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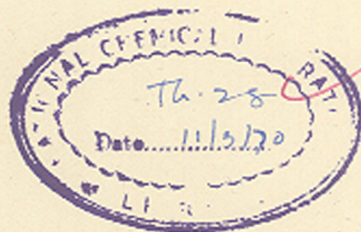
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MICROBIOLOGICAL TRANSFORMATIONS

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by
R. L. HUNTER, M.Sc.

Division of Biochemistry
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FOOD 5
JANUARY 1941

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MICROBIOLOGICAL TRANSFORMATIONS

**OF
TERPENES**

The author is greatly indebted to Dr. P. E. Chatterjee for his valuable guidance throughout this investigation and to Dr. P. N. Bhargava for his cheerful encouragement and helpful suggestions during the later part of the work.

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

The author is indebted to the staff of the spectroscopic and microanalytical sections of this Laboratory for their co-operation.

Finally he wishes to thank the National Chemical Laboratory for permitting him to work in the form of thesis and Council of Higher Education, India for the award of a Junior Research Fellowship during the course of this work.

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The following abbreviations have been used.

ABBREVIATIONS

TLC	:	Thin layer chromatography
PLC	:	Preparative layer chromatography
VPC	:	Vapour phase chromatography
I.R.	:	Infrared
NMR	:	Nuclear magnetic resonance
U.V.	:	Ultraviolet
ATP	:	Adenosinetriphosphate
CMP	:	Chloramphenicol
CoA	:	Coenzyme A
FAD	:	Flavin adenine dinucleotide
LAH	:	Lithium aluminium hydride

CHAPTER 1

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

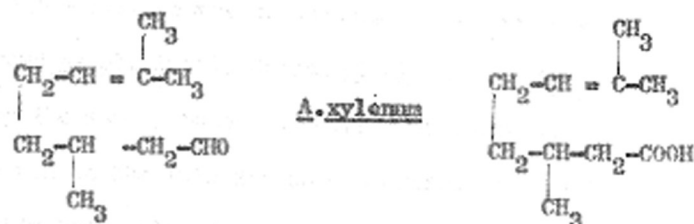
INTRODUCTION

The biological degradation of natural compounds constitutes an important step in the carbon cycle of nature. A considerable amount of information is available today on the biotransformation of compounds such as steroids, alkaloids, aliphatic and aromatic compounds including hydrocarbons, carbohydrates and antibiotics (Tamm, 1962; Pasqualini, 1963, Evans, 1963; Fuhs, 1961; Dagley et al. 1960; Foster, 1962). The aim of these studies is varied ranging from the production of useful compounds such as vitamins, amino acids, antibiotics, etc. through the agency of microorganisms.

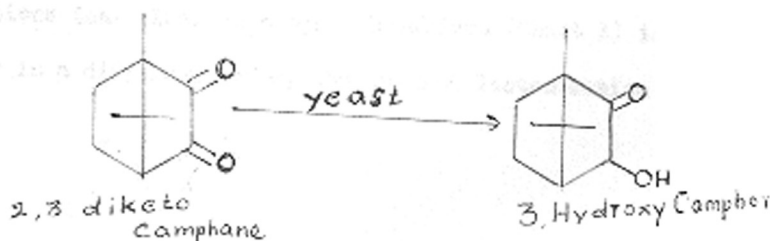
Though investigations on the microbiological transformation of paraffins appeared as early as 1895 (Myoshi, 1895), a considerable interest in this field has been developed with the realization that the hydrocarbon substrates could be among the cheapest potential sources of proteins, amino acids, fatty acids and other useful products (Zobell, 1946, 1950; Johnson, 1964; Miller et al. 1964; Yamada, et al. 1963).

MICROBIOLOGICAL TRANSFORMATION OF TERPENOIDS

A review of literature would indicate that although extensive investigations on the microbiological transformation of steroids have been carried out, relatively little work has been done on microbiological transformation of terpenoids. The chemistry of terpenes has undergone considerable development since they are of great value to the perfumery industry. Though a systematic investigation of the biotransformation of terpenes started from 1959, the work on this line dates back to 1915. The first report on the microbiological transformation of terpenes is by Mayer and Neuberg (1915) who obtained citronellol in 59% yield by reduction of citranellal using bottom yeast. Neuberg and Kerb (1918) later reported the conversion of citral to geraniol in about 30% yield using yeast. Malineri (1929) prepared citronellie acid from citranellal using Acetobacter xylosum.



Neuberg and Pierer (1949), while studying the phytochemical reduction of terpenoid compounds with Baker's yeast, observed that 2,3 diketocamphane (camphor quinone) is reduced in 63% yield to 3 hydroxy camphor.



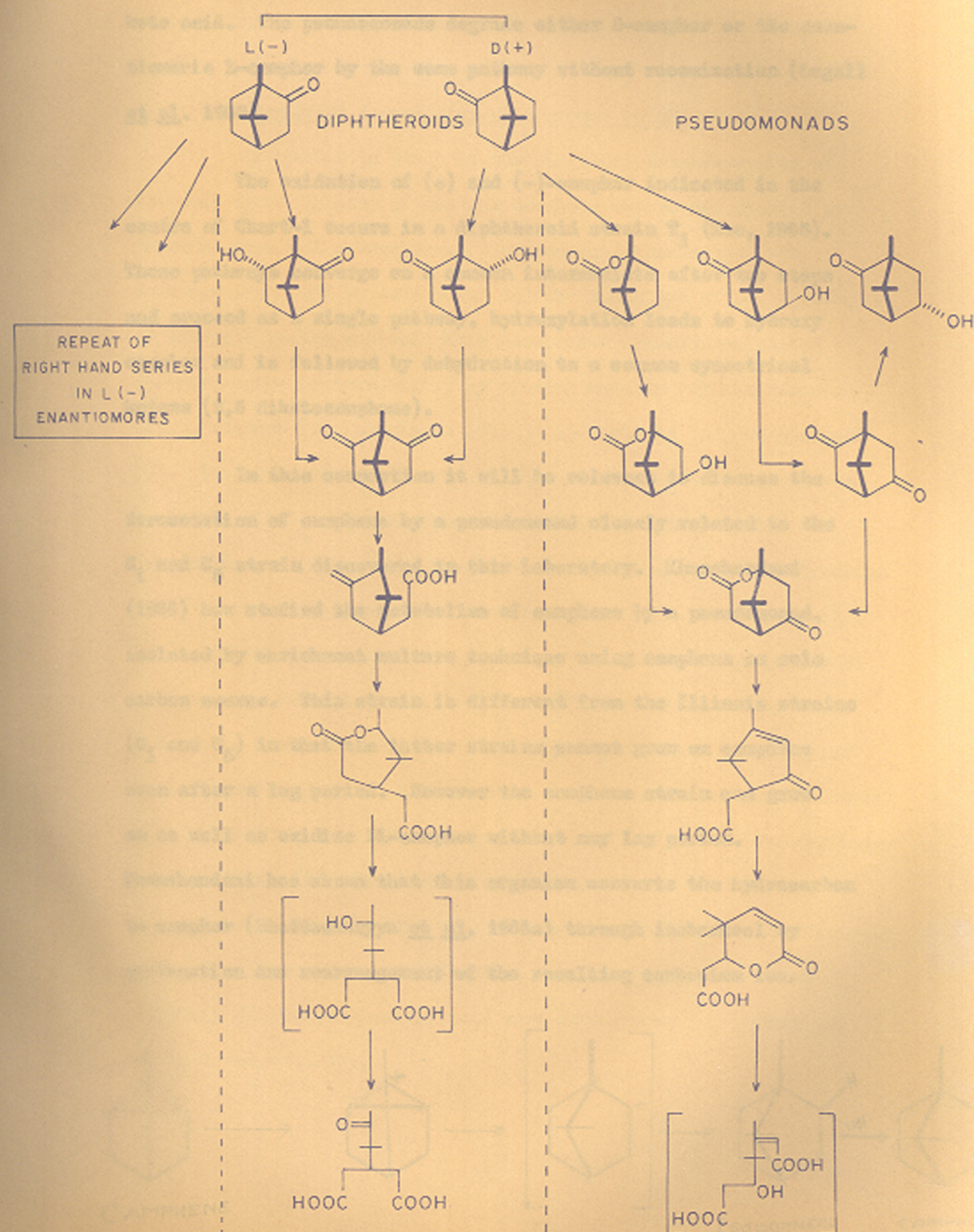
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They also observed that d-camphorquinone gave d-hydroxy camphor, while the racemic compound gave only the corresponding laevo-rotatory hydroxy compound.

Babicca et al. (1955) were able to convert citranellol, pulegol or isopulegol to menthol using Penicillium digitatum. The data available on the microbiological oxidation of terpenes are mostly due to systematic investigations by two groups of workers, one at the University of Illinois, U.S.A. and the other at the National Chemical Laboratory, India.

The first report of the systematic study on the microbial degradation of terpenoids such as camphor was by Corey and Gunsalus (Bradshaw et al. 1959) using a pseudomonad strain C₁ isolated from sewage sludge by enrichment culture techniques using (+)-camphor as the sole carbon source. In the last few years several strains of bacteria by this group have been isolated and shown to utilize camphor as the sole source of carbon. Of these, two strains C₁ and C₅ (Hedegard et al. 1961), Conrad et al. 1961a, 1961b) degrade camphor by the same pathway but differ in their ability to utilize this compound as the sole source of carbon. In strain C₁, camphor is initially hydroxylated and the resulting hydroxyketone is oxidized to diketone. The next step is a Bayer-Villiger oxygenation reaction in which molecular oxygen is inserted between a ketone and the quaternary bridgehead carbon to form a lactone, which hydrolyses and eliminates water to form a conjugated keto acid. The same reactions take place in camphor breakdown (Chart 1) in C₅, but they occur in a different order, the initial lactonization being followed

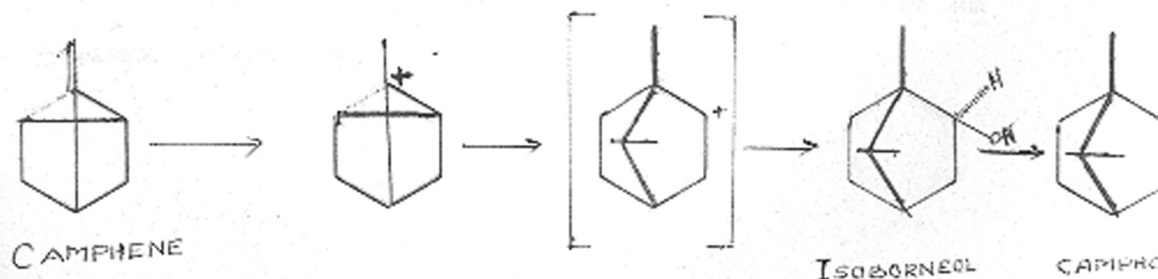
INTERMEDIATES IN INDUCED PATHWAYS OF CAMPHOR OXIDATION

(Gunsalus *et al*, 1965)

by hydroxylation, oxidation and rearrangement to give the conjugated keto acid. The pseudomonads degrade either D-camphor or the enantiomeric L-camphor by the same pathway without racemization (Legall *et al.* 1963).

The oxidation of (+) and (-)-camphor indicated in the centre of Chart-1 occurs in a diphtheroid strain T₁ (Kuo, 1963). These pathways converge on a common intermediate after two steps and proceed as a single pathway, hydroxylation leads to hydroxy camphor and is followed by dehydration to a common symmetrical ketone (2,5 diketocamphane).

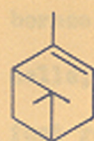
In this connection it will be relevant to discuss the fermentation of camphene by a pseudomonad closely related to the C₁ and C₅ strain discovered in this Laboratory. Khanchandani (1966) has studied the metabolism of camphene by a pseudomonad, isolated by enrichment culture technique using camphene as sole carbon source. This strain is different from the Illinois strains (C₁ and C₅) in that the latter strains cannot grow on camphene even after a lag period. However the camphene strain can grow on as well as oxidize DL-camphor without any lag period. Khanchandani has shown that this organism converts the hydrocarbon to camphor (Bhattacharyya *et al.* 1966a) through isoborneol by protonation and rearrangement of the resulting carbonium ion.



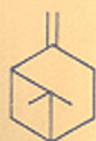
The subsequent steps in the catabolism of camphor seems to be essentially the same as in the case of pseudomonads C_1 and C_5 .

Bacterial degradation of terpenic hydrocarbons

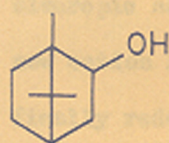
A systematic study of the microbial transformation of terpenic hydrocarbons, pinene and menthene series was started by Bhattacharyya and co-workers. A pseudomonad (PL-strain) was isolated from soil by enrichment culture technique with α -pinene as the sole carbon source. This particular organism exhibited a certain degree of versatility by growing equally well on limonene (1), α -pinene (2), β -pinene (3), Δ^1 -p-menthene (4) and p-cymene (5). The metabolism of pinene by this strain has been worked out by Shukla (1965). He was able to establish the pathway for the degradation of (2) and (3) based on the isolation and identification of the intermediate products from the fermentation broth as well as by sequential adaptation studies. From the fermentation (Chart 2) of α -pinene, borneol (6) and myrtenol (7) were among the neutral components identified and from the acidic fraction a dicarboxylic acid (8) was isolated. Besides these, the presence of myrtenic acid (9), phellandric acid (10) and perillic acid (11) were also demonstrated by VPC. The fermentation of β -pinene gave (-) borneol, the saturated dicarboxylic acid (12) and appreciable amounts of oleuropic acid (13). Two other hydroxy acids, 4 hydroxy phellandric and 4 hydroxy dihydrophellandric acid were formed from α -pinene and β -pinene respectively.

METABOLITES OBTAINED FROM α and β PINENE FERMENTATION

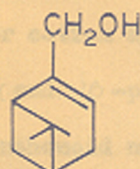
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 α -PINENE



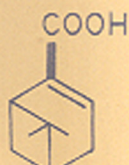
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 β -PINENE



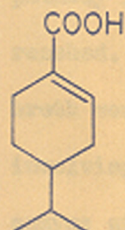
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BORNEOL



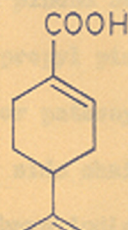
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MYRTENOL



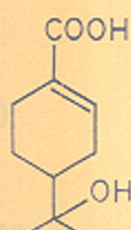
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MYRTENIC ACID



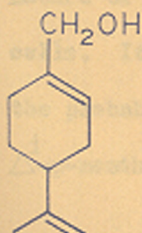
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PHELLANDRIC ACID



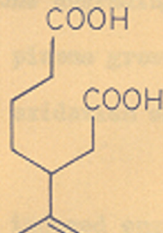
11
PERILLIC ACID



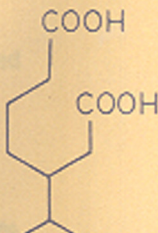
13
OLEUROPIC ACID



14
PERILLYL ALCOHOL



8
 β -ISOPROPENYL
PIMELIC ACID



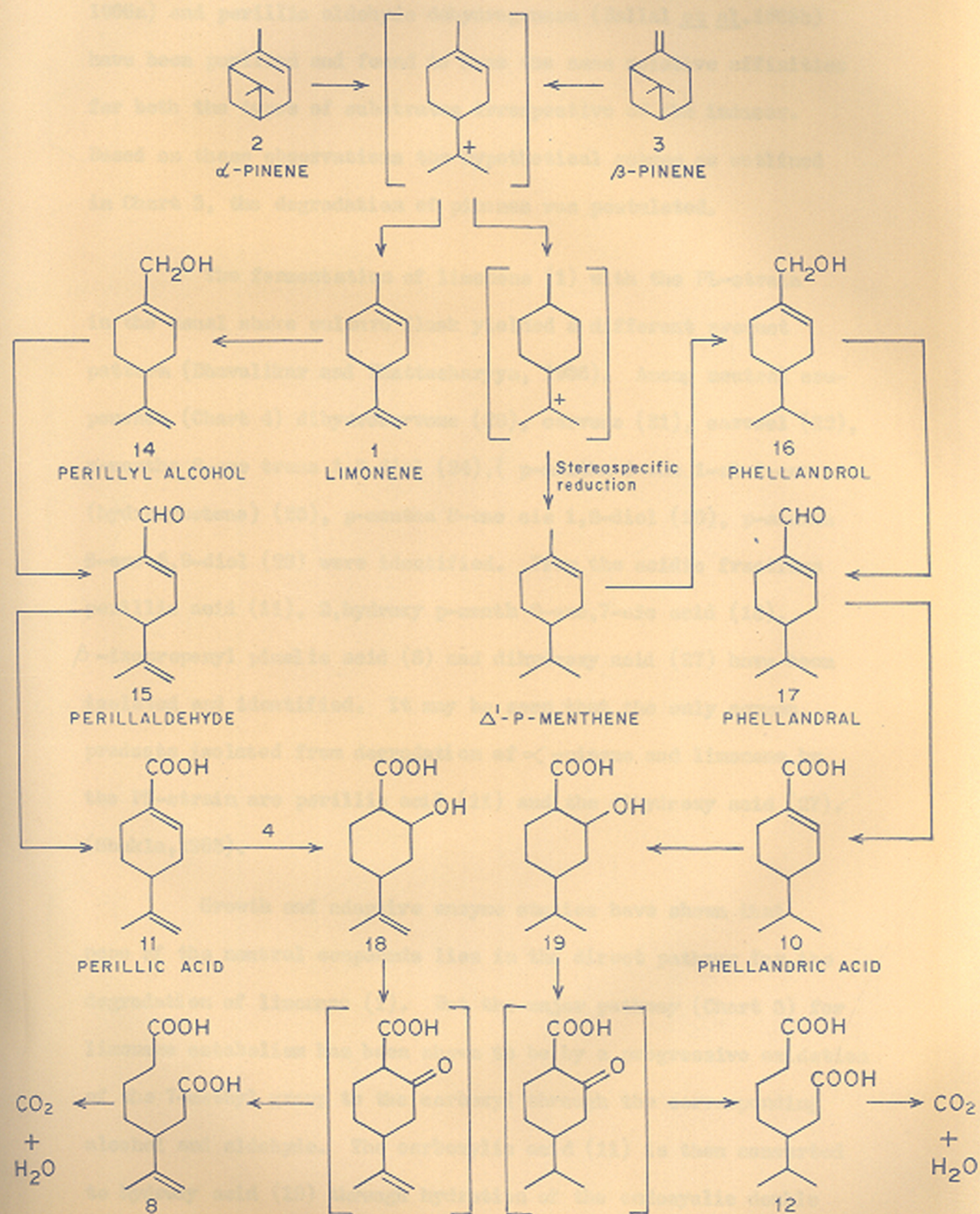
12
 β -ISOPROPYL
PIMELIC ACID

Adaptive enzyme studies have indicated that neither borneol, myrtenol nor cleurepic acid were oxidized by pinene grown cells, whereas the monocarboxylic acid fraction was oxidized at a fast rate. The probable major pathway for the catabolism of pinene nucleus is by a prototropic attack on the double bond and a subsequent Wagner-Meerwin rearrangement which cleaves the cyclobutane ring to give Δ^1 -p-menthenoid cation which can lead to cleurepic acid or can form another cation by hydride shift. This new cation formed from both (+) α and (-) β -pinene is stereospecifically reduced to yield Δ^1 -p-menthenoid nucleus, sterically homologous to (+) limonene (Chart 3).

The pathway was derived based on the data on sequential adaptation. But a clear conclusion as to whether the limonene pathway is of any significance in pinene metabolism has not been reached. The isolation of β -isopropyl pimelic acid (12) from the broth would indicate that the major pathway is via Δ^1 -p-menthene (4) involving the saturated isopropyl side chain. Growth and induced enzyme studies on the other hand have indicated that α and β pinene grown cells are capable of oxidizing limonene and Δ^1 -p-menthene with or without added chloramphenicol indicating the constitutive nature of limonene and Δ^1 -p-menthene degrading enzymes in these cells. It was also observed that pinene grown cells rapidly oxidized the probable intermediates in the oxidation of limonene and Δ^1 -p-menthene.

It is possible that the induced enzyme systems irrespective of whether the inducer is α -pinene or limonene accept both the series of terpenic intermediates, one with the saturated isopropyl side chain

MAJOR PATHWAYS FOR THE CATABOLISM OF α -AND β -PINENES, LIMONENE, AND Δ^1 -P-MENTHENE IN PSEUDOMONAS SP. (PL-STRAIN)

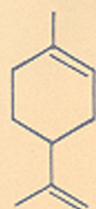


and the other with the unsaturated sidechain. Two of the enzymes of this sequence - perillyl alcohol dehydrogenase (Ballal *et al.* 1966a) and perillic aldehyde dehydrogenase (Ballal *et al.* 1966b) have been purified and found to have the same relative affinities for both the types of substrates irrespective of the inducer. Based on these observations the hypothetical scheme as outlined in Chart 3, the degradation of pinenes was postulated.

The fermentation of limonene (1) with the PL-strain in the usual shake culture flask yielded a different product pattern (Bhavalikar and Bhattacharyya, 1966). Among neutral components (Chart 4) dihydrocarvone (20), carvone (21), carveol (22), p-mentha 8-ene trans 1,2 diol (24), (p-mentha 8-ene 1-ol-2-one (hydroxyketone) (25), p-mentha 8-ene cis 1,2-diol (26), p-mentha 8-ene 8,9-diol (23) were identified. From the acidic fractions perillic acid (11), 2,hydroxy p-mentha 8-ene,7-oic acid (18), β -isopropenyl pimelic acid (8) and dihydroxy acid (27) have been isolated and identified. It may be seen that the only common products isolated from degradation of α -pinene and limonene by the PL-strain are perillic acid (11) and the dihydroxy acid (27). (Shukla, 1965).

