl-3-cyclohexene-2-one, and an alcohol, 1-methyl-3-cyclohexene-2-ol from their physico-chemical properties and comparative IR spectroscopy with authentic samples.

Limonene:

Limonene on incubation with A. niger gave four compounds, which were identified after purification by column chromatography

as (+) carvone, (+) carveol, + α-terpineol and (+) p-mentha-diene 2:8-ol-1 by physico-chemical methods and by mixed and comparative vapour phase chromatography with authentic samples.

l-α-Pinene:

This hydrocarbon on incubation with A.niger was transformed mainly into 3 major components. The compounds were identified as cis-verbenol, verbenone and trans-sobrerol by comparative thin layer chromatography. The verbenol was isolated and characterised as (-) cis-verbenol.

l-β-Pinene:

l-β-Pinene on incubation with A.niger gives rise to mainly 3 products which were separated and identified as (-) pinocarvone, (-) pinocarveol and (-) myrtenol by physico-chemical methods.

The above data have been discussed in the light of some known oxygenation mechanisms involved in biological hydroxylations in the concluding chapter.

CHAPTER X.

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