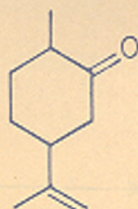
Growth and adaptive enzyme studies have shown that none of the neutral compounds lies in the direct pathway for the degradation of limonene (1). But the major pathway (Chart 5) for limonene catabolism has been shown to be by a progressive oxidation of the 7-methyl group to the carboxyl through the corresponding alcohol and aldehyde. The carboxylic acid (11) is then converted to hydroxy acid (18) through hydration of the endocyclic double

PRODUCTS OF BACTERIAL DEGRADATION OF
(+) LIMONENE



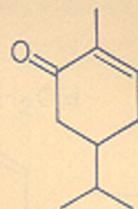
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(+) LIMONENE



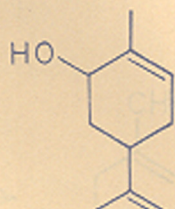
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DIHYDROCARVONE



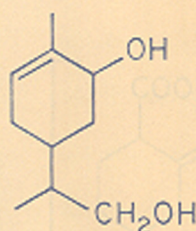
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CARVONE



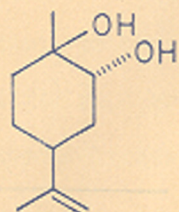
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CARVEOL



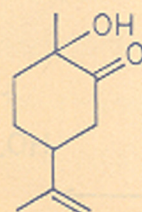
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6,9 DIOL



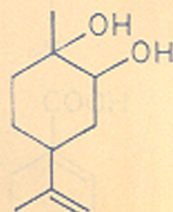
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TRANS 1,2 DIOL



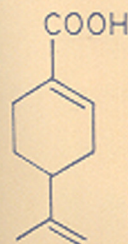
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HYDROXY KETONE



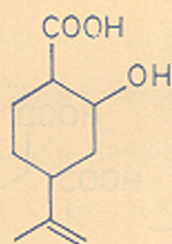
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CIS-1,2 DIOL



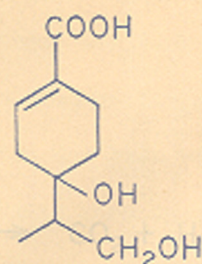
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PERILLIC ACID



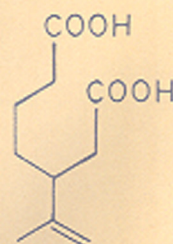
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HYDROXY ACID



27

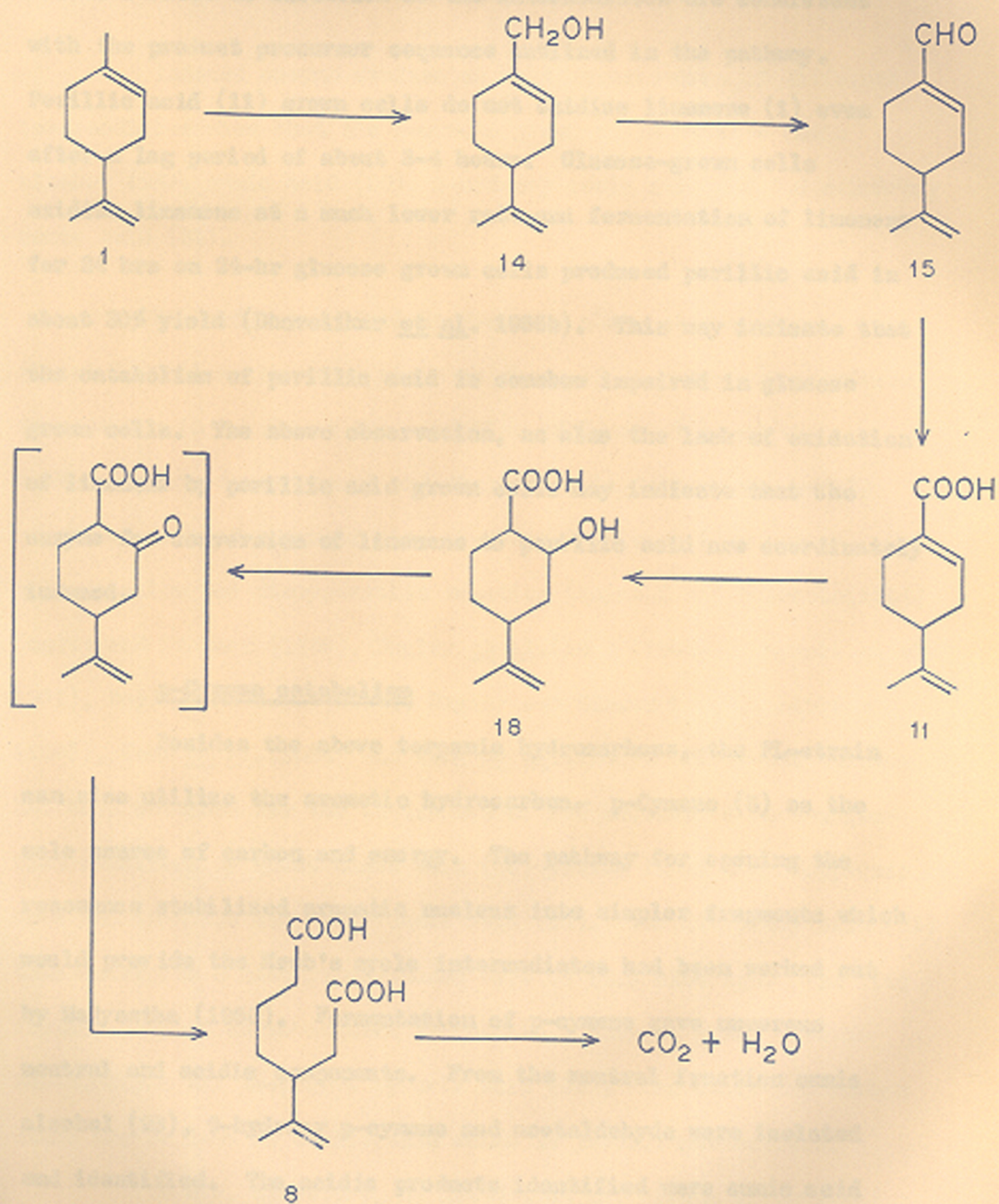
DIHYDROXY ACID



8

DICARBOXYLIC ACID

PATHWAY FOR THE DEGRADATION OF LIMONENE



bond. The hydroxy acid is oxidized to a β -keto acid and cleaved to β -isopropenyl pimelic acid (8) (Dhavalikar *et al.* 1966b). All the probable intermediates in this pathway support the growth of the organism and also they are oxidized without any lag. The relative rates of oxidation of the intermediates are consistent with the product precursor sequence outlined in the pathway. Perillic acid (11) grown cells do not oxidize limonene (1) even after a lag period of about 3-4 hours. Glucose-grown cells oxidize limonene at a much lower rate and fermentation of limonene for 24 hrs on 24-hr glucose grown cells produced perillic acid in about 30% yield (Dhavalikar *et al.* 1966b). This may indicate that the catabolism of perillic acid is somehow impaired in glucose grown cells. The above observation, as also the lack of oxidation of limonene by perillic acid grown cells may indicate that the enzyme for conversion of limonene to perillic acid are coordinately induced.

p-Cymene catabolism

Besides the above terpenic hydrocarbons, the PL-strain can also utilize the aromatic hydrocarbon, p-Cymene (5) as the sole source of carbon and energy. The pathway for opening the resonance stabilized aromatic nucleus into simpler fragments which would provide the Kreb's cycle intermediates had been worked out by Madyastha (1966). Fermentation of p-cymene gave numerous neutral and acidic components. From the neutral fraction cumic alcohol (28), 9-hydroxy p-cymene and acetaldehyde were isolated and identified. The acidic products identified were cumic acid (30), 3-hydroxy cumic acid, 2,3-dihydroxy cumic acid (31),

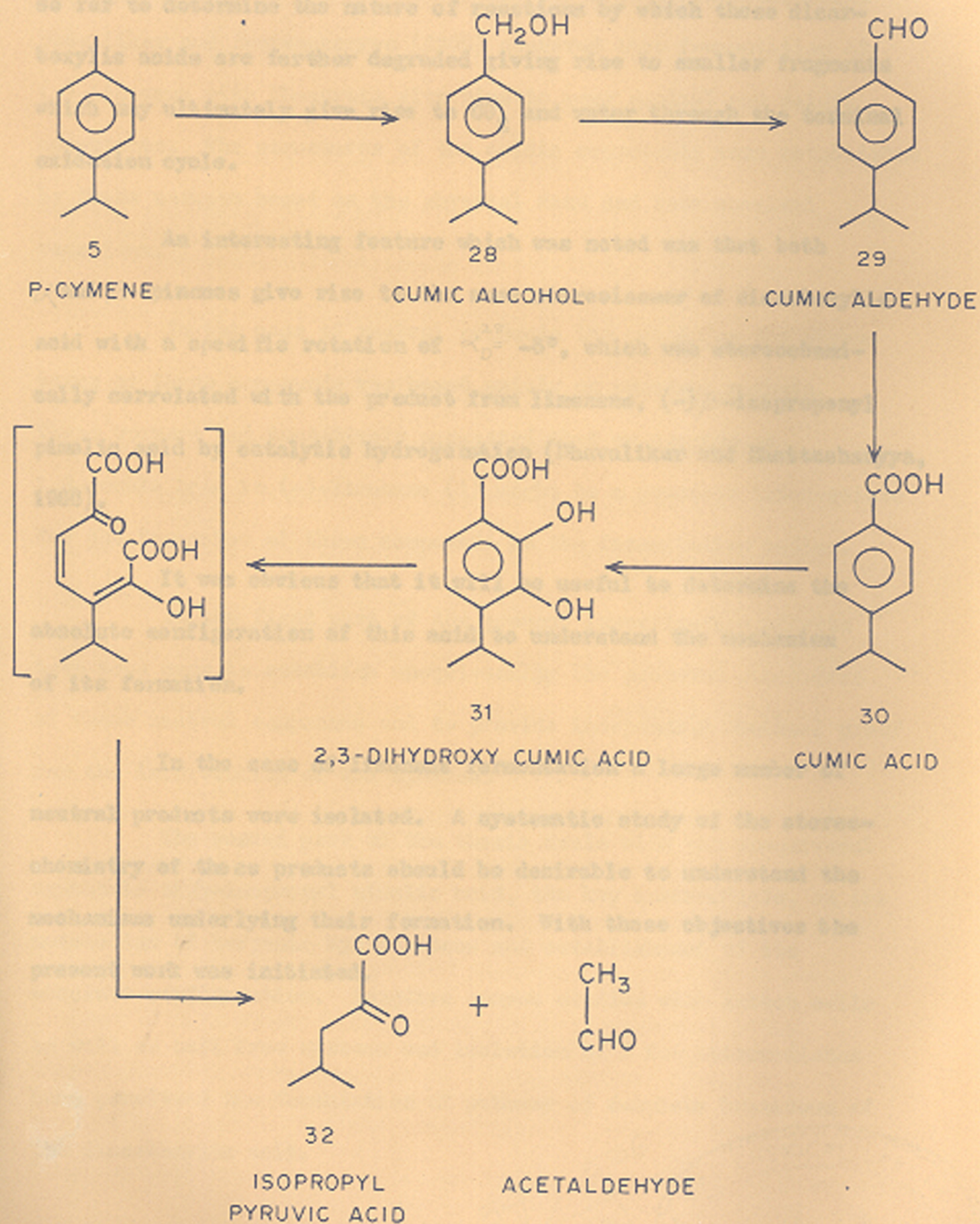
α -p-tolyl propionic acid and isopropyl pyruvic acid (32). Growth and oxidation studies have shown that 9-hydroxy p-cymene and α -p-tolyl propionic acids are side products. Incubation of 2,3-dihydroxy cuminic acid with cell-free extracts yielded considerable amount of isopropyl pyruvic acid and acetaldehyde.

Induced enzyme studies as well as oxidation of different intermediates show that the p-cymene grown cells oxidize cuminic alcohol (28), cuminic acid (30), 3-hydroxy cuminic acid and 2,3-dihydroxy cuminic acid (31).

The pathway arrived at from the above observations (Chart 6) follows the same steps as those of limonene or p-menthene degradation in so far as the oxidation of 7-methyl group, the carboxyl group is concerned and thereafter it follows the widely known sequence of aromatic ring cleavage involving high energy hydroxylation and dioxygenation reaction (Hayashi and Hoshimoto(1959), Dagley and Stophar (1959), Dagley *et al.*(1960)(1965a), Hayashi *et al.* (1961), Dagley and Wood (1965), Dagley and Gibson (1964), Ribbons (1966), Toso *et al.*(1964) and King *et al.*(1964). Clear-cut evidence has been obtained for the occurrence of two successive monooxygenation reactions by which cuminic acid (30) yields first 3-hydroxycuminic acid and then 2,3 dihydroxy cuminic acid (31). The dihydroxy acid (31) then undergoes a metapyrocatechase type of ring cleavage. The ring fission product on subsequent hydrolytic cleavage, gives rise to acetaldehyde and isopropyl pyruvic acid (32). The other two carbon atoms may be lost as oxalic acid which is further metabolized.

It may, therefore, be noted that all groups of workers working on *Pl*-strain have worked out pathways for degradation upto

PROPOSED PATHWAY FOR THE DEGRADATION OF p-CYMENE IN
PSEUDOMONAS IN SP. PL-STRAIN



the intermediate stage of two dicarboxylic acids. Limonene yields unsaturated dicarboxylic acid, whereas the pinenes are degraded to the acyclic saturated dicarboxylic acid. No efforts had been made so far to determine the nature of reactions by which these dicarboxylic acids are further degraded giving rise to smaller fragments which may ultimately give rise to CO_2 and water through the terminal oxidation cycle.

An interesting feature which was noted was that both α and β -pinenes give rise to the same stereoisomer of dicarboxylic acid with a specific rotation of $[\alpha]_D^{25} -5^\circ$, which was stereochemically correlated with the product from limonene, $(-)\beta$ -isopropenyl pimelic acid by catalytic hydrogenation (Dhavalikar and Bhattacharyya, 1966).

It was obvious that it will be useful to determine the absolute configuration of this acid to understand the mechanism of its formation.

In the case of limonene fermentation a large number of neutral products were isolated. A systematic study of the stereochemistry of these products should be desirable to understand the mechanisms underlying their formation. With these objectives the present work was initiated.

PRESENT INVESTIGATION

During the course of the microbiological investigations Dhavalikar and Bhattacharyya (1966) observed that fermentation of (+)-limonene with PL-strain give rise to many neutral and acidic components. The structures of the acidic components were established by these workers based on the physical data and some chemical reactions.

In the case of neutral products the proof for the structures of these compounds was based mainly on spectral methods. These workers had tentatively suggested that an epoxide at 1,2-double bond in (+)-limonene (1) might be a possible intermediate for the formation of these compounds in the fermentation medium.

In the first part of the present investigation efforts have been made to establish unequivocally the proposed structures of these neutral compounds and to provide independent chemical proof for the intervention of the epoxide.

The second part of the thesis deals with the unequivocal synthesis of β -isopropyl pimelic acid, the key intermediate, on the metabolism of terpenic hydrocarbons and establishment of its absolute configuration. Adaptive enzyme studies with intact cells as well as cell free systems and isolation of a few intermediates have permitted the formulation of pathway of complete breakdown of the dicarboxylic acid.

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The first chapter of the thesis gives a resume of the subject and the second describes the materials and methods. In the third chapter an investigation of the reaction of (+)-limonene with peracetic acid has been reported. This investigation has been helpful in two ways viz. (a) a detailed study of the chemistry and the stereochemistry of the products substantiated the possibility that limonene epoxide was an intermediate in the fermentation, and (b) chemically the epoxide has been converted to several other products which ^{were} helpful in the correlation of structures of synthetic products with those obtained in limonene fermentation.

In order to meet the first requirement, the reaction was run under mild and close to biological conditions existing in the fermentation itself. For this, the epoxide was not isolated and the reaction was carried out between (+)limonene and excess of peracetic acid, in which a reaction period of fifty hours corresponding to that used for the fermentation of limonene was maintained. Like the fermentation process, the peracetic acid oxidation of (+)limonene yielded a number of neutral products. (+)Dihydrocarvone (20), (-)carvone (33), (+)carveol (22), p-mentha-8-ene-1-ol, 2-one (25) and p-mentha-8-ene-trans 1,2-diol (24) were among those which have been characterized (Chart 7). The mechanism for the formation of these transformation products and the obvious parallelism of this chemical degradation of (+)limonene with the microbiological transformation of (+)limonene are discussed. A new synthetic method for the preparation of p-mentha-8-ene cis 1,2 diol (26), another biotransformation product of (+)limonene, is described. The method employed is that developed for cis hydroxylation in the

synthetic steroids by Woodward and Brucher (1957). It involves the interaction of an olefin with iodine-silver acetate and wet acetic acid to give by way of neighbouring group participation (Weinstein, 1942), the *cis* hydroxyacetate in one operation. Subsequent hydrolysis yields the free diol. (+) Limonene when subjected to this reaction gave the product mentioned earlier. Synthesis of the hitherto unknown diol, *p*-mentha-2-ene,6,9 diol (23) is also described. The addition of diborane and its alkyl derivatives to olefins is a fast quantitative reaction of an unusual broad generality and remarkably free from undesirable rearrangements or other side reactions. Moreover, the reaction involved a stereochemical *cis*-addition from the less hindered side of the double bond, the boron atom becoming attached preferentially to the less substituted of the two carbon atoms of the double bond. Finally the resulting organoboranes, intermediates of high reactivity which are readily transformed into other products of interest in organic synthesis, without rearrangement (or loss of stereospecific structure) of the original hydroboration product. The products being of a defined stereochemistry should permit reliable configurational assignments and structural correlations. Thus by selective hydroboration of (+)carveol with diisiamyl borane it has been possible to obtain the (stereospecific) diol identical with the one formed during fermentation of limonene.

In Chapter IV a total synthesis of β -isopropyl pimelic acid, a key intermediate in the degradation of both α and β -pinenes as well as Δ^1 -*p*-menthene, has been developed. A second synthesis from (+) Δ^1 -*p*-menthene by ozonolysis and Willgerodt reaction

establishes the absolute configuration of the fermentation product and correlates with (+)limonene.

In Chapter V isolation of a few more intermediates such as a dicarboxylic acid and methyl isopropyl ketone has been described.

Chapter VI deals with sequential adaptation with various intermediates in the degradation of the dicarboxylic acid and enzymic studies with cell-free systems are reported. These experiments indicate that the dicarboxylic acid is degraded by progressive β -oxidation to methyl isopropyl ketone which may be oxidized to isobutyric acid. It has been shown that in cell-free system isobutyrate goes to acetone and pyruvate thereby linking it with the terminal oxidative cycles.

Chapter VII gives a summary of the entire work.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

All chemicals used for the preparation of media were of chemically pure grade and those used for enzymatic studies were of analytically pure grade. (+)- Δ^1 -p-Menthene which was prepared by catalytic hydrogenation of (+)limonene obtained from M/s. Kelkar Brothers, Bombay, was kindly supplied by Mr B. G. Pujar of our Department. Δ^1 -p-Menthene $\alpha_D^{25} +110^\circ$ was further purified by ^{vacuum} distillation over sodium metal. Based on VPC analysis, (+) Δ^1 -p-menthene used in these studies was 99.5% pure. Neutral alumina, Gr-I and silica gel supplied by the Fine Chemicals Project of this Laboratory were used for column chromatography. Silica gel for thin layer and preparative layer chromatography was also supplied by the Fine Chemicals Project. (+) Limonene used in our experiments was supplied by M/s. Kelkar Bros. Bombay and had a specific rotation $\alpha_D^{25} +118^\circ$. Carvone used for hydroboration was Fluka product and showed a specific rotation $\alpha_D^{25} +58.6^\circ$. LAH reduction of carvone gave cis-carveol $\alpha_D^{25} +34.1^\circ$. Isobutyric acid (British Drug Houses), cyanoacetamide (E. Merck) and isobutyraldehyde (Fluka) were used.

Maintenance and propagation of culture

The organism was originally isolated from soil by Shukla and was found to be Gram-negative, motile rods having the characteristics of the pseudomonas species. This organism growing on Δ^1 -p-menthene as the sole source of carbon was propagated on a nutrient agar slant of the following composition (Mackie and McCartney, 1949):

Peptone (Armour).....	1 gm
Sodium chloride (BDH).....	0.5 gm
Yeast extract (Oxoid).....	0.5 gm
Beef extract (Difco).....	0.5 gm
Agar	2.0 gm
Total volume	100 ml

The first four constituents were dissolved in water and the pH was adjusted to 7 with 4N sodium hydroxide. The final volume was then made to 100 ml. Two per cent of agar (Alcaden or Difco) was added and the mixture was steamed for one hour. For preparation of slants 6-7 ml aliquots were distributed into 10 x 150 mm pyrex test tubes, autoclaved at 15 psi (120°) for 20 min and were slanted. The slants were resteamed for one hour after 24 hrs to destroy any germinated spores and reslanted again. These slants were inoculated either from broth or stock culture and incubated at 23° for 24 to 48 hrs.

For purification of the culture by the streaking method, nutrient agar of the above composition was poured while hot aseptically in previously autoclaved (20 psi for 1 hr) petri dishes. The culture could also be propagated even on glucose mineral salt-agar slants. The composition of the mineral salts will be described below. To this basal medium were added 1% glucose and 2% agar and the slants were prepared in the usual way.

Maintenance of the culture

It is possible to maintain the organism and to keep viable for very long periods on the surface of nutrient agar covered with a layer of sterile liquid paraffin. These paraffin covered slants were stored at room temperature (26-28°C). For routine work the culture was maintained by regular transfer into liquid medium.

Mineral salt medium for growth and fermentation

Since the microbial oxidation of hydrocarbons is usually accompanied by acid production, the presence of carbonate or phosphate

in the medium is desirable to buffer it to a favourable hydrogen ion concentration. The organism was, therefore, grown in a chemically defined medium as described by Seubert and Werner (1960) having the following composition:

1) Dipotassium hydrogen phosphate (Danpha grade, India)	63.0 gm
2) Potassium dihydrogen phosphate	18.2 gm
3) Ammonium nitrate (E. Merck)	10.0 gm
4) Magnesium sulphate (B.D.H.) anhydrous	1.0 gm
5) Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, E. Merck)....	1.0 gm
6) Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, B.D.H.).....	1.0 gm
7) Manganese sulphate anhydrous (Riedel de Hahn, A.G.)	6 mg
8) Sodium molybdate (B.D.H.)	6 mg

made upto 10 litres, pH 7

All the constituents were dissolved separately excepting the phosphate, added to the phosphate solution and the total volume was made upto 10 litres. Aliquots of 100 ml of the medium were taken in 500 ml Erlenmeyer flasks which were plugged and autoclaved. One to two per cent of inoculum and 1 ml of the respective carbon sources were added to each flask. The flasks were incubated at $28^\circ \pm 1^\circ$ on a rotary shaker at 220 rpm for 24-48 hrs.

Extraction and separation of compounds

At the end of the incubation period (24-48 hrs) contents of the flasks were pooled together, acidified with 6N HCl to pH 2.0 and extracted twice or thrice with ether. The ether layers were pooled together and shaken with hyflosuperceel to remove the suspended material.

The clear ether extracts were separated into acidic and neutral fractions by washing with aqueous 5% sodium carbonate solution. The acidic components were liberated by acidifying the aqueous sodium carbonate layer and recovered by extracting with ether.

The acids forming acetone-insoluble cyclohexylamine salts were separated by treatment of the total acid fraction with cyclohexylamine in dry acetone. The insoluble cyclohexylamine salts were filtered and washed with dry acetone and free acids were generated by a treatment of the cyclohexylamine salt with 6N hydrochloric acid. The acids liberated were taken up in ether and were converted into methyl esters by treatment with diazomethane in ether. The separation and identification of these compounds were monitored by various physicochemical methods such as TLC, VPC and other spectroscopic methods as detailed in subsequent chapter.

Chromatography

TLC was carried out with 85% silica gel using 15% of plaster of Paris as the binder.

Resolution of neutral compounds and methyl esters of acids was achieved by using hexane-ethyl-acetate 95:5 (System I), 90:10 (System II) or 85:15 (System III). For the resolution of dihydroxy compound (neutral) hexane:ethyl acetate 70:30 (System IV) was used. The compounds were detected either by spraying the plate with conc. H_2SO_4 and developing the spots by heating for 10-15 min at 110° or by keeping the plate in iodine chamber.

Column chromatography

The neutral components were separated by chromatography over alumina or silica gel as the case may be using the following

sequence as the routine procedure: pet-ether; pet-ether:ether mixture a) 95:5, b) 90:10, c) 80:20, d) 50:50 and e) 100% ether and finally with methanol.

Preparative layer chromatography

Preparative 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 plate placed horizontally in a square trough and the gel allowed to set. After drying in air the plates were activated in an oven for 6 to 8 hours at 110°C. The chamber used for the development of the plates was 30 cm x 30 cm x 30 cm cubic aquarium tank in which the solvent system was poured one hour prior to the experiment and the chamber was allowed to saturate with the vapors of the solvent. The plates were then streaked with the sample with the help of a fine capillary and placed in the chamber on a glass rack above the solvent layer which were allowed to saturate for one hour 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 system. This operation was repeated once again in order to get compact bands. Better resolutions were usually obtained by multiple runs. The plates were then dried and portions of the plate (1 cm) on either sides were marked out. The middle portion of the plates were covered with rectangular glass plates. The strips on both the sides were sprayed with conc. sulphuric acid which were developed by careful heating of the sides of the plates mounted on a tripod stand over a micro 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 out with ether to get the desired compound.

Identification of 2,4 dinitrophenylhydrazones derivatives of oxo acids by TLC and paper chromatography

The oxo acid as well as the neutral carbonyl compounds were isolated by converting them into their corresponding 2,4-dinitrophenylhydrazones and identified by TLC and paper chromatography. The following systems were used for TLC.

Solvent system for oxo acid

Benzene:tetrahydrofuran:acetic acid 60:38:2 (system IV)

For neutral carbonyl compounds

Benzene:tetrahydrofuran 60:40 (system V)

Ascending unidimensional paper chromatography was carried out on Whatman No.1 sheets n-butanol ethanol water (7:1:2) (Dagley and Gibson, 1964) was used as the solvent system.

Preparation of 2,4-dnp solution

2,4 Dinitrophenylhydrazine (0.5 gm) was dissolved in 500 ml 3N HCl (approx.) in the usual manner. The solution was filtered and left in the refrigerator when not in use.

Physical and spectral measurements

All the melting points and boiling points are uncorrected. Optical rotations were taken in chloroform as solvent unless otherwise specifically mentioned in a Hilger standard polarimeter using 1 dm tube. The Infrared spectra were recorded on a Perkin-Elmer model 221 or a Grubb-Parson double beam spectrometer. The

phases used were mostly liquid film or mull and in some cases in carbon tetrachloride or chloroform solutions. The ultraviolet spectra were taken on a Beckman Model DU spectrophotometer with spectroscopically pure rectified spirit as a solvent. Gas chromatography was carried out on a Griffin-Tatlock model G, Mark III or on Perkin-Elmer vapor Aerograph, analytical model (A 350B) using hydrogen as the carrier gas. All NMR spectra were recorded on a Varian Associates A-60 spectrometer using CCl_4 and in some cases CHCl_3 and deuteriochloroform CDCl_3 and TMS (tetramethylsilane) as the internal standard. All NMR signals are recorded in δ values.

Growth studies

In the experiments where growth pattern of the organism was studied on different substrates, the cells adapted to Δ^1 -p-menthene were used. Δ^1 -p-Menthene-grown cells were freed from adhering substrates by centrifugation, centrifuged cells washed with 0.05 M phosphate buffer and resuspended in sterile medium. Centrifugations and washings were done under aseptic conditions. Suitable aliquots (2 ml) of cell suspension with a turbidity reading of 250-280 (Klett reading) at 660 m μ (red filter) were inoculated into flasks containing the respective substrates (100-200 mg) and incubated in the usual manner.

Enzymatic studies

Protein estimations: Protein measurements were made by the Folin-Ciocalteu phenol reagent according to the procedure described by Lowry *et al.* (1951). Crystalline bovine serum albumin (Sigma) was used as the working standard and the colour was read at 500 m μ in a Beckman DU spectrophotometer.

Protein measurements were also made by the optical method of Warburg and Christian (1942) modified by Jagannathan et al. (1956) using the following empirical equation:

$$\frac{4}{7} [2.3 \times (\text{O.D. at } 280 \text{ m}\mu) - (\text{O.D. at } 260 \text{ m}\mu)] = \text{mg protein per ml of solution}$$

This method was used when the ratio of O.D.s at 280 mμ and 260 mμ was more than 0.9 to 1.0. In crude preparations protein determination was made by the method of Lowry et al. (1951).

Manometric studies

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

The flasks, in a total volume of 3 ml, containing 0.2 ml 20% KOH in the central well, ^{had} 1.5 ml of cell suspension containing 1.2 mg dry weight of cells and 1 ml of M/20 phosphate buffer. The substrates 5-20 μmoles and other substances were added either by tipping from the side arm or incorporating them directly into the main flask (in each case the volume was kept at 3 ml). The temperature of the bath was maintained at 28° and flasks were shaken in this bath (110 strokes/min with 2 cm strokes).

PREPARATION OF THE SUBSTRATES

- 1) Preparation of ethyl isobutyrylacetic acid
(Krecker and MacElvin, 1934)

a) Isobutyryl acetonitrile: In a one-litre, three-necked flask, fitted with a dropping funnel, reflux condenser and vacuum sealed stirrer were placed 46 gm of sodium metal sand (powdered under xylene and washed twice with anhydrous ether) and 350 cc anhydrous ether. During the course of 2-3 hours 92 gm (2 moles) of absolute

ethanol were added from the dropping funnel. The mixture was stirred without heating until the reaction ceased and ether was removed by distillation. To the remaining sodium ethoxide, was added a mixture of 77.3 gm (0.67 moles) of ethyl isobutyrate and 27.3 gm (0.67 moles) of acetonitrile. The mixture was heated with stirring for three hours at 115-120° whereupon the alcohol formed in the reaction was distilled off under slightly reduced pressure. Another portion (0.67 ml) of each reactant was added the mixture refluxed for additional three hours and the alcohol removed as before. This process was repeated until a total of 2 moles of each of ethyl-isobutyrate and acetonitrile had been added. The mixture was then cooled, decomposed with ice-water containing a slight excess of acetic acid and extracted with ether, washed with 5% sodium bicarbonate solution, dried over anhydrous sodium sulphate and evaporated. The residue distilled under diminished pressure to yield straw coloured oil B.P. 102-104°C/12-13 mm (yield 98 gm, 44%).

b) Ethyl isobutyryl acetate: A solution of 222 gm (2 moles) of isobutyreacetonitrile in 92 gm (2 moles) of absolute alcohol was cooled in ice salt bath and dry HCl gas passed in until 71 gm (2 moles) had been absorbed. Upon standing in ice bath overnight, the mixture set to a mass of crystals of imino ester hydrochloride. This product was washed with ether to remove unchanged nitrile and hydrolysed by dissolving in water and allowing to stand at room temperature (28°) for several hours. The resulting ester layer was taken up in ether, dried over anhydrous sodium sulphate and after removal of ether distilled. Yield 251 gm (81%), B.P. 90-92°/15-16 mm.

c) Methyl-isopropyl ketone: Since the isobutyryl acetic acid is unstable, the ester of isobutyryl acetic acid after hydrolysis

with 10% KOH, neutralization of the resultant product with 3N HCl gave the methyl isopropyl ketone which was extracted with ether, washed with water dried over anhydrous sodium sulphate. Product after removal of ether was treated with equimolar amount of 2,4 dnp solution in 3N HCl giving 2,4 dnp derivative of methyl isopropyl ketone, m.p. 121°C.

d) Preparation of methyl malonic acid: Sodium ethoxide was prepared in a 500 ml three-necked flask fitted with a condenser, mercury sealed stirrer and a dropping funnel, by dissolving 12 gm of finely divided metallic sodium in 250 ml of dry ethanol. Then 80 gm of diethyl malonate were added to sodium ethoxide solution with stirring for about 15 min. To this mixture was added 71 gm of methyl iodide (freshly distilled) slowly over a period of 1 hr. Reaction proceeded smoothly with the separation of crystalline sodium iodide and the mixture becomes pale orange and faintly alkaline. It was refluxed with stirring for half an hour, neutralized with glacial acetic acid and cooled. Sodium iodide was filtered off, washed with cold alcohol. Major amount of alcohol was removed from the pooled filtrates and washings by distillation under atmospheric pressure. The residue was dissolved in 600-700 ml of water acidified with 2.5 ml conc. HCl and shaken with the residue from distillation. The ester layer was removed and the aqueous layer was extracted twice with ether. The ether extract and ester layers were combined and shaken quickly with CaCl_2 and filtered at once. Ether was removed and the ester rapidly shaken exactly for one minute with an ice-cold solution of 2.5 gm of sodium hydroxide in 8 ml of water to remove the unreacted malonic ester. The alkali was drained off and the ester layer was washed with dilute acid, dried over CaCl_2 and distilled under vacuum, B.P. 70°/6 mm.

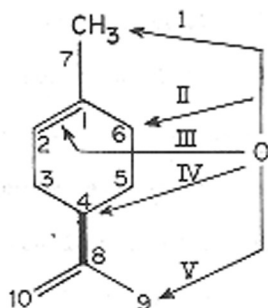
e) Saponification: A mixture of 3.5 gm of diethyl methyl malonate and a solution of 1.6 gm of NaOH in 10 ml of water were heated on water bath for 4 hours. Sodium methyl malonate separated out. This salt as such was used for further experimental work.

CHAPTER 3

PERACETIC ACID OXIDATION OF (+)-LIMONENE

DISCUSSION

From the structures of the isolated products from limonene fermentation, Dhavalikar and Bhattacharyya (1966) concluded that there are several foci in the limonene nucleus which are susceptible to oxygenation by the microorganism. The situation can be summarized structurally as shown below.



The attack on the methyl group (I) leads to the formation of perillyl alcohol which is in the main energy yielding pathway. Oxygenation by (II) at allylic position -6 is responsible for the formation of (+)cis carveol and possibly (+) carvone. Pathway (III) may account for 8-p-menthene-1,2-trans diol, 8-p-menthene 1,2-cis diol and possibly (+) dihydrocarvone. An oxygenation at position 4 (IV) will account for the formation of 4,9-dihydroxy-1-p-menthene-7-oic acid from limonene (1) and the pinenes (2) and (3). However Shukla (1966) postulated the mediation of Δ^1 -p-menthenoid cation with the charge at position -4. This may also have been responsible for the 4 hydroxylated acids from α and β -pinenes. The evidence for the hydroxylation at position 9(V) comes from the isolation of 9-hydroxylated

products such as 4,9 dihydroxy-1-p-menthene,7-oic acid, both from limonene (1) and α -pinene (2), the 9-hydroxy p-cymene from p-cymene (5) and the isolation of 6,9 dihydroxy Δ^1 -p-menthene,1,p-menthene,7,9 dicarboxylic acid (in the present investigation). However in the case of limonene the appearance of a 9-hydroxy group could also have been due to a non-Markownikoff addition of water to the 8,9 double bond.

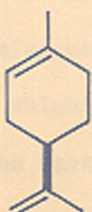
The question whether (+) cis-carveol, (+)carvone could also be derived through the epoxidation of 1,2 double bond was discussed by Dhavalikar and Bhattacharyya (1966). From stereochemical considerations the formation of these compounds by allylic shift of the double bond in the epoxide ring opening was ruled out. However in the current investigation it was considered desirable to develop a chemical model system which is likely to yield the same type of oxidation products as with the fermentation.

It was also considered necessary to synthesize all the neutral metabolites of limonene in order to establish conclusively the structure and stereochemistry of those products. It was anticipated that such studies might give an insight on the oxygenation mechanism involved.

Action of peracetic acid on limonene

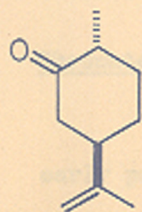
After prolonged peracetic acid oxidation of (+)limonene, the product was hydrolysed and it showed the presence of eight components on TLC examination. A chromatographic separation was, therefore, necessary. In order to avoid high basicity of alumina, which may result in formation of artifacts, silica gel was preferred as the medium for chromatography.

CHART - 7

PRODUCTS FROM PERACETIC ACID
OXIDATION OF (+) LIMONENE

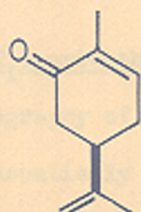
1

(+) LIMONENE



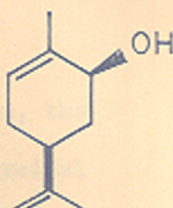
20

(+) DIHYDROCARVONE



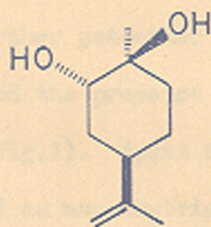
33

(-) CARVONE

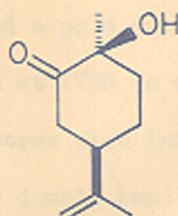


22

(+) CIS-CARVEOL



24

(+) P-MENTHA-8-ENE
TRANS-1,2 DIOL

25

(+) P-MENTHA-8-ENE
1-OL, 2-ONE

Isolation of products

The reaction products after hydrolysis of the acetates were separated into polar and nonpolar fractions by a modified Craig distribution method, a four transfer partitioning between light-petroleum ether and 90% methanol which has been used very successfully by earlier workers to separate the bacterial metabolites. The petroleum ether layer (N_I) mainly consisted of unreacted limonene and nonpolar transformation products. The aqueous methanol (N_{II}) contained more polar hydroxylated transformation products. These two fractions were separately subjected to column chromatography in order to resolve their individual components.

Chromatography of nonpolar (N_I) pet-ether fraction

By a careful chromatography of the nonpolar (N_I) fraction, the early pet-ether eluate was essentially a single compound, the physical properties of which clearly indicated that it was unchanged (+)limonene.

In subsequent pet-ether fractions small amounts of a crystalline compound m.p. 91°C, could be isolated. This however could not be characterized due to paucity of material.

Further pet-ether fractions furnished a pale yellow liquid which showed the presence of a carbonyl band at 1720 in the infrared spectrum (Fig.1). Apart from this, the spectrum also indicated the presence of an unsymmetrically disubstituted double bond at 889 cm^{-1} and 1660 cm^{-1} . A comparison of the physical properties, infrared spectra and the retention times on VPC with an authentic sample established beyond doubt that this compound was (+)dihydrocarvone(20).

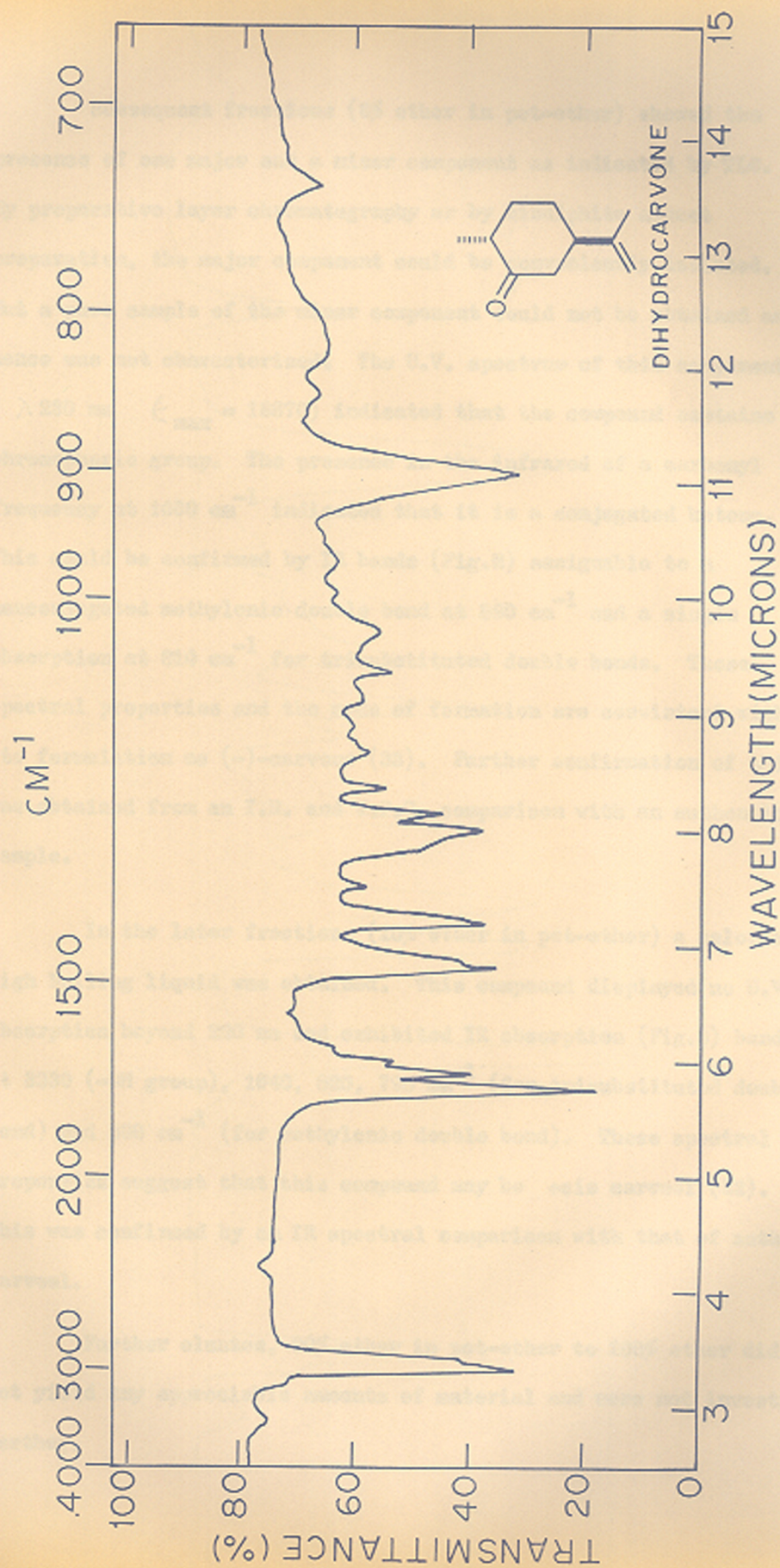


Fig.1

Subsequent fractions (5% ether in pet-ether) showed the presence of one major and a minor component as indicated by TLC. By preparative layer chromatography or by bisulphite adduct preparation, the major component could be conveniently isolated. But a pure sample of the minor component could not be obtained and hence was not characterized. The U.V. spectrum of this component ($\lambda_{230 \text{ m}\mu}$ $\epsilon_{\text{max}} = 18670$) indicated that the compound contains a chromophoric group. The presence in the infrared of a carbonyl frequency at 1660 cm^{-1} indicated that it is a conjugated ketone. This could be confirmed by IR bands (Fig.2) assignable to a nonconjugated methylenic double bond at 890 cm^{-1} and a single absorption at 810 cm^{-1} for trisubstituted double bonds. These spectral properties and the mode of formation are consistent with its formulation as (-)-carvone (33). Further confirmation of this was obtained from an I.R. and V.P.C. comparison with an authentic sample.

In the later fractions (10% ether in pet-ether) a colorless high boiling liquid was obtained. This compound displayed no U.V. absorption beyond $220 \text{ m}\mu$ and exhibited IR absorption (Fig.3) bands at 3330 (-OH group), 1640 , 820 , 790 cm^{-1} (for trisubstituted double bond) and 890 cm^{-1} (for methylenic double bond). These spectral properties suggest that this compound may be +cis carveol (22). This was confirmed by an IR spectral comparison with that of authentic carveol.

Further eluates, 20% ether in pet-ether to 100% ether did not yield any appreciable amounts of material and were not investigated further.

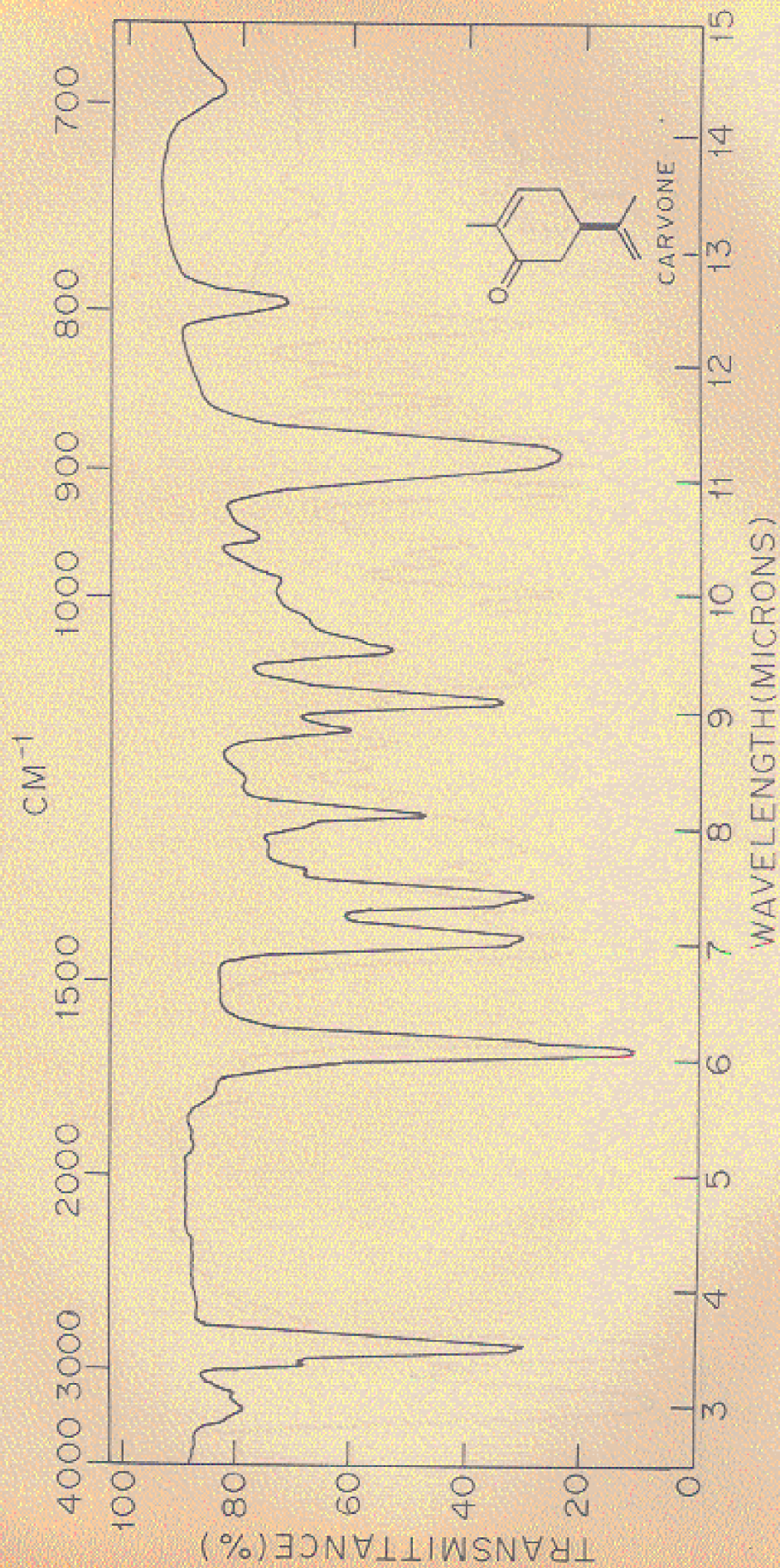


Fig. 2

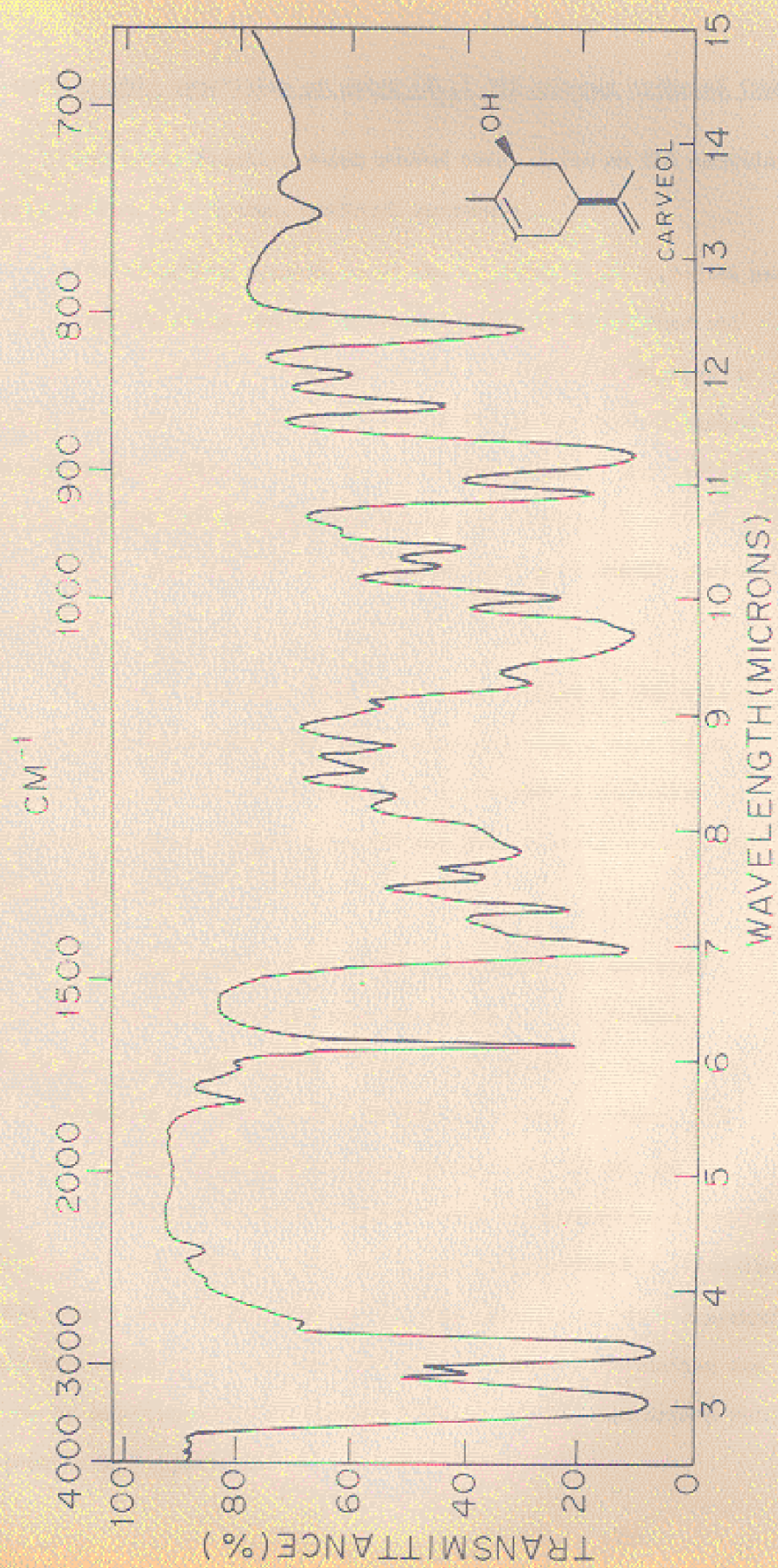


Fig. 3

Chromatographic separation of polar (N_{II}) 90% aqueous methanol fraction

The polar fraction which showed three spots on TLC contained mainly hydroxy and hydroxy carbonyl compounds.

The effective separation of the expected trans 1,2-diol was achieved by following the technique developed by Dhavalikar and Bhattacharyya (1966) as described in the experimental section by taking advantage of the insolubility of the trans diol in carbon tetrachloride. After removal of the trans 1,2 diol, the remaining mixture was chromatographed over silica gel (1:50). The first eluates (pet-ether to 10% ether in pet-ether) gave mostly nonpolar impurities.

The tail fractions (20% ether in pet-ether to 50% ether) though mostly pure were however contaminated with a more polar impurity. In the following fractions (pet-ether + 50% ether to 100% ether) the more polar compound was persistently present in higher amounts.

The purification of these two compounds was achieved effectively by following the same technique as used before (Dhavalikar and Bhattacharyya, 1966). The purity of the faster moving compound was established by TLC in several systems. It displayed no high intensity of absorption above 220 m μ and revealed in its IR spectrum (Fig.5c) bands assignable to a hydroxyl (3450 cm^{-1}) the carbonyl (1704 cm^{-1}) and unsymmetrically disubstituted double bond ($1645, 888\text{ cm}^{-1}$). The identity of this compound (hydroxyketone) with that isolated from limonene fermentation was shown by superimposable IR spectra (Fig.5) and TLC comparison with authentic hydroxyketone.

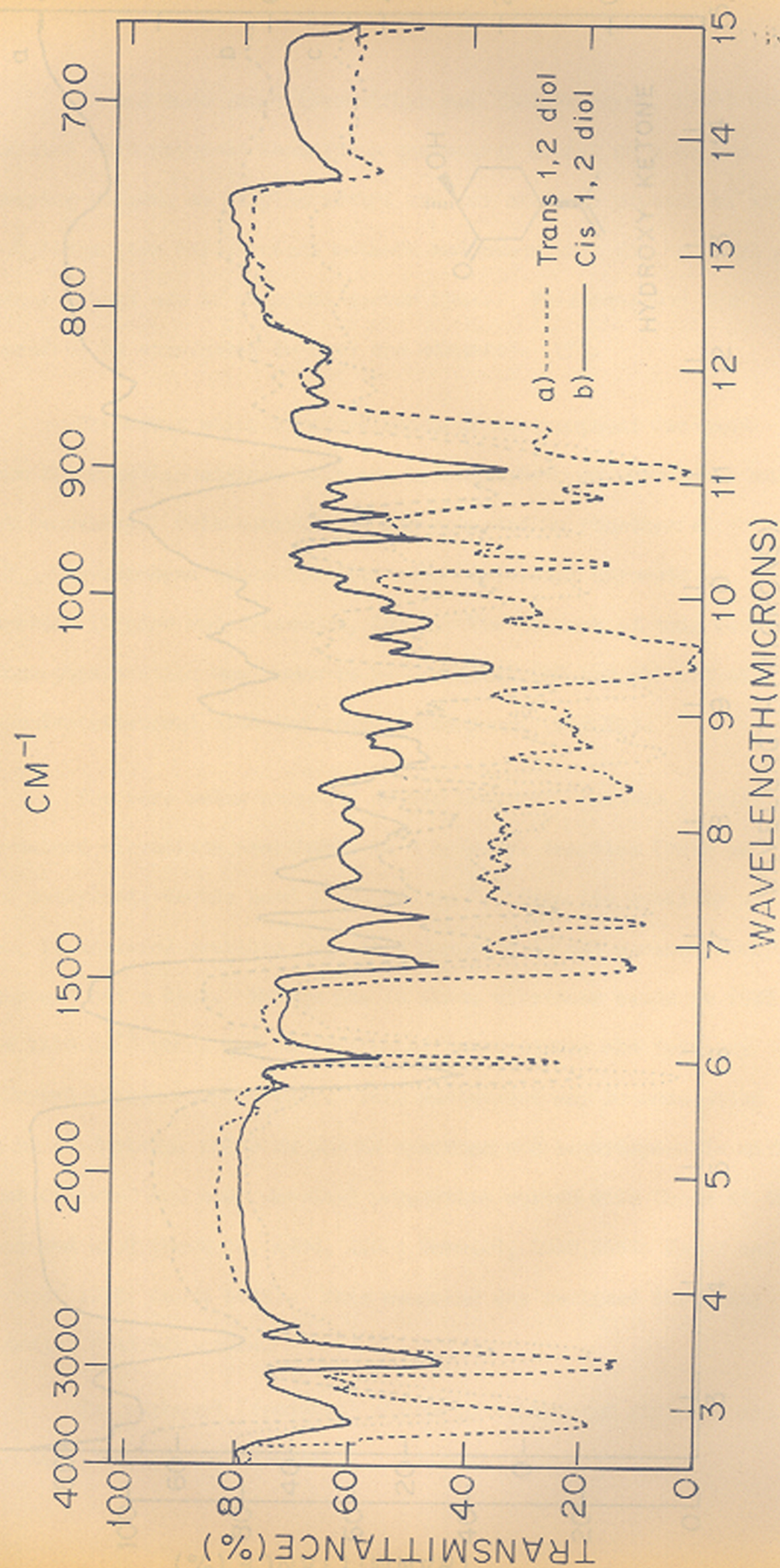


Fig. 4

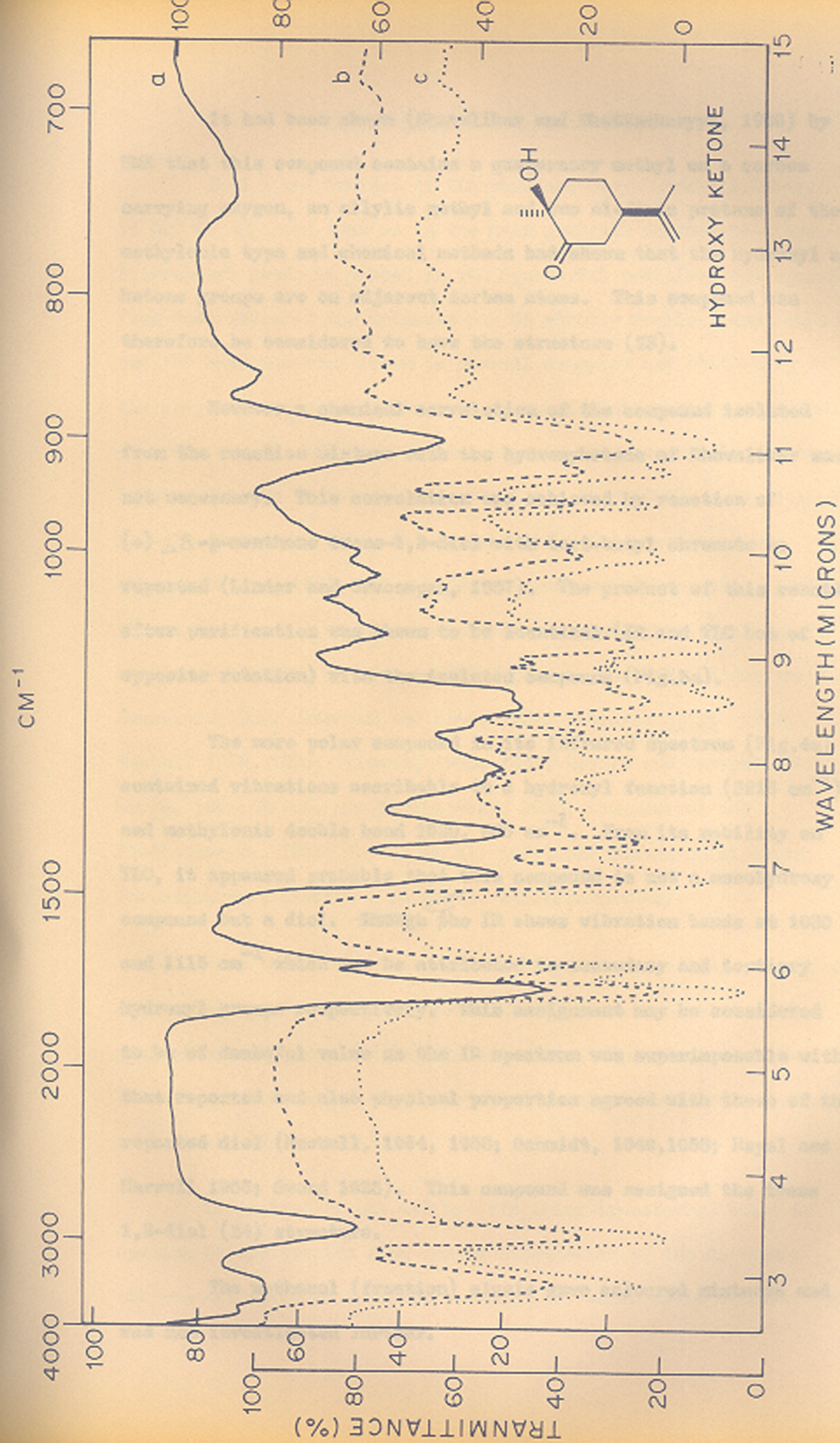


Fig. 5

It had been shown (Dhavalikar and Bhattacharyya, 1966) by NMR that this compound contains a quaternary methyl on a carbon carrying oxygen, an allylic methyl and two olefinic protons of the methylenic type and chemical methods had shown that the hydroxyl and ketone groups are on adjacent carbon atoms. This compound can therefore be considered to have the structure (25).

However a chemical correlation of the compound isolated from the reaction mixture with the hydroxyketone of Dhavalikar was not necessary. This correlation was achieved by reaction of (+) Δ^8 -p-menthene trans-1,2-diol with tert-butyl chromate as reported (Linder and Greenspan, 1957). The product of this reaction after purification was shown to be identical (IR and TLC but of opposite rotation) with the isolated compound (Fig.5a).

The more polar compound in its infrared spectrum (Fig.4a) contained vibrations ascribable to a hydroxyl function (3215 cm^{-1}) and methylenic double bond $1630, 880\text{ cm}^{-1}$. From its mobility on TLC, it appeared probable that this compound is not a monohydroxy compound but a diol. ~~Though~~ The IR shows vibration bands at 1030 cm^{-1} and 1115 cm^{-1} which may be attributed to secondary and tertiary hydroxyl groups respectively. This assignment may be considered to be of doubtful value as the IR spectrum was superimposable with that reported and also physical properties agreed with those of the reported diol (Newhall, 1964, 1958; Schmidt, 1949, 1958; Royal and Harwell 1955; Sword 1925). This compound was assigned the trans 1,2-diol (24) structure.

The methanol (fraction) eluate gave coloured mixtures and was not investigated further.

MECHANISM OF PERACID OXIDATION

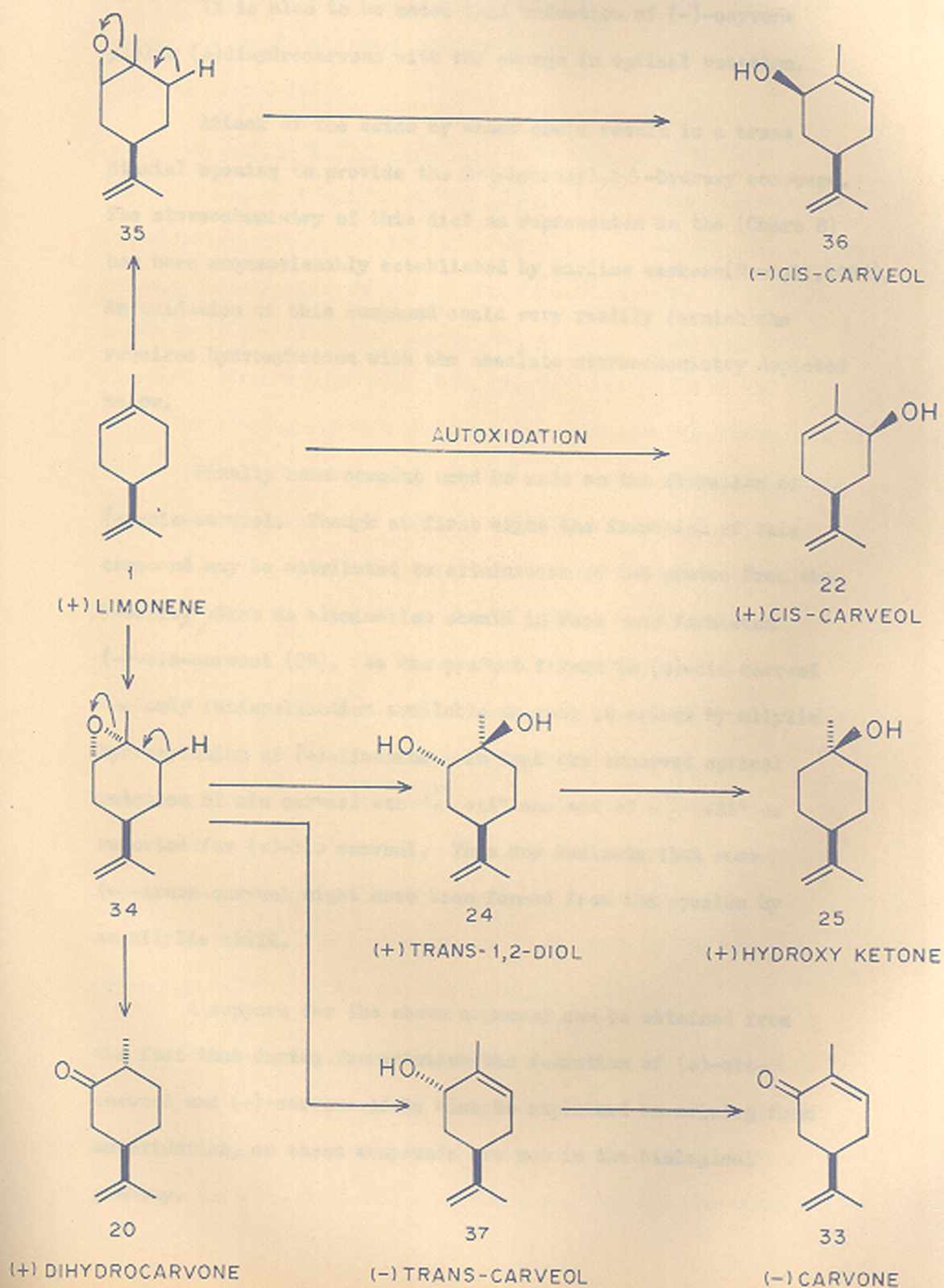
Both α and β oxides of limonene are known (Newhall, 1964).

It may be supposed that as in the most probable conformation the isopropenyl group is placed in the equatorial position, the approach from both sides of the molecule may be equally facile. That this is not the case has been shown in several examples and that entry of the reagent is controlled even by an equatorial substituent which is bulky enough, has been demonstrated in the case of the compound arising through Woodward's hydroxylation. In fact the formation of (+)-dihydrocarvone (20), (-)-carvone (33), (+)-p-mentha-8-ene 1-ol, 2-one (hydroxyketone) (25), all can conveniently arise from the α -oxide (Chart 8). As all of these compounds have rotations equal in magnitude to those earlier reported (Simenson, 1947; Linder and Greenspan, 1957; Johnston and Read, 1934; Scheunck *et al.* 1964; Newhall 1958, 1964; Schmidt, 1949, 1958; Royals and Harwell, 1955; Sword, 1925; Royals and Loffingwell, 1964; Chakravarti and Bhattacharyya, 1954; Royals *et al.* 1951) the mechanism of their formation must involve a concerted reaction pathway.

Thus in the formation of (+)-dihydrocarvone (20), migration of the C-2 hydride (Chart 8) must take place with retention of stereochemistry to yield (+)-dihydrocarvone. The stereochemistry of such acid catalyzed ring opening of epoxides have been studied extensively (Karta 1965).

If instead of the C-2 hydride being transferred there is opening of the epoxide ring due to elimination of the C₆-proton the product would be (-)-trans carveol (37) which could understandably

MECHANISM OF PERACID OXIDATION OF (+) LIMONENE



be oxidized to (-)-carvone (33).

It is also to be noted that reduction of (-)-carvone yields (+)-dihydrocarvone with the change in optical rotation.

Attack of the oxide by water could result in a trans diaxial opening to provide the 2- α -hydroxyl, 1- β -hydroxy compound. The stereochemistry of this diol as represented in the (Chart 8) has been unquestionably established by earlier workers (Newhall, 1964). An oxidation of this compound could very readily furnish the required hydroxyketone with the absolute stereochemistry depicted below.

Finally some comment need be made on the formation of (+)-cis-carveol. Though at first sight the formation of this compound may be attributed to elimination of C-6 proton from the β -oxide, such an elimination should in fact have furnished (-)-cis-carveol (36). As the product formed is (+)-cis-carveol the only rationalisation available is that it arises by allylic hydroxylation of (+)-limonene. In fact the observed optical rotation of cis carveol was $\alpha_D^{25} +11^\circ$ instead of $\alpha_D^{25} +21^\circ$ as reported for (+)-cis carveol. This may indicate that some (-)-trans carveol might have been formed from the epoxide by an allylic shift.

A support for the above argument can be obtained from the fact that during fermentation the formation of (+)-cis-carveol and (+)-carvone could also be explained as arising from autooxidation, as these compounds are not in the biological pathway.

The only remaining difficulty in the mechanism is why in autoxidation the entering -OH group should be cis-placed with respect to the isopropenyl function. No ready explanation for this can be furnished.

SYNTHESIS OF p-MENTHA-8-ENE-CIS-1,2 DIOL (26)

In an approach to the cis glycol one can readily conceive of oxidation with osmium tetroxide to provide a cis-glycol. However in such an oxidation the glycol function is expected to be trans oriented to the directing isopropenyl group. As the required compound does not have this geometry, this method would not be useful in the present case in which not only have the two hydroxyls to be cis to each other, but they must be also cis in relation to the directing isopropenyl group. A useful reaction for achieving this type of geometry has been described by Woodward and Brucher (1937) and Brucher and Evans (1958). This involves the interaction of an olefin with iodine, silver acetate and wet acetic acid to give by way of a neighbouring group participation reaction (Weinstein *et al.* 1942) cis-hydroxyacetate in one operation. Subsequent hydrolysis yields the free diol. It was therefore decided to try this reaction with (+) limonene in the present case. The product obtained from such a reaction was identical in every respect (TLC, IR) with the one isolated from the bacterial fermentation (Dhavalikar and Bhattacharyya, 1966).

This method of achieving this objective was preferred to that reported (Schmidt, 1949, 1959) because even though Schmidt has reported a cis glycol from (+) limonene itself, its mode of formation is very difficult to rationalize. At the same time its

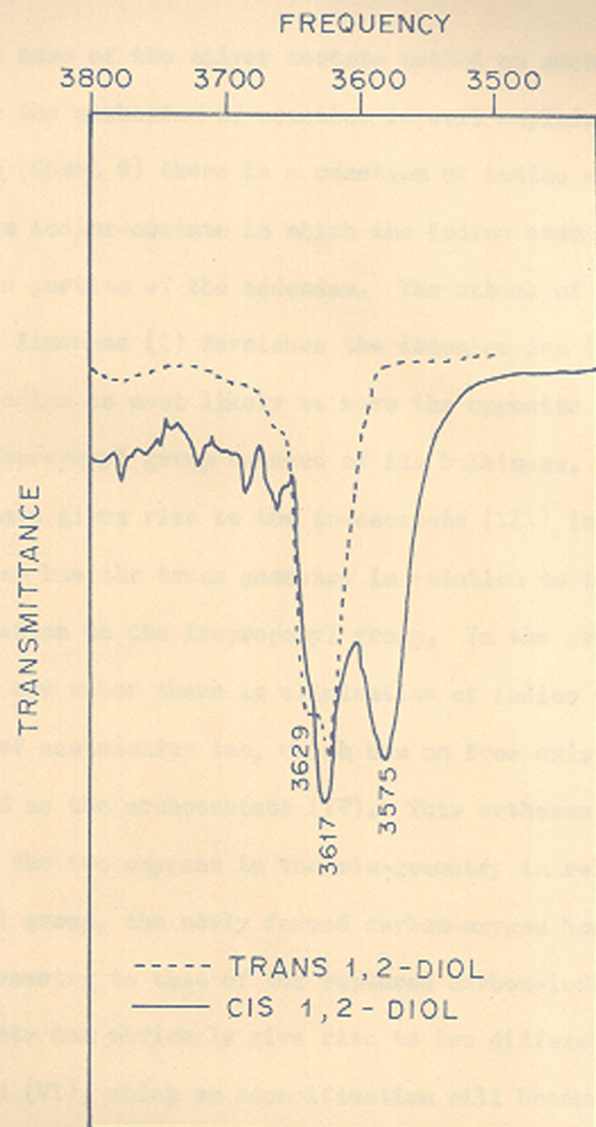


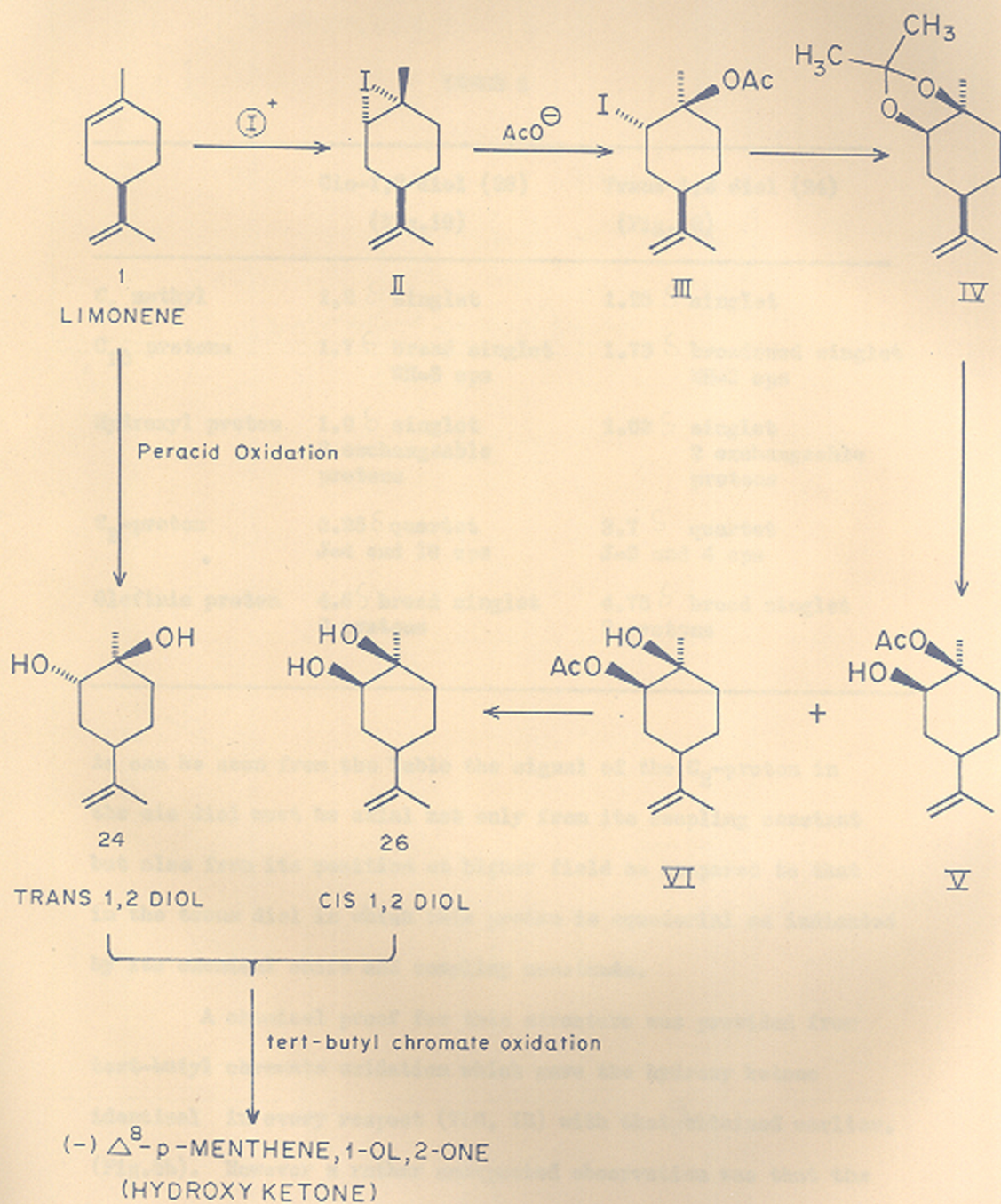
Fig. 6

relationship to the isopropenyl group is not indicated.

MECHANISM FOR CIS-HYD

In the case of the silver acetate method no such ambiguity exists because the mechanism of reaction is well explained. In the first step (Chart 9) there is a reaction of iodine with silver acetate to give iodine-acetate in which the iodine atom will serve as the positive portion of the addendum. The attack of this iodine acetate on (+) limonene (1) furnishes the iodonium ion (II) in which the entering iodine is most likely to have the opposite geometry to that of the isopropenyl group because of its bulkiness. A SN_2 attack by acetate gives rise to the iodoacetate (III) in which the acetate function has the trans geometry in relation to the iodine and cis in relation to the isopropenyl group. In the presence of silver acetate and water there is elimination of iodine through the participation of acetoxonium ion, which has no free existence but can be isolated as the orthoacetate (IV). This orthoacetate will naturally have the two oxygens in the cis-geometry in relation to the isopropenyl group, the newly formed carbon-oxygen bond having the opposite geometry to that of the ruptured carbon-iodine bond. This orthoacetate can obviously give rise to two different hydroxy-acetate (V) and (VI), which on saponification will become the identical glycols in which the two hydroxyls and the isopropenyl group are cis-related (26). Compound (26) in I.R. (Fig.4b) showed hydroxy groups at ν_{max} 3335, 1056, 1112 cm^{-1} and an exocyclic double bond at 886 cm^{-1} . The most noteworthy distinguishing feature between the trans and the cis diols is the appearance of two distinct hydroxyl bands (Perkin-Elmer, in carbon tetrachloride) in the case of cis diol. In that of the trans diol only one band is observed (Fig.6).

MECHANISM FOR CIS-HYDROXYLATION



A comparison of the PMR spectrum of the diol obtained by Woodward's hydroxylation method and that obtained during peracetic acid oxidation of (+) limonene is shown in Table 1.

TABLE 1

	Cis-1,2 diol (26) (Fig.10)	Trans 1,2 diol (24) (Fig. 9)
C ₁ methyl	1.2 δ singlet	1.26 δ singlet
C ₁₀ protons	1.7 δ broad singlet WH=3 cps	1.73 δ broadened singlet WH=3 cps
Hydroxyl proton	1.9 δ singlet 2 exchangeable protons	1.63 δ singlet 2 exchangeable protons
C ₂ -proton	3.25 δ quartet J=4 and 10 cps	3.7 δ quartet J=2 and 4 cps
Olefinic proton	4.6 δ broad singlet 2 protons	4.75 δ broad singlet 2 protons

As can be seen from the Table the signal of the C₂-proton in the cis diol must be axial not only from its coupling constant but also from its position at higher field as compared to that in the trans diol in which this proton is equatorial as indicated by its chemical shift and coupling constants.

A chemical proof for this structure was provided from tert-butyl chromate oxidation which gave the hydroxy ketone identical in every respect (TLC, IR) with that obtained earlier. (Fig.5b). However a rather unexpected observation was that the rotation of the hydroxyketone was opposite to that obtained from

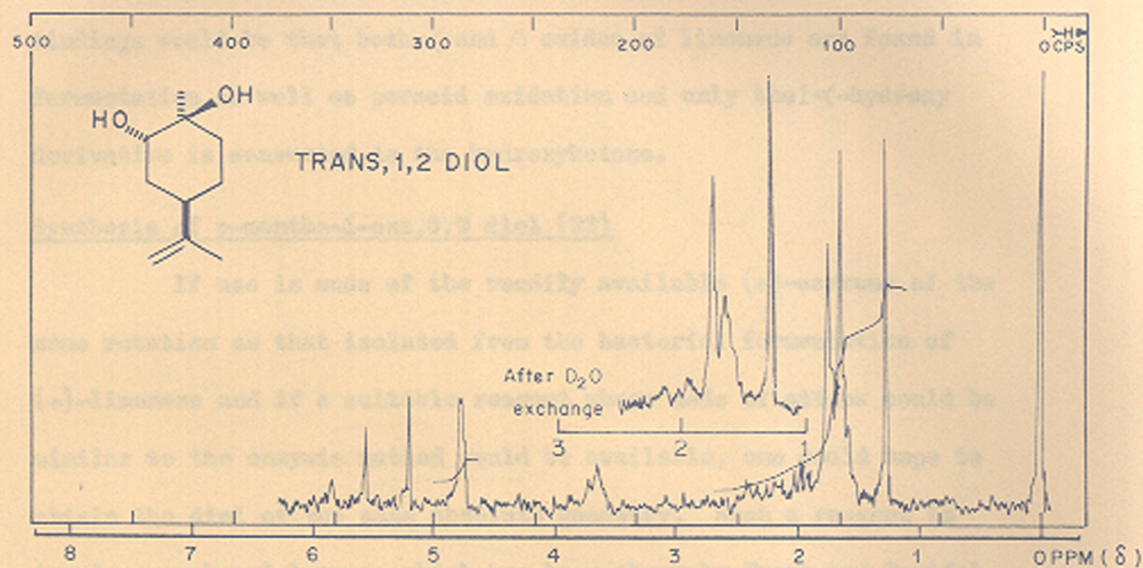


FIG. 9.

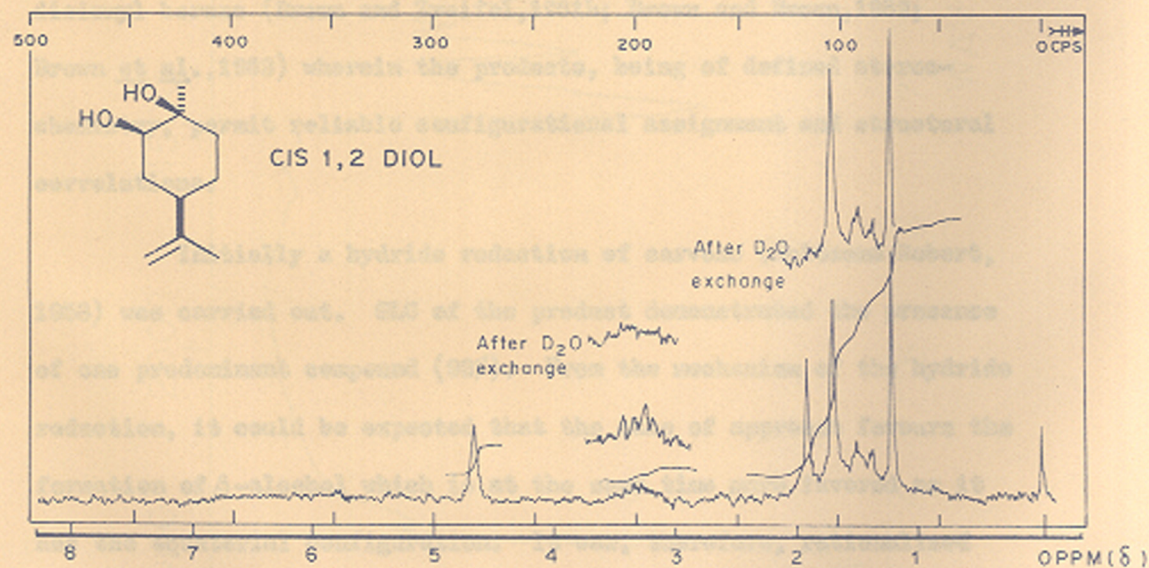


FIG. 10.

fermentation of limonene. The fermentation product showed positive rotation. The only conclusion that would satisfy these findings would be that both α and β oxides of limonene are found in fermentation as well as peracid oxidation and only the α -hydroxy derivative is converted to the hydroxyketone.

Synthesis of p-mentha-1-ene,8,9 diol (23)

If use is made of the readily available (+)-carvone of the same rotation as that isolated from the bacterial fermentation of (+)-limonene and if a suitable reagent whose mode of attack could be similar to the enzymic method would be available, one could hope to obtain the diol of the same absolute geometry. Such a reagent is isopinocampheyl borane, which has been shown by Brown and Zweifel (1961a) to yield optically active products from optically active starting materials. However there was a possibility that this reagent would attack either or both the double bonds in carveol. From this consideration preference was given to the more selective disiamyl borane (Brown and Zweifel, 1961b; Brown and Brown, 1962; Brown *et al.*, 1963) wherein the products, being of defined stereochemistry, permit reliable configurational assignment and structural correlations.

Initially a hydride reduction of carvone (Reitsem Robert, 1953) was carried out. GLC of the product demonstrated the presence of one predominant compound (93%). From the mechanism of the hydride reduction, it could be expected that the ease of approach favours the formation of β -alcohol which is at the same time more favored as it has the equatorial configuration. It was, therefore, rationalized that the major compound was the cis alcohol. Experimental confirmation

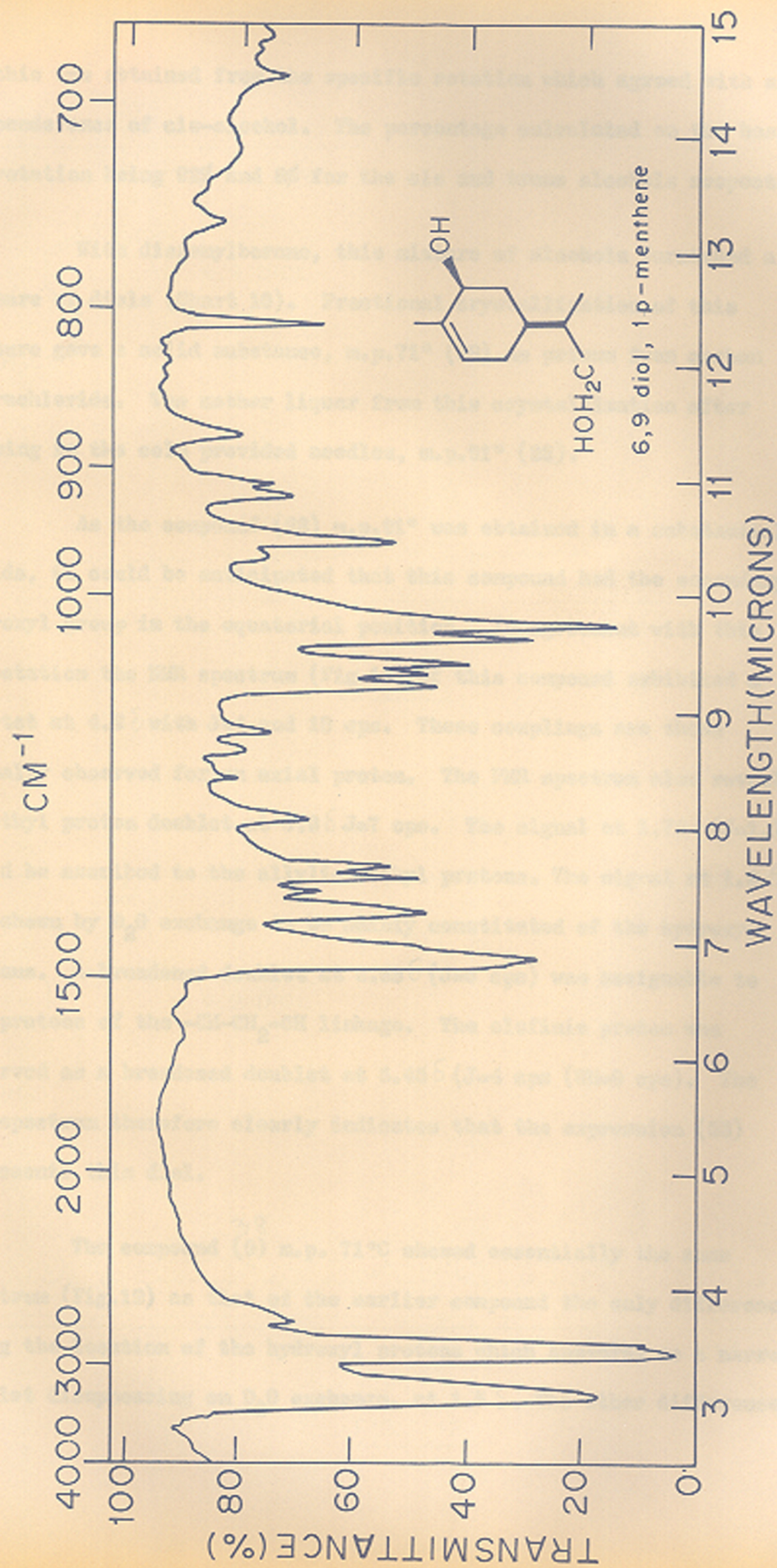


Fig. 7

of this was obtained from the specific rotation which agreed with a preponderance of cis-alcohol. The percentage calculated on the basis of rotation being 92% and 8% for the cis and trans alcohols respectively.

With disiamylborane, this mixture of alcohols furnished a mixture of diols (Chart 10). Fractional crystallization of this mixture gave a solid substance, m.p. 71° (38) as prisms from carbon tetrachloride. The mother liquor from this crystallization after keeping in the cold provided needles, m.p. 91° (23).

As the compound (23) m.p. 91° was obtained in a substantial yields, it could be anticipated that this compound had the secondary hydroxyl group in the equatorial position. In agreement with this expectation the NMR spectrum (Fig. 11) of this compound exhibited a quartet at 4.2 δ with $J=4$ and 10 cps. These couplings are those normally observed for an axial proton. The PMR spectrum also revealed a methyl proton doublet at 0.9 δ ($J=7$ cps). The signal at 1.75 δ ($J=1.5$ cps) could be ascribed to the allylic methyl protons. The signal at 1.8 δ was shown by D₂O exchange to be mainly constituted of the hydroxyl protons. A broadened doublet at 2.53 δ ($J=6$ cps) was assignable to the protons of the -CH-CH₂-OH linkage. The olefinic proton was observed as a broadened doublet at 5.45 δ ($J=4$ cps (WH=9 cps)). The PMR spectrum therefore clearly indicates that the expression (23) represents this diol.

The compound (9) ³³m.p. 71°C showed essentially the same spectrum (Fig. 12) as that of the earlier compound the only difference being the location of the hydroxyl protons which appeared as a narrow singlet disappearing on D₂O exchange, at 1.5 δ . The other difference

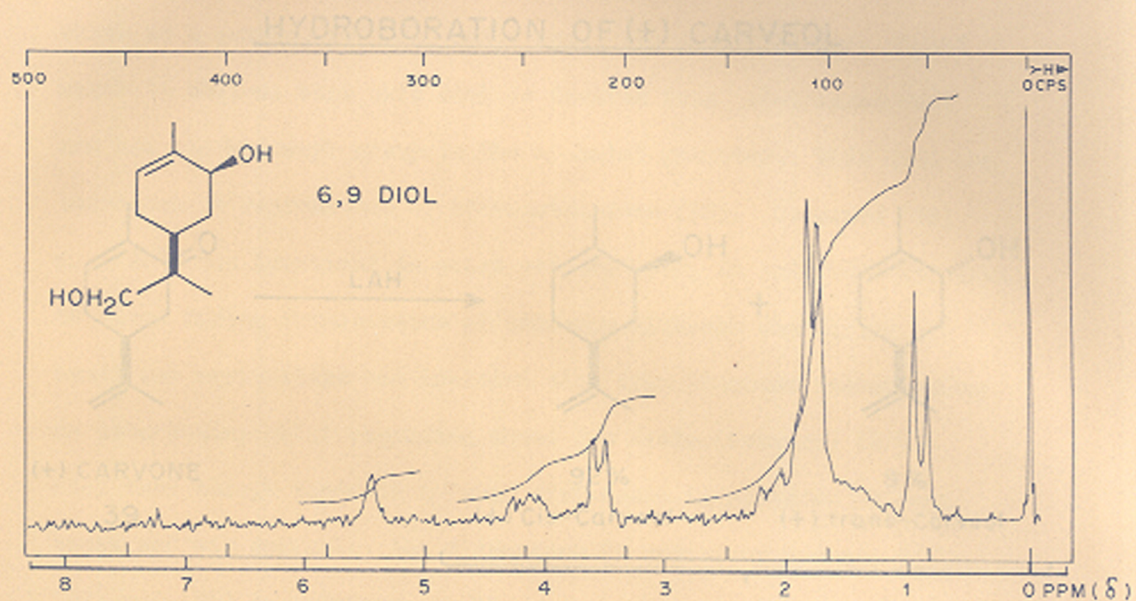


FIG. 11.

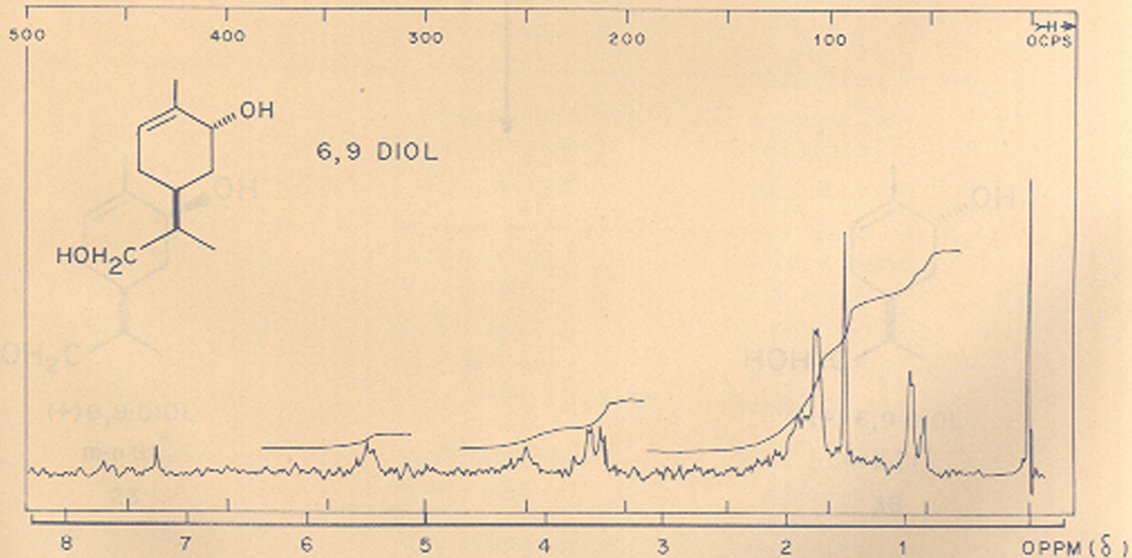
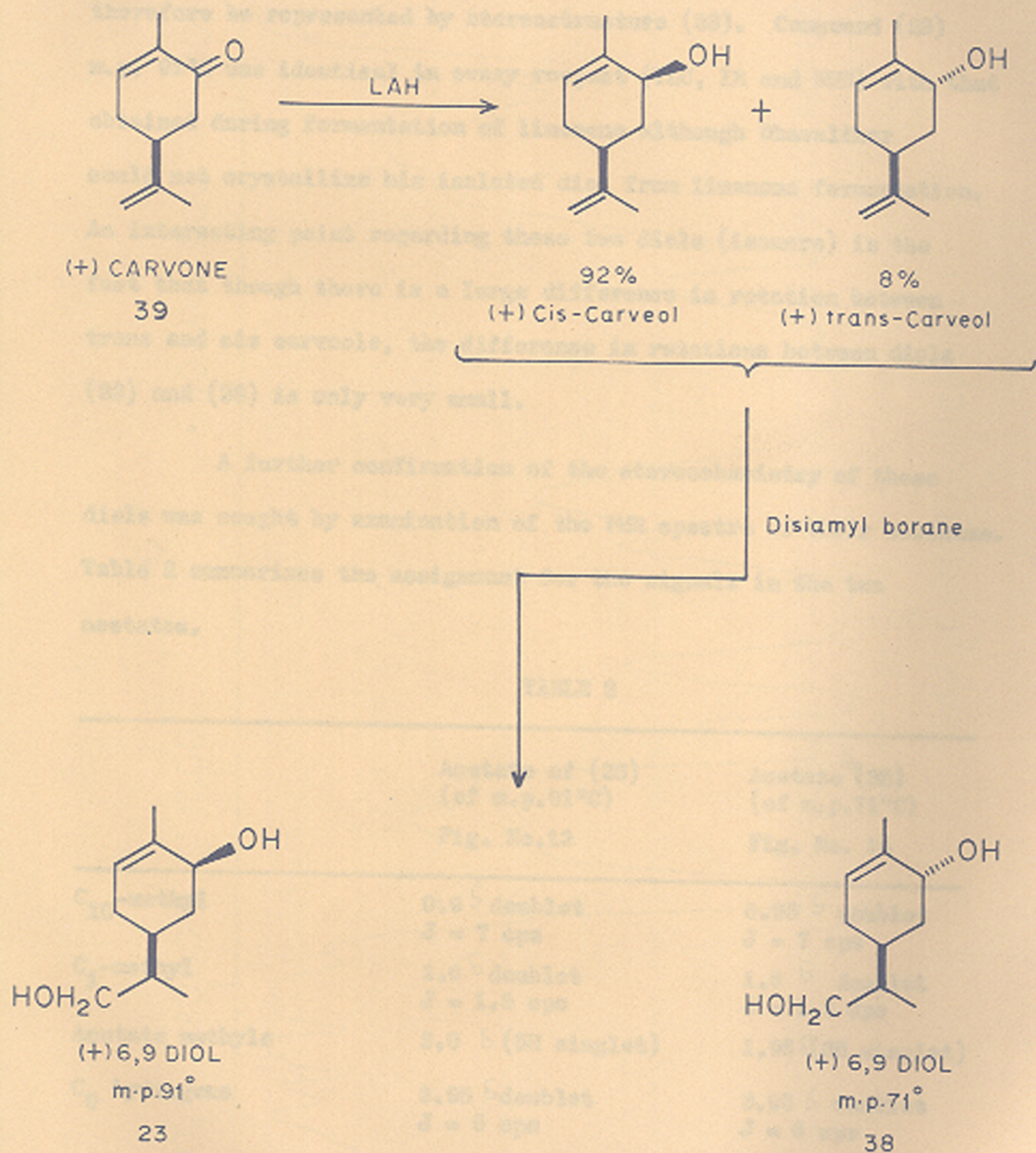


FIG. 12.

HYDROBORATION OF (+) CARVEOL



from the earlier spectrum is in the nature of the signal at 4.2 δ which is a quartet with $J=3$ and 6 cps. The nature of this signal makes it obvious that this diol is derived from (+)-transcarveol and has its hydroxyl group in the α -axial geometry. This diol can therefore be represented by stereostructure (38). Compound (23) m.p. 91°C was identical in every respect (TLC, IR and NMR) with that obtained during fermentation of limonene although Dhavalikar could not crystallize his isolated diol from limonene fermentation. An interesting point regarding these two diols (isomers) is the fact that though there is a large difference in rotation between trans and cis carveols, the difference in rotations between diols (23) and (38) is only very small.

A further confirmation of the stereochemistry of these diols was sought by examination of the PMR spectra of their acetates. Table 2 summarizes the assignment for the signals in the two acetates.

TABLE 2

	Acetate of (23) (of m.p. 91°C) Fig. No. 13	Acetate ^a (38) (of m.p. 71°C) Fig. No. 14
C ₁₀ -methyl	0.9 δ doublet J = 7 cps	0.95 δ doublet J = 7 cps
C ₁ -methyl	1.6 δ doublet J = 1.5 cps	1.6 δ doublet J = 1.5 cps
Acetate methyls	2.0 δ (6H singlet)	1.96 δ (6H singlet)
C ₉ hydrogens	3.95 δ doublet J = 6 cps	3.95 δ doublet J = 6 cps
C-2 proton C-6 proton	Two proton signal around 5.45 δ (singlet)	Two proton singlet around 5.45 δ

(Comparative NMR studies of acetates - 40 and 41)

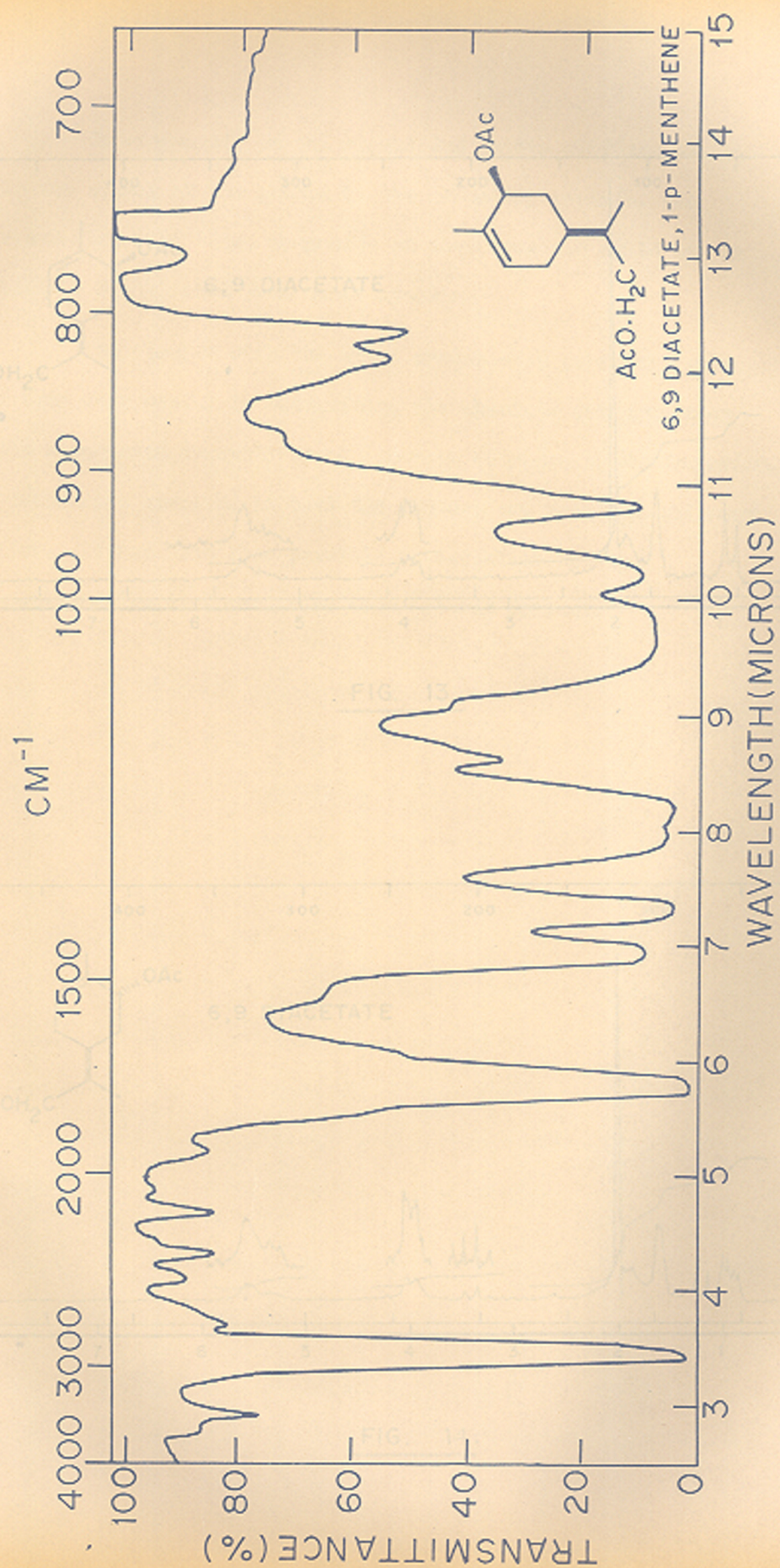


Fig. 8

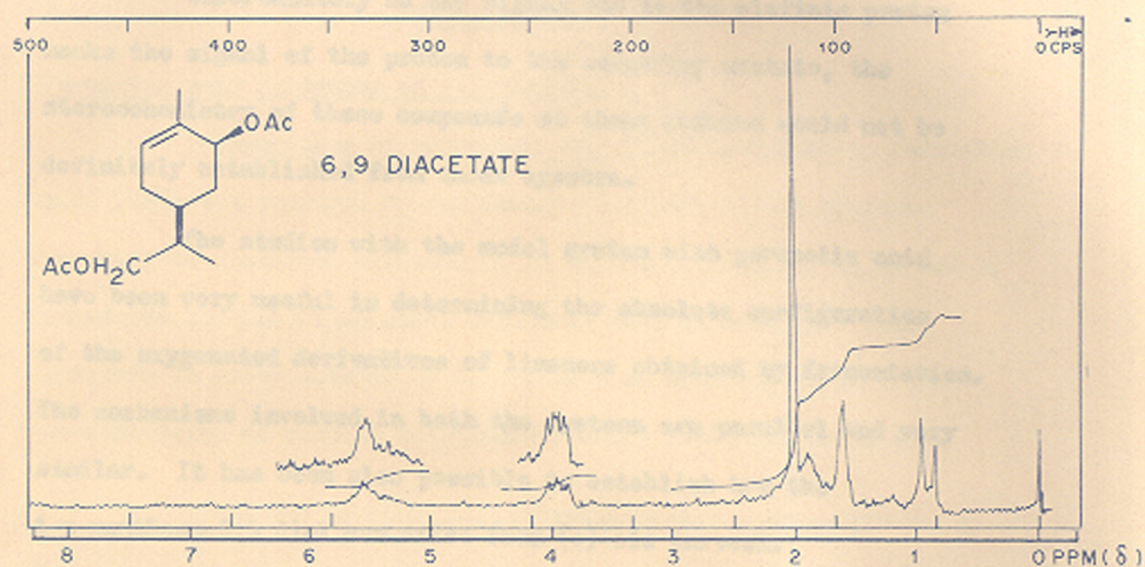


FIG. 13.

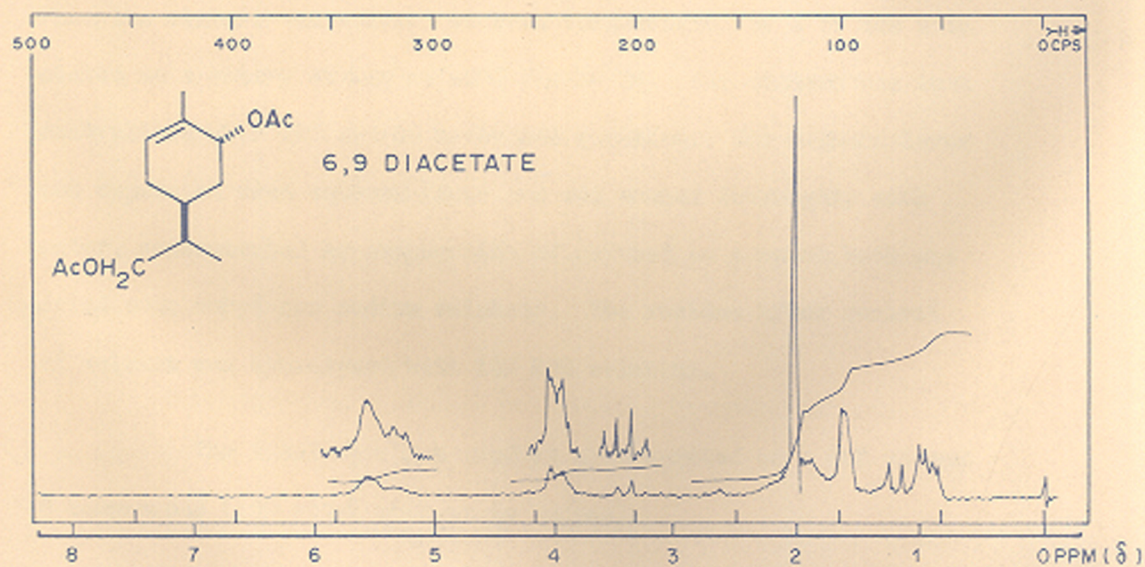


FIG. 14.

Unfortunately as the signal due to the olefinic proton masks the signal of the proton to the secondary acetate, the stereochemistry of these compounds at these carbons could not be definitely established from these spectra.

The studies with the model system with peracetic acid have been very useful in determining the absolute configuration of the oxygenated derivatives of limonene obtained by fermentation. The mechanisms involved in both the systems are parallel and very similar. It has been also possible to establish how the 1-p-menthene-5,9 diol can arise from (+)-cis carveol.

EXPERIMENTAL

Peracetic acid oxidation of (+)-limonene

Preparation of peracetic acid (Greenspan, 1946):- Peracetic acid (40%) was prepared by cautiously adding (40 ml) 30% hydrogen peroxide to a stirred solution of 10 ml acetic acid, 10 ml of acetic anhydride and 0.5 ml of conc. sulphuric acid mixture in a flask immersed in water bath at 25°C. After 4 hr standing at 15°C the concentration of peracid was iodimetrically estimated to be 40%.

In a 250 ml three-necked flask fitted with a stirrer, a thermometer and a dropping funnel were placed 20 gm of freshly distilled (+) limonene in 100 ml chloroform. To this mixture pre-cooled to 15°C were added 40 ml of 40% peracetic acid slowly through the dropping funnel with vigorous stirring, maintaining the temperature below 40°C. The entire lot of peracid was added within 45 min. Then the mixture was stirred at room temperature for 2 hr and then shaken on a rotary shaker at 28°C for 30 hr. The mixture was then neutralized with 10% sodium carbonate solution. The organic layer was separated from aqueous layer and was washed thoroughly with water, concentrated by evaporation of solvent on a water bath and dried over anhydrous sodium sulphate. The residue after removal of solvent was hydrolysed with 10% KOH solution.

The total reaction product when spotted on a TLC showed 8 components (10% ethyl acetate in hexane).

The product was separated into polar and nonpolar fractions by a modified four transfer Craig partitioning between

light petroleum ether and 90% aqueous methanol (Dhavalikar and Bhattacharyya, 1966).

The petroleum ether layer consisting mainly of unreacted limonene and nonpolar (N_I) fraction was passed over silica gel (1:50). The aqueous methanol layer (N_{II}) containing more polar hydroxylation transformation products similarly subjected to silica gel chromatography.

Chromatographic separation of nonpolar (N_I) products

A total of 6 gm of the neutral fraction (N_I) was chromatographed over silica gel (300 gm) and was eluted with the usual solvents (as described in Materials and Methods).

The first fraction eluted with pet ether was a liquid which was purified by distillation under reduced pressure b.p. 90°-95°/1 mm and identified as unreacted limonene (4.8 gm).

In the subsequent pet-ether eluates a crystalline compound (180 mg) separates out which was crystallized from pet. ether chloroform as prisms, m.p. 91°C.

IR ν max 3000, 1480, 1390, 1380, 1285, 1240, 1200, 1010, 958, 900, 855 (not shown).

This compound could not be further characterized due to paucity of material.

The next pet-ether eluate fraction showed a single spot (5% ethyl-acetate in hexane). This was distilled under reduced pressure 85-87°C/0.5 mm giving 150 mg of the pure material (20).

$\alpha_D^{25} +11^\circ$. Reported $\alpha_D^{25} +23^\circ$.

Found: C 78.93, H 10.88. $C_{10}H_{16}O$ requires: C 78.89, H 10.59%.

IR (Fig. 1) $\nu_{\max}$ 1712, 1645, 1450, 1378, 1320, 1267, 1250, 1220, 1182, 1142, 1110, 1085, 1055, 1020, 958, 890, 840, 792 cm^{-1} .

The physico-chemical and spectral properties of this compound were in agreement with those reported for dihydrocarvone (Compound 20) (Simenson, 1947; Royals and Leffingwell, 1964; Chakravarti, 1954).

Subsequent fractions (5% ether in pet-ether) showed the presence of one major and one minor component as indicated by TLC (10% ethyl acetate in hexane). Either by preparative layer chromatography or by sodium bisulphite adduct formation, the major component could be conveniently isolated since the mixture showed an UV absorption at λ_{235}^{max} (E_{max} 18670) suggesting the presence of α,β -unsaturated carbonyl compound which are known to form the adduct with bisulphite. Total eluate (200 mg) was taken up in ether (20 ml) and extracted with sodium sulphite and sodium hydrogen carbonate. The aqueous extract after hydrolysis with sodium carbonate (10 ml) gave a red coloured compound which was extracted with ether. After distillation, α,β -unsaturated ketone was obtained (33) (180 mg)

b.p. 85-90° bath at 0.5 mm. $\alpha_D^{25} = -54^\circ$; reported $\alpha_D = -62.46^\circ$.

Found: C 80.23, H 9.86. $C_{10}H_{14}O$ requires: C 79.95, H 9.35%.

IR (Fig. 2) $\nu_{\max}$ 1675, 1645, 1450, 1248, 1365, 1245, 1140, 1112, 1057, 1050, 1027, 1000, 960, 890, 800, 703 cm^{-1} .

On the basis of these physical properties this compound was identified as (-)-carvone (33) (Eschinazi, 1953; Simenson, 1947; Linder and Greenspan, 1957; Royals et al. 1951).

The fraction eluted with 10% ether in pet-ether was found to be a liquid (165 mg) and contained three components which were separated into individual components by preparative layer chromatography (10% ethyl acetate in hexane). The fastest moving compound was found to be carveol (22) by comparative TLC, VPC and IR spectroscopy with an authentic sample of carveol which has been further purified by distillation under reduced pressure b.p. 90-95° bath/0.75 mm to give compound (22).

$$\alpha_D^{25} = +11^\circ; \text{ reported } \alpha_D = +21^\circ.$$

Found: C 78.57, H 10.92, $C_{10}H_{16}O$ requires: C 78.89, H 10.59%.

I.R. (Fig.3) $\nu_{\max}$ 3355, 1645, 1440, 1372, 1080, 1057, 1030, 888, 810 cm^{-1} (Simenson, 1947; Linder and Greenspan, 1957; Johnston and Read, 1934; Schenck *et al.* 1964).

Later fraction, 20% ether in pet-ether to 100% ether did not yield any appreciable amount of material.

Chromatographic separation of polar fraction 90% methanol (N_{II})

The polar fraction which contained mainly hydroxy and ketohydroxy compounds showed 3 spots on TLC (15% ethyl acetate in hexane).

The separation of the expected trans p-menth 8-ene 1,2 diol (a dihydroxy compound) was achieved by taking the advantage of the insolubility of the dihydroxy compound in carbon tetrachloride in cold. To a 3.5 gm aliquot of polar fraction 25 ml of light petroleum ether was added. The mixture was cooled in ice-salt bath and treated with 25 ml of cold carbon tetrachloride with scratching. A colourless crystalline compound (24) crystallized

out (1.8 gm) which was filtered off. Recryst. benzene-chloroform
m.p. 66°C.

$\alpha_D^{25} +36^\circ$.

Found: C 70.68, H 10.79. $C_{10}H_{18}O_2$ requires: C 70.54, H 10.66%.

U.V. absorption λ_{max} 209 m μ (E_{max} 3600). IR (Fig. 4a) ν_{max} 3475, 1637, 1450, 1370, 1290, 1255, 1236, 1180, 1158, 1128, 1050, 1037, 1000, 960, 945, 910, 888, 863, 820, 780, 725 cm^{-1} . (NMR Fig. 9).

This compound was assigned the structure (24) (Schmidt, 1949; 1958; Newhall, 1964; Royals and Harwell, 1955; Newhall, 1958; Sword, 1925).

The mother liquor (1.7 gm) after removing diol was concentrated on water bath and chromatographed over silica gel (1:50) and the column was eluted with usual solvents. The column was first eluted with pet-ether to remove traces of nonpolar impurities (0.130 gm).

The fractions eluting with 10% and 20% ether in pet-ether were found to contain traces of carvone, carveol and dihydrocarvone (0.470 gm). The next fraction eluting with 20% to 50% ether in pet-ether was found to contain two components, one in major amount. The major component was purified by removing more polar diol by following the procedure described before to separate the diol. After removing the traces of diol (0.090 gm) the pet-ether soluble compound was distilled under vacuum (0.450 gm) and was characterized to be hydroxy ketone (25) by a comparison of its physico-chemical and spectral properties with those of authentic hydroxy ketone obtained by tert. butyl chromate oxidation of trans diol.

$\alpha_D^{25} +49^\circ$.

Found: C 71.43, H 9.87%. Calculated for $C_{10}H_{16}O_2$ C 71.39, H 9.59%.

IR (Fig. 5c) $\nu_{\max}$ 3450, 1700, 1645, 1450, 1375, 1175, 1125, 1050, 1032, 910, 887, 800, 732 cm^{-1} .

The last fraction eluted with ether was found to be pure diol (0.500 gm), m.p. 66°C.

Tert.butyl chromate oxidation of Δ^8 -p-menthene trans 1,2 diol
(Linder and Greenspan, 1957)

Chromium trioxide (1.293 gm) in 3 ml benzene was added in small portions to 6.5 ml of tert.butyl alcohol (dry) with cooling. The solution was then diluted with 2 ml dry benzene and dried over anhydrous magnesium sulphate. This solution was added slowly under cooling to 2 gm of anhydrous diol (24) in 10 ml of dry benzene, keeping the temperature between 25-30°C for 2 hrs. The complex was then hydrolyzed by addition in succession 12 ml of water, 1.6 gm of oxalic acid and 12 ml of 20% sulphuric acid with stirring at 25°C. After 3 hr the mixture separated into two layers. The aqueous layer was extracted with 5 ml of dry benzene. The pooled organic layer was then washed with alkali and water, dried over anhydrous magnesium sulphate and concentrated. The product after distillation was found to be single on TLC.

$\alpha_D^{25} = -52^\circ$.

Found: C 71.37, H 9.95. $C_{10}H_{16}O_2$ requires, C 71.39, H 9.59%.

I.R. (Fig. 5a) $\nu_{\max}$ 3450, 1700, 1645, 1450, 1375, 1175, 1125, 1050, 1032, 710, 887, 850, 800, 732 cm^{-1} .

On the basis of these properties the product was identified as hydroxy ketone (8-p-menthene 1-ol 2-one) (25).

cis-Hydroxylation of (+) Limonene

(+) Limonene (5.657 gm; 0.0416 moles) was dissolved in 90.5% glacial acetic acid (150 ml) in a three-necked flask and treated with 16.43 gm (0.098 ml) of silver acetate followed by 11.89 gm (0.0461 mole) of iodine (powdered) crystals in small portions under vigorously stirring reaction mixture over a period of 30 min at room temperature. When all the iodine had been absorbed as indicated by the disappearance of colour of the mixture i.e., (45 min) aqueous acetic acid (19.7 ml prepared by dilution of 2 ml of water upto 50 ml with acetic acid) was added. The reaction mixture was heated upto 90-95° on a boiling water bath for about 3 hr with vigorous stirring. The reaction mixture was then cooled, treated with excess of sodium chloride and filtered. The precipitate was washed with hot benzene. The pooled filtrate was evaporated to a residue which was taken up in methanol, filtered, neutralized with methanolic-KOH and then treated with 3.1 gm of KOH in 30 ml of methanol. The hydrolysis was allowed to proceed overnight in a nitrogen atmosphere. The reaction mixture was neutralized with dil. HCl ('AnalaR') at ice bath temperature. Methanol was removed to give a residue weighing about 6.8 gm. The reaction product was chromatographed over neutral alumina grade-II (1:30 ratio). The impurities were removed by eluting with petroleum ether to 50% ether in pet-ether. The eluate coming in 100% ether gave a colourless TIC pure solid (26). Traces of colouring matter was removed by trituration of this solid with hexane. Crystallized from hexane, as massy needles. m.p. 71.5-73.5°C. Mixed melting point with trans 1,2 diol showed a depression in m.p. i.e., mixture starts melting at 35°C and completely melts at 43°C.

$\begin{array}{c} \diagup \\ \text{C} \\ \diagdown \end{array} + 18^\circ \text{ (10\% acetone)}.$

Found: C 70.47, H 10.66. $C_{10}H_{18}O_2$ requires C 70.54, H 10.66%.

IR (Fig. 4b) γ max 3450, 1655, 1460, 1385, 1180, 1115, 1060, 1030, 970, 900, 863, 820 cm^{-1} . (IR spectrum Perkin-Elmer - Fig. 6a and 6b).

(NMR Fig. 10). On the basis of these properties the structure was assigned to be (26) (Schmidt, 1949, 1958; Bluman and Wood, 1952).

Tertiary butyl chromate oxidation of cis diol

Tertiary butyl chromate oxidation of cis diol resulted in the same hydroxy ketone (25) which was also obtained by tertiary butyl chromate oxidation of trans 1,2 diol (24). The IR spectrum was superimposable (Fig. 5b).

$$\alpha_D^{25} = -51.98^\circ.$$

Lithium Aluminium Hydride reduction of (+)-Carvone

To 3.1 gm of LAH (0.33 eq) in 75 ml dry ether was added dropwise 45 gm (0.3 mole) of (+) carvone in 75 ml of anhydrous ether. After all the carvone had been added the mixture was refluxed for an additional hour. About 15 ml of water was added cautiously to this mixture followed by 200 ml of 10% HCl. The ether layer was separated and the pooled extracts were dried over anhydrous sodium sulphate. Ether was evaporated leaving 41 gm (95%) oil $\alpha_D^{25} = +31.5^\circ$. Distillation of the oil gave about 40 gm of carveol. b.p. 109-111°/12 mm. $\alpha_D^{25} = +33.2^\circ$. Lit. (Johnston and Read, 1934) d-cis carveol b.p. 101°/11 mm. $\alpha_D^{25} = +23.9^\circ$.

Selective hydroboration of (+)-carveol

Preparation of 2-methyl,2-butene (Norris, 1927)

tert-Amyl alcohol (300 gm) was added to an ice-cold mixture of 200 ml conc. sulphuric acid and 400 ml of water. On distillation 180 gm of 2-methyl 2-butene (92%) was obtained, b.p. 36.7 - 38.7°C.

Purification of 2-methyl,2-butene

The distillate washed with water, dried over calcium chloride (anhydrous) and distilled. It was next refluxed for about four hours with metallic sodium until the latter was unaffected. When it was finally distilled the hydrocarbon distilled at 38-40°C.

For hydroboration of carveol double the theoretical amount of disiamyl borane was used.

Purification of diglyme, tetrahydrofuran, boron trifluoride etherate and the diborane and disiamyl borane were carried out as described in earlier studies (Brown and Zweital, 1961; Brown and Brown, 1962).

Hydroboration

Preparation of disiamylborane: Disiamylborane (0.2 mol) was prepared by passing diborane gas (prepared by addition of 5.7 gm (0.15 mole) of sodium borohydride in 20 ml dry diglyme to 28.5 gm of BF_3 -etherate in 20 ml of diglyme) through a pre-cooled solution (0-5°) (28 gm) of freshly distilled 2-methyl,2-butene (0.4 mole) in 80 ml of tetrahydrofuran (perfectly dry) which was kept under nitrogen atmosphere, over a period of 30 min. The semi-solid reaction mixture containing 0.2 mole disiamylborane was allowed to remain at 0°-5° for 20 hr and then used for hydroboration of carveol.

Hydroboration of carveol

To 0.2 mole disiamylborane in tetrahydrofuran at 0°C 15.2 gm (0.1 mole) of carveol was added in nitrogen atmosphere. The mixture was allowed to remain at room temperature for 3 hr. It was then treated with 5 ml of water to hydrolyse any residual hydride followed by 60 ml of 3N sodium hydroxide and 60 ml of 30% hydrogen peroxide. The latter was added slowly (vigorous reaction) maintaining the reaction temperature between 25°-40°. After 2 hr at room temperature, the reaction mixture was extracted with ether, washed with brine followed by ice cold water and then dried over anhydrous sodium sulphate. The solvent was distilled to give a gummy residue weighing about 20 gm.

By passing over alumina grade-I (1:30) unreacted carveol was removed and the diol fraction eluting out in ether was taken up in 20 ml of carbon tetrachloride cooled to -5°C. By scratching the sides of the tube a solid (38) separated which was filtered and washed with cold carbon tetrachloride. It was crystallized from carbon tetrachloride as prisms m.p. 71°C. $E_{\text{max}}^{28} 1753$.

$\alpha_D^{28} = +49^\circ$.

Found: C 70.26, H 10.67. $C_{10}H_{18}O_2$ requires C 70.54, H 10.66%.

IR (Fig. 7) ν_{max} 3300, 2925, 2850, 1460, 1380, 1345, 1325, 1305, 1240, 1140, 1085, 1070, 1065, 1038, 1025, 955, 920, 800 cm^{-1} .

(NMR Fig. 12).

Mother liquor after evaporation and crystallization from chloroform and pet-ether (23) separates out as needles m.p. 91°C.

$E_{\text{max}}^{28} 1645$. $\alpha_D^{28} = +25.25^\circ$.

Analysis: Found C 70.26, H 10.67. $C_{10}H_{18}O_2$ requires C 70.54, H 10.66%.
 IR spectrum is superimposable with the one of compound melting at 71°C.
 (NMR Fig.11).

Acetylation of Diol (23)

To diol (23) (80 mg) pyridine (1 ml) and acetic anhydride (0.8 ml) were added and the mixture was kept overnight at 25°C.
 After working up in the usual manner the product obtained was a liquid (96 mg) b.p. 120-125° bath/4 mm. $\alpha_D^{23} = +32.9^\circ$.
 IR (Fig.8) ν_{max} 1725, 1650, 1440, 1370, 1250, 1170, 1120, 1030, 1000, 950, 920, 860, 802, 790, 740 cm^{-1} . (NMR Fig.13).

Acetylation of Diol (38)

Diol (38) (80 mg) acetylated by following the same procedure. After working up in the usual manner the product was distilled to give (75 mg) gummy material which was purified by distillation. b.p. 115-126°/4 mm. $\alpha_D = +48.39^\circ$.
 IR spectrum (not shown) was identical with the diacetate of (23).
 (NMR Fig.14).

CHAPTER 4

SYNTHESIS, STRUCTURE AND STEREOCHEMISTRY OF β -ISOPROPYL

PIMELIC ACID

SYNTHESIS, STRUCTURE AND STEREOCHEMISTRY OF β -ISOPROPYL PIMELIC ACID

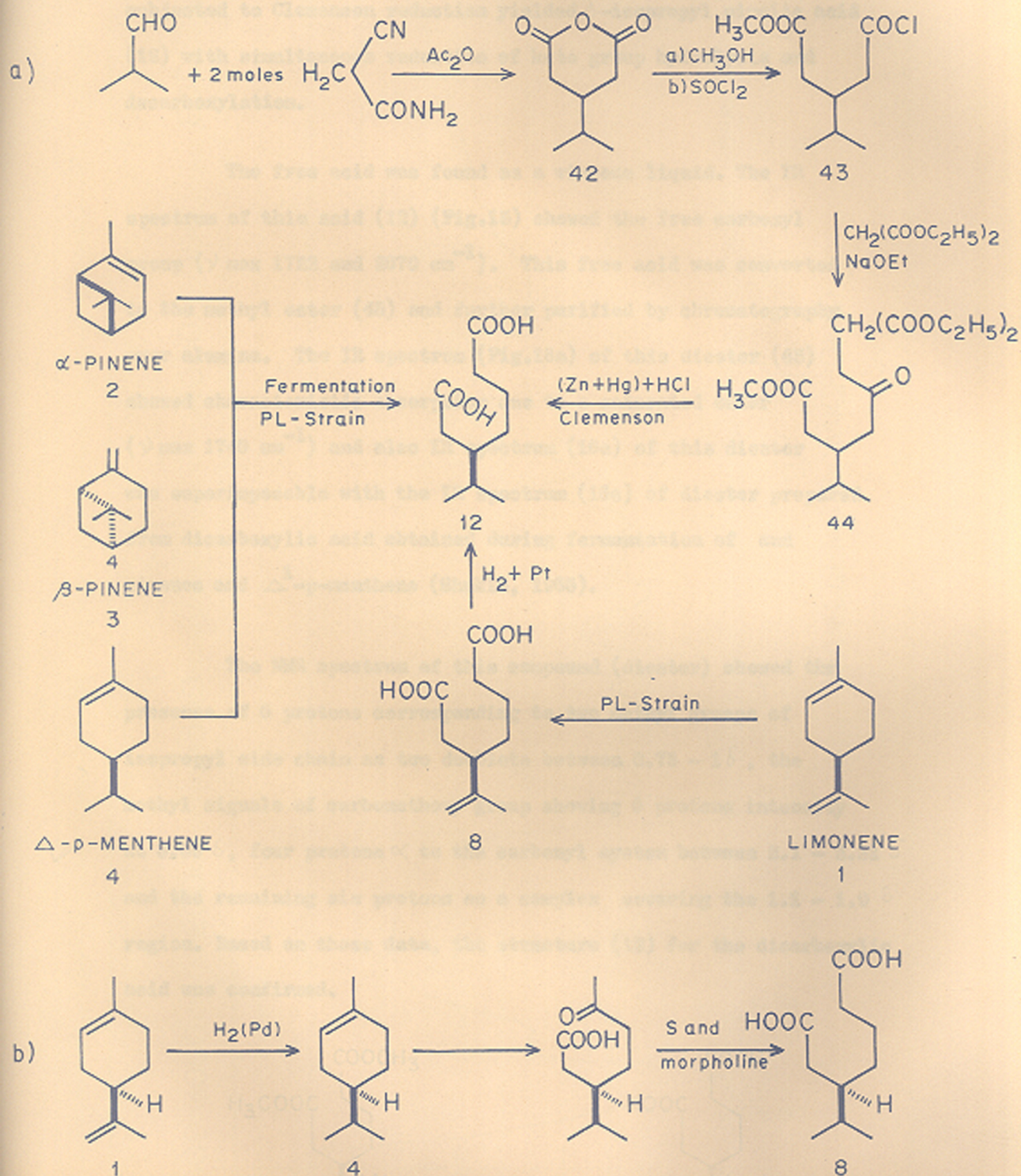
During the studies on the fermentation of limonene and α and β pinenes, two acyclic dicarboxylic acids were isolated and were considered to be the key intermediates in the degradation of these hydrocarbons. The $(-)\beta$ -isopropenyl pimelic acid (8) isolated by Dhavalikar and Bhattacharyya (1966) from limonene was shown by these workers to be structurally and stereochemically correlated with the saturated $(-)\beta$ -isopropyl pimelic acid (12), a degradation product of both α and β pinenes. It may be noted that all the other products obtained from limonene and α -pinene had positive specific rotation, whereas those from β -pinene had negative specific rotations. The β -isopropyl pimelic acid (12) on the other hand had identical specific rotation irrespective of its origin from $(+)\alpha$ or $(-)\beta$ -pinene. It was, therefore, considered necessary to establish the structure of these important intermediates by unequivocal methods of synthesis and to correlate stereochemically both the structures of d-limonene and α -pinene which have been established rigorously.

Further the paucity of material obtainable by fermentation made work on the further biodegradation rather slow and time-consuming. From these considerations also the development of a chemical synthesis of β -isopropyl pimelic acid (12) became urgent.

A. Total synthesis of β -isopropyl pimelic acid (12)

Condensation of isobutyraldehyde with two moles of cyanoacetamide (Chart 11a) gave β -isopropyl glutaric acid which yielded its anhydride (42) by treating with acetic anhydride. The anhydride (42) was methanolysed to the monomethyl ester and this monoester

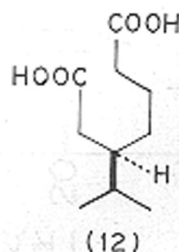
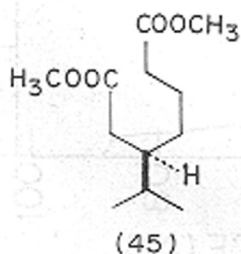
SYNTHESIS OF DICARBOXYLIC ACID



was converted to the corresponding acid chloride (43) by refluxing with thionyl chloride. Compound (43) when condensed with one mole of malonic ester gave a ketotetracarboxylic ester (44), which, when subjected to Clemenson reduction yielded β -isopropyl pimelic acid (12) with simultaneous reduction of keto group hydrolysis and decarboxylation.

The free acid was found as a viscous liquid. The IR spectrum of this acid (12) (Fig.15) showed the free carboxyl group (ν max 1722 and 2670 cm^{-1}). This free acid was converted to the methyl ester (45) and further purified by chromatography over alumina. The IR spectrum (Fig.16a) of this diester (45) showed characteristic absorption due to a saturated ester (ν max 1740 cm^{-1}) and also IR spectrum (16a) of this diester was superimposable with the IR spectrum (16c) of diester prepared from dicarboxylic acid obtained during fermentation of and pinenes and Δ^1 -p-menthene (Shukla, 1965).

The NMR spectrum of this compound (diester) showed the presence of 6 protons corresponding to two methyl groups of isopropyl side chain as two doublets between 0.75 - 1.6 δ , the methyl signals of carbomethoxy group showing 6 protons intensity at 3.59 δ , four protons α to the carbonyl system between 2.1 - 2.31 δ and the remaining six protons as a complex covering the 1.1 - 1.9 δ region. Based on these data, the structure (12) for the dicarboxylic acid was confirmed.



Dicarboxylic acid Methyl Ester

Dicarboxylic acid

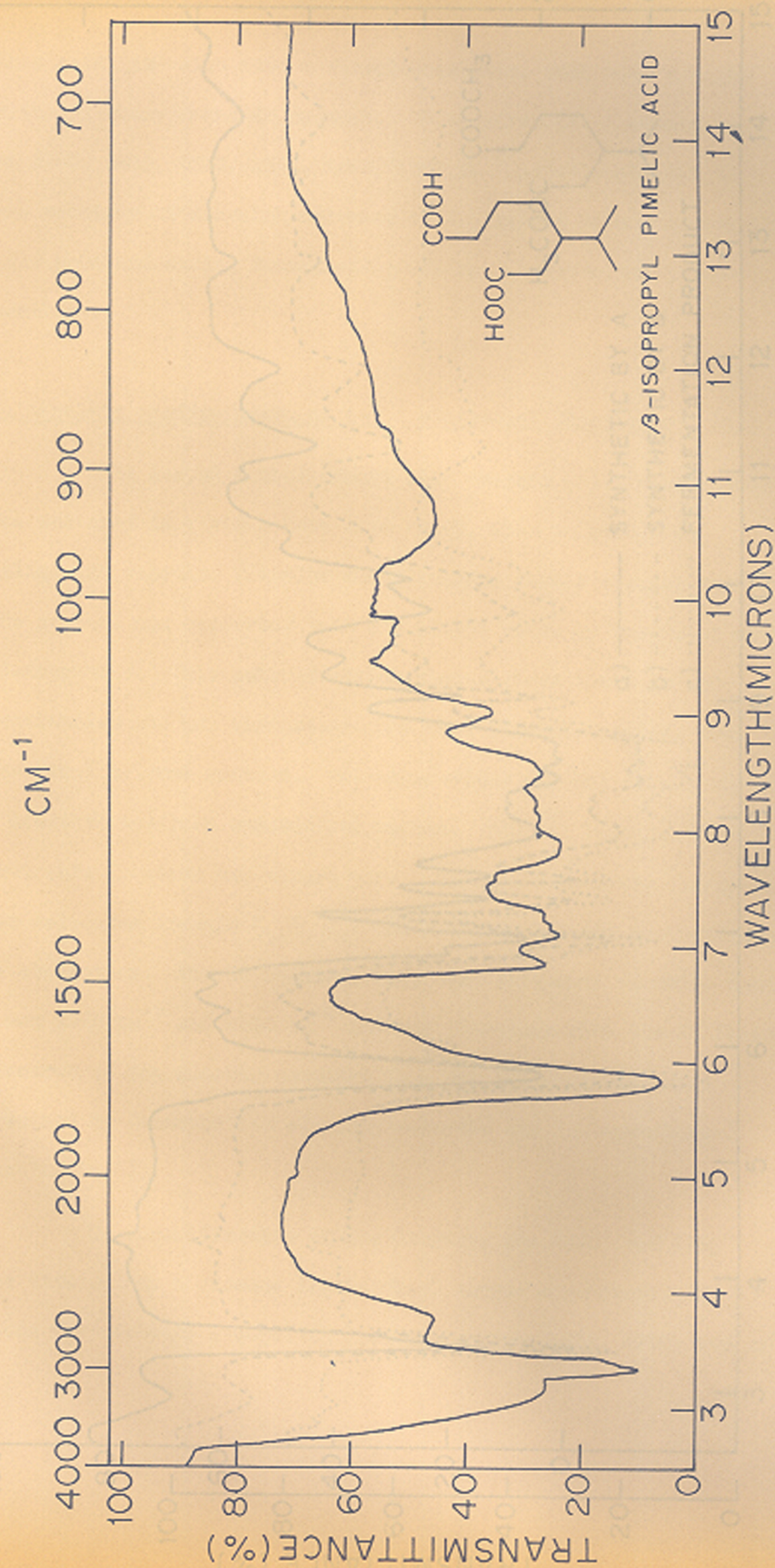
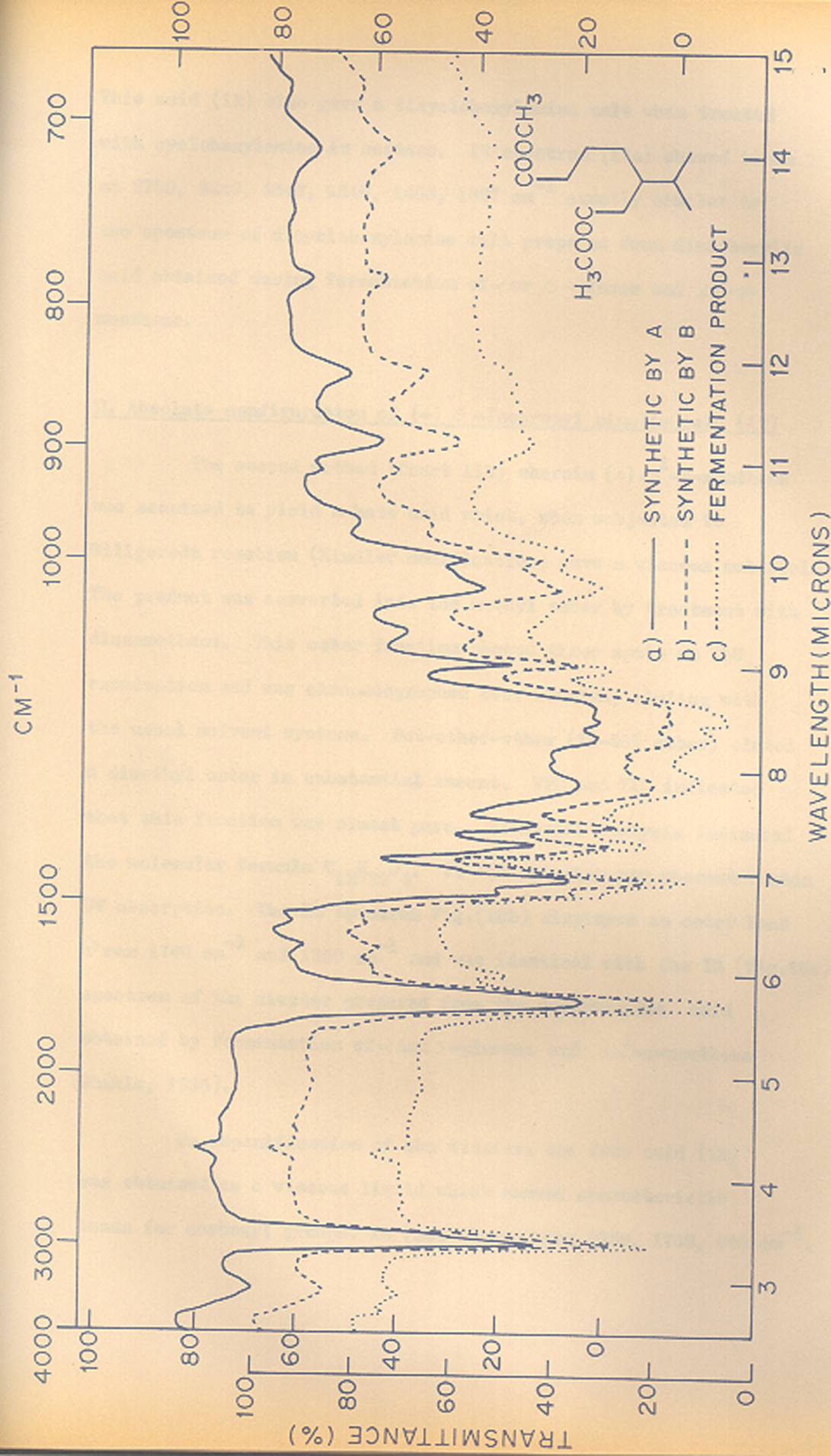


Fig. 15

Fig.16

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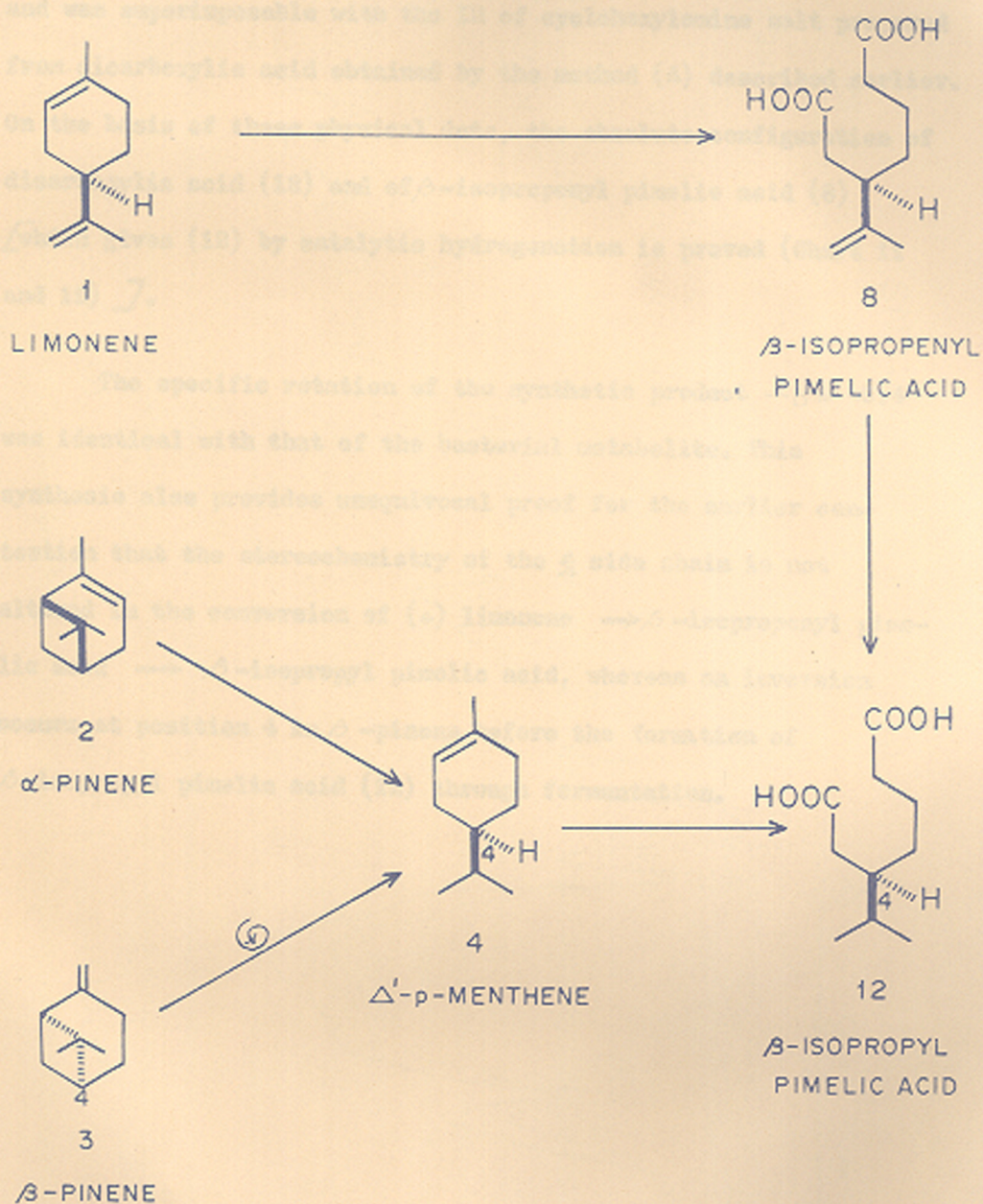
This acid (12) also gave a dicyclohexylamine salt when treated with cyclohexylamine in acetone. IR spectrum (17a) showed bands at 2700, 2240, 1648, 1546, 1465, 1397 cm^{-1} exactly similar to the spectrum of dicyclohexylamine salt prepared from dicarboxylic acid obtained during fermentation of α or β -pinene and Δ^1 -p-menthene.

B. Absolute configuration of (-) β -Isopropyl pimelic acid (12)

The second method (Chart 11b) wherein (+) Δ^1 -p-menthene was ozonized to yield a keto acid which, when subjected to Willgerodt reaction (Kindler modification) gave a viscous material. The product was converted into the methyl ester by treatment with diazomethane. This ester fraction showed three spots on TLC examination and was chromatographed over alumina, eluting with the usual solvent systems. Pet-ether-ether (10-50% ether) eluted a dimethyl ester in substantial amount. VPC and TLC indicated that this fraction was almost pure. Elemental analysis indicated the molecular formula $\text{C}_{12}\text{H}_{22}\text{O}_4$. It did not show any characteristic UV absorption. The IR spectrum Fig.(16b) displayed an ester band ν_{max} 1740 cm^{-1} and 1250 cm^{-1} and was identical with the IR (Fig.16c) spectrum of the diester prepared from the dicarboxylic acid obtained by fermentation of α and β -pinenes and Δ^1 -p-menthene (Shukla, 1965).

On saponification of the diester, the free acid (12) was obtained as a viscous liquid which showed characteristic bands for carboxyl groups. IR (not shown) ν_{max} 2650, 1708, 940 cm^{-1} .

ABSOLUTE CONFIGURATION OF β -ISOPROPYL PIMELIC ACID (12)
AND β -ISOPROPENYL PIMELIC ACID (8)



The dicyclohexylamine salt of the dicarboxylic acid was obtained as colourless crystalline solid by treating the acid with cyclohexylamine in acetone (m.p. 155-160°C). The IR spectrum (Fig. 17b) indicated typical carboxylate salt bands (2280, 1645 cm^{-1}) and was superimposable with the IR of cyclohexylamine salt prepared from dicarboxylic acid obtained by the method (A) described earlier. On the basis of these physical data, the absolute configuration of dicarboxylic acid (12) and of β -isopropenyl pimelic acid (8) [which gives (12) by catalytic hydrogenation is proved (Chart 11 and 12)].

The specific rotation of the synthetic product $[\alpha]_D = -0.4^\circ$ was identical with that of the bacterial metabolite. This synthesis also provides unequivocal proof for the earlier contention that the stereochemistry of the 4 side chain is not altered in the conversion of (+) limonene $\rightarrow \beta$ -isopropenyl pimelic acid $\rightarrow \beta$ -isopropyl pimelic acid, whereas an inversion occurs at position 4 in β -pinene before the formation of β -isopropyl pimelic acid (12) through fermentation.

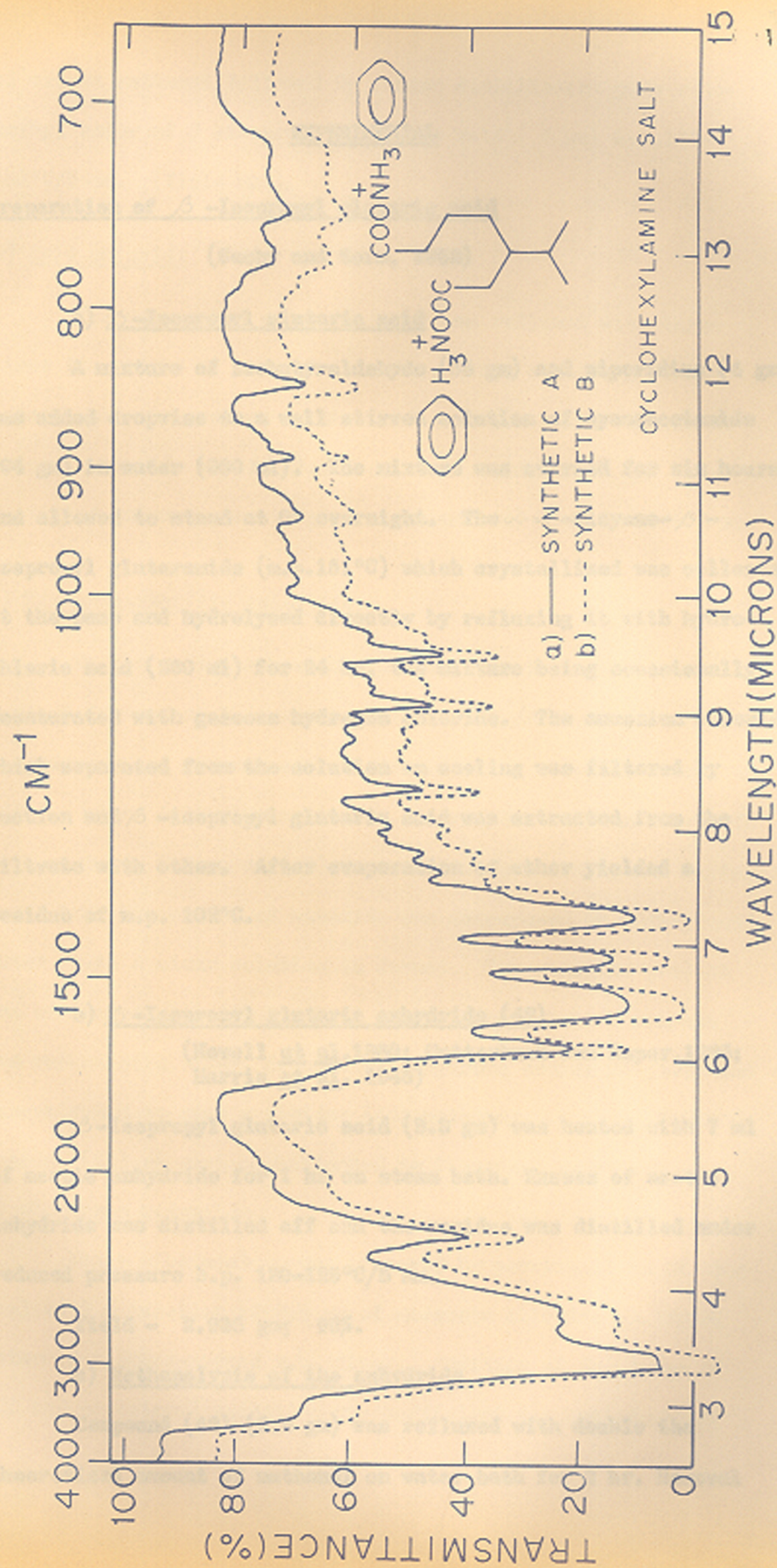


Fig. 17

EXPERIMENTALPreparation of β -Isopropyl glutaric acid

(Suchy and Sorm, 1958)

a) β -Isopropyl glutaric acid

A mixture of isobutyraldehyde (35 gm) and piperidine (4 gm) was added dropwise to a well stirred solution of cyanoacetamide (94 gm) in water (500 ml). The mixture was stirred for six hours and allowed to stand at 0° overnight. The α,α' -dicyano- β -isopropyl glutaramide (m.p. 151°C) which crystallized was collected at the pump and hydrolysed directly by refluxing it with hydrochloric acid (250 ml) for 24 hr, the mixture being occasionally resaturated with gaseous hydrogen chloride. The ammonium chloride which separated from the solution on cooling was filtered by suction and β -isopropyl glutaric acid was extracted from the filtrate with ether. After evaporation of ether yielded a residue of m.p. 102°C.

b) β -Isopropyl glutaric anhydride (42)

(Howell et al. 1890; Cutterbuck and Raper, 1925;
Harris et al. 1945)

β -Isopropyl glutaric acid (3.3 gm) was heated with 7 ml of acetic anhydride for 1 hr on steam bath. Excess of acetic anhydride was distilled off and the residue was distilled under reduced pressure b.p. 120-125°C/5 mm.

Yield - 2.935 gm; 90%.

c) Methanolysis of the anhydride

Compound (42) (2.8 gm) was refluxed with double the theoretical amount of methanol on water bath for 2 hr. Removal

of excess methanol followed by vacuum distillation gave monomethyl ester of β -isopropyl glutaric acid (3 gm) b.p. 125-130°C/5 mm. (Yield 80%).

d) Acid chloride

The monomethyl ester (2.5 gm) was refluxed with three times its weight of thionyl chloride. When evolution of HCl ceased, the mixture was further refluxed for 1 hr. Excess of thionyl chloride was distilled off and residue (2.8 gm) was distilled under vacuum to give compound (43) b.p. 80-90°C/5 mm.

e) Ketotricarboxylic ester (44)

To the sodium ethoxide prepared from 1 gm of sodium and 8 ml of dry methanol in a 100 ml three-necked flask fitted with a condenser and mercury sealed stirrer and a dropping funnel was added 2 ml of diethylmalonate and stirred for half an hour. To this mixture, 2.5 gm of (43) was added with stirring and refluxed for 2 hr. The excess of ethoxide was decomposed by adding cold water until a clear solution is formed. The reaction product was extracted with 50 ml x 3 of ether. The ethereal extract was washed with water, dried over anhydrous sodium sulphate and concentrated to give a residue (44) weighing 2.8 gm. This residue (44) as such, was subjected to Clemenson reduction.

f) Clemenson reduction

Mossy zinc amalgam was prepared by covering 1 gm of zinc wool with a solution of 0.5 gm of mercuric chloride in 100 ml water in a three-necked r.b. flask. Addition of few drops of HCl and occasional agitation was sufficient for amalgamation.

The amalgam was decanted from water and washed twice or thrice with water. It was then covered with 25 ml of conc. hydrochloric acid (25 ml) and water (25 ml). The reaction flask was then equipped with condenser and a stirrer and ketotricarboxylic ester (2.5 gm) in 30 ml of toluene was added to the amalgamated zinc and refluxed for 10 hr with vigorous stirring. Simultaneous reduction of keto group and hydrolysis of the ester took place to give a tricarboxylic acid, which was heated at 180°C for few minutes to give the dicarboxylic acid (12). The product was extracted with ether, ethereal fraction was treated with 10 ml of sodium carbonate solution. The aqueous sodium carbonate layer was acidified to give the free dicarboxylic acid which was taken up in ether, washed with water, dried over anhydrous sodium sulphate and finally recovered after removal of solvent (800 mg). The IR spectrum of the product was found to be superimposable with that of the dicarboxylic acid obtained during the fermentation of α -pinene, β -pinene and Δ^1 -p-menthene (Fig.15) ν max 2670, 1722 cm^{-1} . The methyl ester of the product prepared by treatment with diazomethane in dry ether was found to be 95% pure on TLC.

The methyl ester was further purified by passing over a neutral alumina grade II (1:30) column. The impurities were removed by eluting the column thoroughly with petroleum ether. The pure dicarboxylic acid methyl ester was eluted from the column with petroleum ether-ether (9:1 mixture) and the fractions eluted with this system was distilled under reduced pressure (b.p. 140-150 bath/3 mm). The IR (Fig.16a) spectrum of this ester was superimposable with that of ester prepared from esterification

of β -isopropyl pimelic acid obtained during fermentation of Δ^1 -p-menthene and α and β -pinenes ν max 2945, 1740, 1440, 1200, 1170, 1020 cm^{-1} .

Found: C 62.74, H 9.77. $\text{C}_{12}\text{H}_{22}\text{O}_4$ requires C 62.58, H 9.63%.

Hydrolysis of ester

The methyl ester was refluxed with excess of 5% methanolic KOH solution on a water-bath for 4 hr. Methanol was removed under vacuum and the residue after dilution with water, was extracted with ether to remove any unreacted methyl ester. The acid liberated after acidification of the aqueous layer with hydrochloric acid was taken up in ether. The ether layer was washed, dried and concentrated to give free acid.

Found: C 59.29, H 8.86. $\text{C}_{10}\text{H}_{18}\text{O}_4$ requires: C 59.36, H 8.97%.

IR (Fig.15) ν max 2670, 1722 cm^{-1} .

Preparation of cyclohexylamine salt

The acid obtained (600 mg) after hydrolysis was dissolved in dry acetone (5 ml) and treated dropwise with 1 ml of freshly distilled cyclohexylamine in acetone (5 ml) with stirring. The resulting mixture was warmed on a water-bath for a few minutes, cooled and allowed to remain at 0°C overnight when the mixture set to a solid mass. The precipitate was filtered and washed thoroughly with dry acetone to remove adhering impurities and finally dried m.p. 155-160°. There was no depression of m.p. when mixed with the Cy-salt of β -isopropyl pimelic acid obtained during fermentation.

IR spectrum (Fig.17a) showed bands at 2700, 2240, 1648, 1546, 1465, 1397 cm^{-1} .

Method II (The absolute configuration)

Partial synthesis of β -isopropyl pimelic acid from Δ^1 -p-menthene

a) Ozonolysis of Δ^1 -p-menthene

Δ^1 -p-Menthene (10 gm) in 30 ml of ethylacetate was ozonised. The ozonoid intermediate after removal of the solvent was hydrolysed with 25 ml of 5% acetic acid and refluxed on a water bath for three hr. The product was extracted with chloroform (3 x 20 ml), washed with water and extracted from the chloroform layer with 10% aqueous sodium carbonate solution. The aqueous carbonate layer was acidified to get the free acid which was taken up in chloroform. The chloroform extract was washed with water, dried over anhydrous sodium sulphate and the residue distilled under reduced pressure after removal of the solvent. 12.5 gm. b.p. 174-174.9°/9 mm.

b) Willgerodt reaction with keto acid (Kindler modification)

To 1.8 gm of keto acid placed in a 50 ml r.b. flask fitted with a reflux condenser with a drying tube was added 1 gm of morpholine and 0.4 gm of sulphur and the mixture was refluxed at a moderate rate for 2 hr on oil bath at 140° and then vigorously for 15 hr. The reaction product was taken up in ether, washed successively with an equal volume of water and dilute hydrochloric acid to remove unreacted morpholine, finally with water again. The solvent was distilled off and the residue of crude thioamide was refluxed with 100 ml of 10% KOH solution for 12 hr. The reaction product after acidification was taken up in ether by extracting the product several times with ether. The combined ethereal extract was washed with water, dried over anhydrous sodium sulphate

and evaporated to yield dicarboxylic acid (300 mg).

$$\alpha_D = -6.4^\circ.$$

Found: C 62.67, H 9.68. $C_{12}H_{22}O_4$ requires C 62.54, H 9.63%.

This dicarboxylic acid had identical IR spectrum as that of β -isopropyl pimelic acid obtained during fermentation of Δ^1 -p-menthene and α and β pinenes and the synthetic material (Dhavalikar and Bhattacharyya, 1966; Shukla, 1965).

The methyl ester of this acid prepared by the method described earlier had IR spectrum superimposable with that of fermentation product b.p. 125-130°/1 mm (Fig. 16b).

Cyclohexylamine salt

The diester after hydrolysis with 10% KOH for 4 hr on water bath gave the free dicarboxylic acid which gave cyclohexylamine salt when treated with cyclohexylamine in dry acetone by the method reported earlier (IR spectrum, Fig. 17b). m.p. 148-150°, mixed m.p. 149-152°.

CHAPTER 5

METABOLIC PATHWAY FOR THE DEGRADATION OF

β -ISOPROPYL PIMERIC ACID

METABOLIC PATHWAY FOR DEGRADATION OF β -ISOPROPYL PIMELIC ACID

Isolation of methyl isopropyl ketone

It will be noticed that in β -isopropyl pimelic acid (12) all the ten carbons of the terpenoid substrates are intact. This substrate is finally degraded to carbon dioxide and water through smaller units possibly two carbon fragments. The earlier workers could not locate or isolate any unit smaller than C_{10} . It was therefore necessary to look more carefully in the fermentation broth for the possible presence of smaller units other than the first ring cleaved product i.e., β -isopropyl pimelic acid.

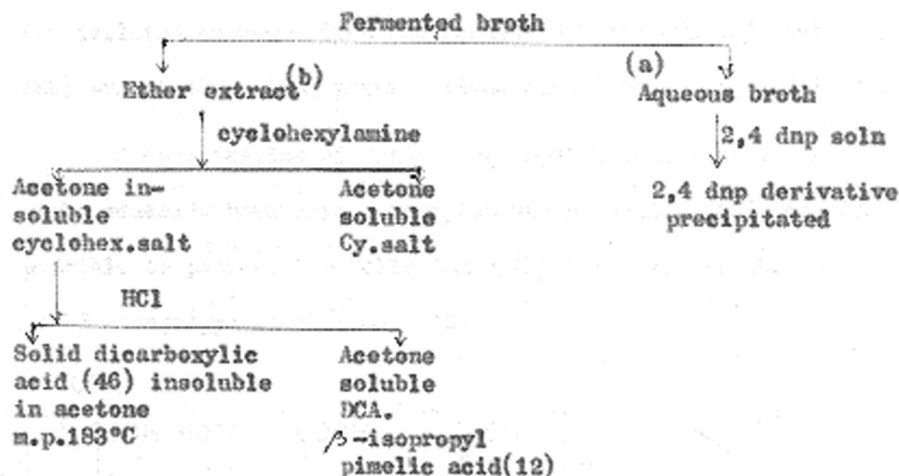
β -Isopropyl pimelic acid accumulated as the cleaved product when α or β pinene or Δ^1 -p-menthene was used as the hydrocarbon source. Since the accumulation of the dicarboxylic acid was substantially greater in the case of Δ^1 -p-menthene, this was used as the preferred hydrocarbon source in fermentation and the fermentation broth was analysed more carefully to obtain possible intermediates of shorter chain length other than the dicarboxylic acid (12).

The large scale fermentation of Δ^1 -p-menthene was carried out with 0.2 to 0.3% of the added substrate i.e., Δ^1 -p-menthene was added to the pre-sterilized flasks (500 ml) containing usual mineral salt medium (100 ml) along with the inoculum and kept for shaking on rotary shaker for 48 hr.

After 48 hr of fermentation the broth from all the flasks was pooled together, acidified and worked out according to the scheme shown below. The aqueous broth after extraction with

other several times, was tested for any water soluble keto compounds with acidic 2,4 dinitrophenyl hydrazine solution.

SCHEME I

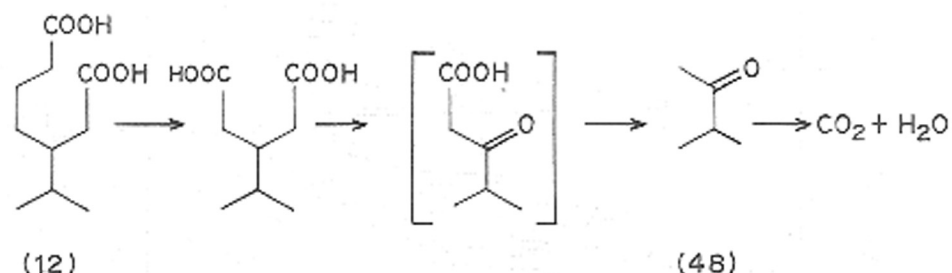


a) Aqueous layer (Scheme I) after centrifugation to remove the suspended cells, was treated with acidic 2,4 dinitrophenyl hydrazine solution. After 24 hr standing at room temperature an orange yellow precipitate separated out. The precipitated 2,4 dinitrophenylhydrazones derivatives were taken in ethyl acetate and was separated into acidic (nil) and neutral (268 mg) derivatives using 10% sodium carbonate solution. The substrate blank and cell blanks failed to give any derivative excepting unreacted 2,4 dnp itself.

Neutral 2,4 dinitrophenyl hydrazones (268 mg) on TLC examination showed three spots among which one was in major amount. The major component was separated out by preparative layer chromatography. It was crystallized from aqueous methanol

to pale yellow crystals. m.p. 118-120°. On TLC the purified hydrazone had an identical mobility pattern as that of 2,4 dinitrophenyl hydrazone of authentic methyl-isopropyl ketone. The presence of methyl isopropyl ketone was finally proved by spectral comparison of the hydrazones of authentic as well as the isolated compound from the fermented broth (Fig. 18a and 18b) and mixed melting point - mixed m.p. 119°C, reported 121°C.

Identification of methyl isopropyl ketone (48) which might probably have arisen from isobutyryl acetic acid made it possible to propose the following pathway for the metabolism of β -isopropyl pimelic acid (12).



In order to find the validity of the proposed pathway growth, oxidation and adaptive enzyme studies were undertaken.

b) The ether extract was directly treated with cyclohexylamine in order to separate the dicarboxylic acids. The acetone-insoluble cyclohexylamine salts after acidification yielded two products, among which one was an acetone insoluble solid m.p. 183°C. The IR (Fig. 19) and UV spectra indicated the presence of an α/β -unsaturated carboxyl group (ν_{max} 1678, 1647, 2550 cm^{-1}), UV λ_{max} 217 nm. The IR spectrum of methyl ester (Fig. 20) exhibited IR bands at 1720, 1660, 1263 cm^{-1} (α/β -unsaturated ester). The NMR spectrum of the methyl ester showed

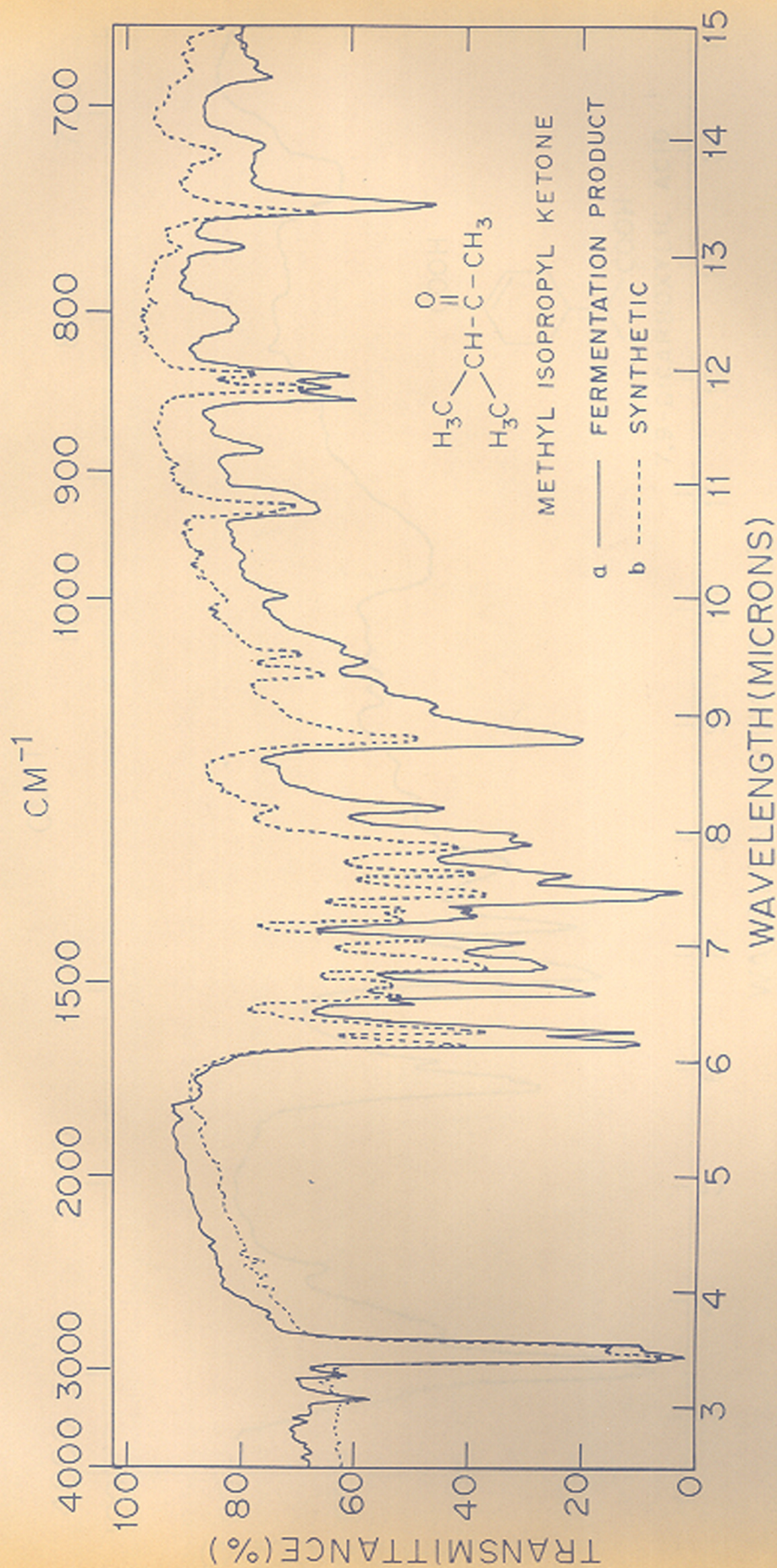


Fig. 18

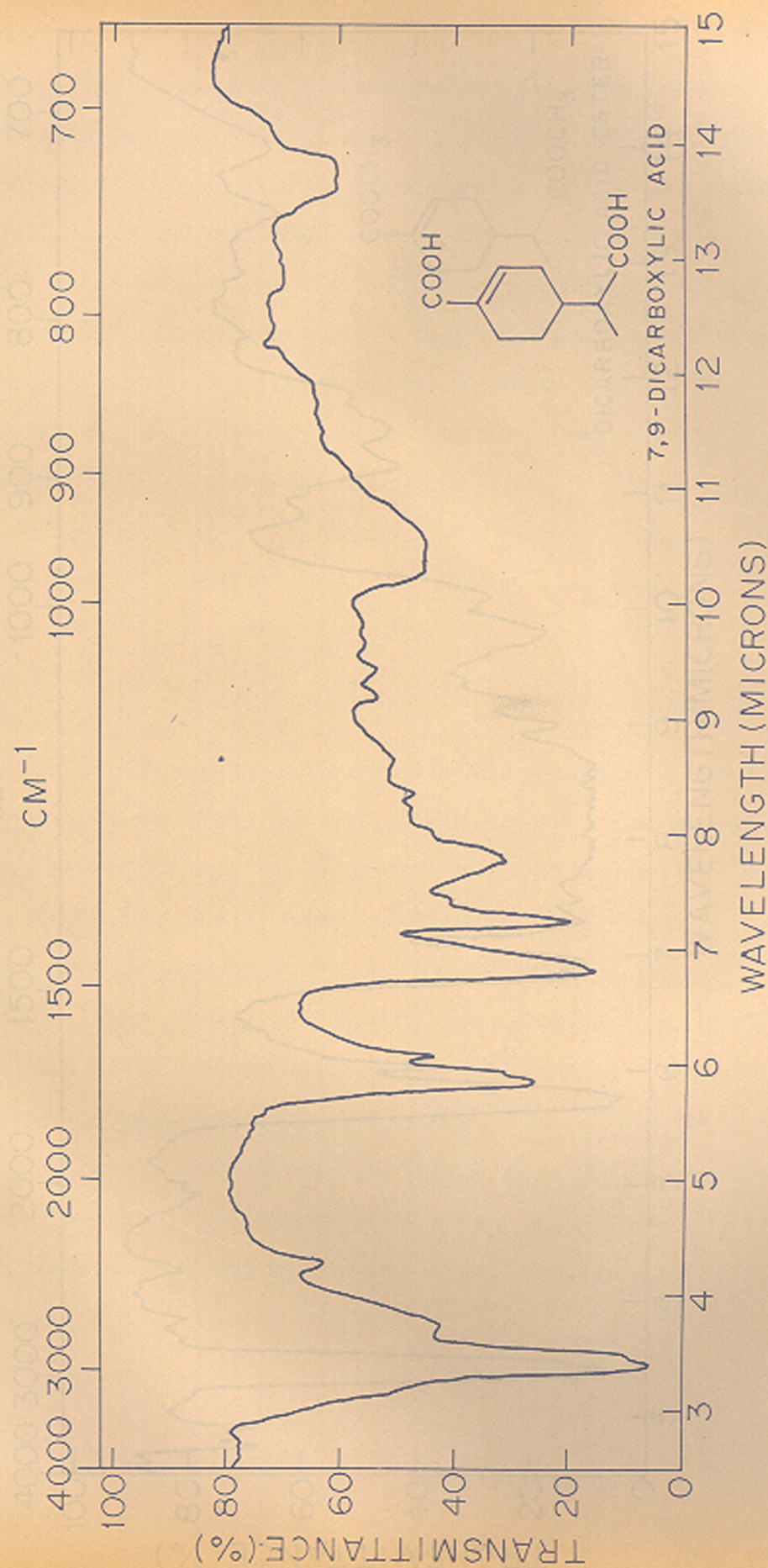


Fig.19

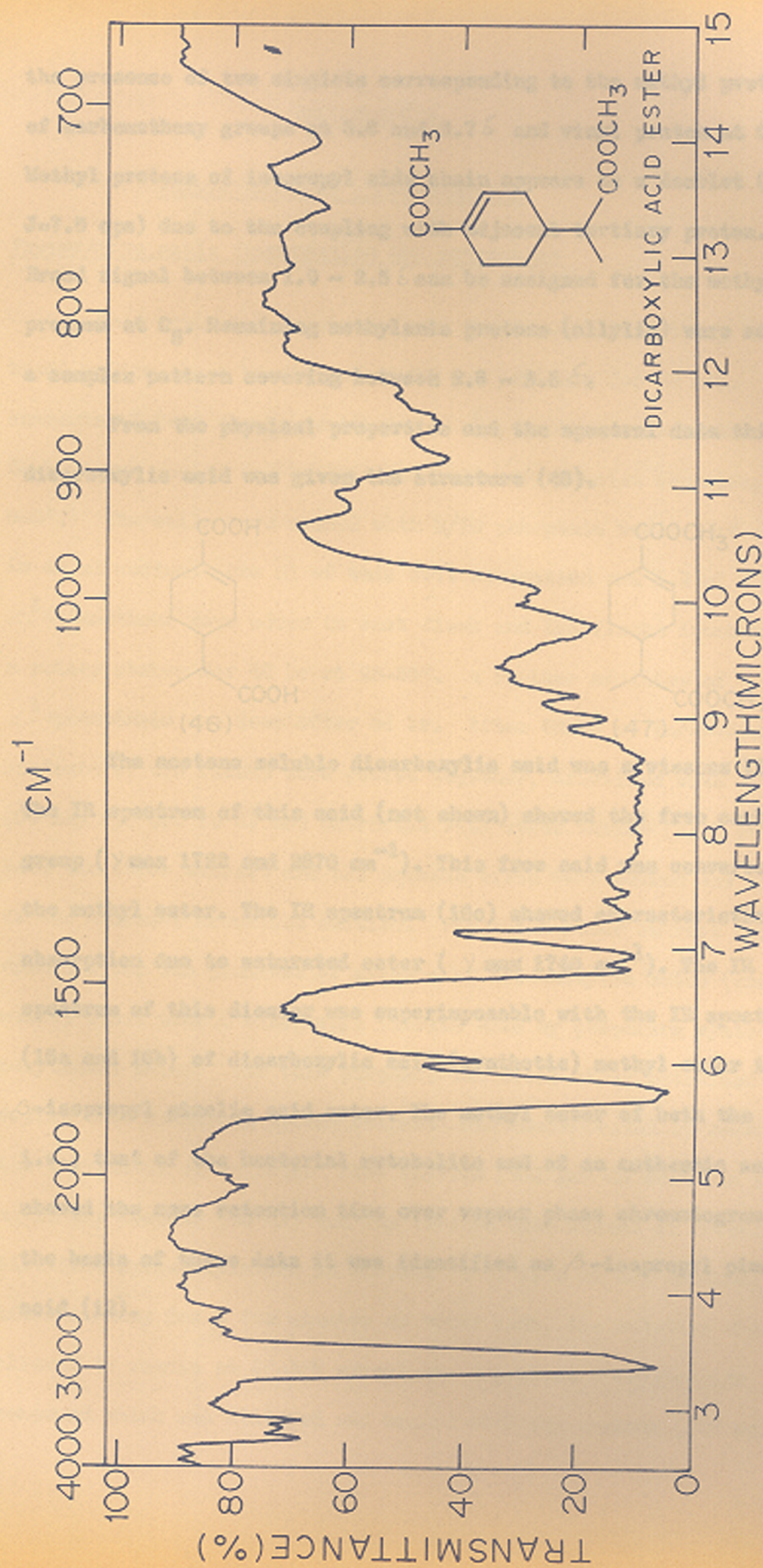
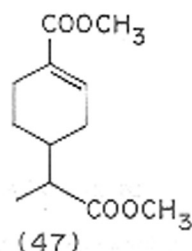
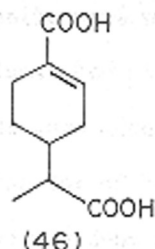


Fig. 20

the presence of two singlets corresponding to the methyl protons of carbomethoxy groups at 3.6 and 3.7 δ and vinyl proton at 6.89 δ . Methyl protons of isopropyl side chain appears as a doublet (1.15 δ , $J=7.5$ cps) due to the coupling with adjacent tertiary proton. Broad signal between 1.9 - 2.5 δ can be assigned for the methylenic protons at C_5 . Remaining methylenic protons (allylic) were seen as a complex pattern covering between 2.6 - 3.5 δ .

From the physical properties and the spectral data this dicarboxylic acid was given the structure (46).



The acetone soluble dicarboxylic acid was a viscous liquid. The IR spectrum of this acid (not shown) showed the free carboxyl group ($\nu_{\max}$ 1722 and 2670 cm^{-1}). This free acid was converted to the methyl ester. The IR spectrum (16c) showed characteristic absorption due to saturated ester ($\nu_{\max}$ 1740 cm^{-1}). The IR spectrum of this diester was superimposable with the IR spectrum (16a and 16b) of dicarboxylic acid (synthetic) methyl ester i.e., β -isopropyl pimelic acid ester. The methyl ester of both the acids i.e., that of the bacterial metabolite and of an authentic sample showed the same retention time over vapour phase chromatogram. On the basis of these data it was identified as β -isopropyl pimelic acid (12).

EXPERIMENTAL

FERMENTATION OF Δ^1 -p-MENTHENE

Preparative scale fermentation and extraction

In a typical fermentation, 40 flasks each containing 100 ml basal medium, were sterilized at 15 psi pressure for 20 min. The contents of four flasks containing Δ^1 -p-menthene grown cells for 24 hr, were pooled and centrifuged in a refrigerated centrifuge at 0-2°. The cells were washed with M/20 phosphate buffer and suspended in 40 ml medium. One ml of this cell suspension and 0.3 ml of Δ^1 -p-menthene were added to each flask and the flasks incubated on a rotary shaker for 48 hr at 28-30°. A further addition of Δ^1 -p-menthene was done after 24 hr. After 48 hr of incubation, the contents of the flasks were pooled together, acidified with 6N HCl (20 ml) (pH 2.5 to 3) and extracted with ether thrice (1/10 vol.). Both the ether extract and the aqueous broth were worked out according to the Scheme-I described earlier. The total ether extract was examined for the presence or absence of dicarboxylic acids (smaller units) other than β -isopropyl pimelic acid by preparing cyclohexylamine salts. For this purpose, the total ether extracted after drying over anhydrous sodium sulphate was concentrated by removal of the solvent on water bath. This residue (4.8 gm) as such was taken up in dry acetone (20 ml) and was treated with excess of freshly distilled cyclohexylamine in acetone (3 ml in 8 ml dry acetone). After warming for a few minutes on water bath, the solution when allowed to remain at 0°-5°C overnight, deposited a crystalline compound which was filtered and washed with dry acetone (800 mg).

Regeneration of dicarboxylic acids

A slurry of the cyclohexylamine salt (800 mg) in water was acidified with 6N HCl and the liberated acids were extracted with chloroform. The combined extracts (60 ml) were washed with small amount of water, dried over anhydrous sodium sulphate and evaporated to give a gummy mass. This residual mass when treated with dry acetone deposited a white crystalline (200 mg) compound which was filtered at the pump, washed thoroughly with dry acetone and recrystallized from pet-ether-chloroform separating out as plates. It showed m.p. 183°C. IR (Fig.19) $\nu_{\max}$ 2670, 1725, 1680, 1640, 1480, 1370, 1285, 950 cm^{-1} .

Preparation of methyl ester

The free acid (200 mg) was esterified with diazomethane to give dicarboxylic acid methyl ester (47) which was found to be 96% pure on VPC. This was further purified by vacuum distillation. It showed IR bands (Fig.20) $\nu_{\max}$ 1745, 1650, 890, 810 cm^{-1} .
Found: C 63.26, H 8.06. $\text{C}_{12}\text{H}_{18}\text{O}_4$ requires C 63.1, H 7.89%.

β -Isopropyl pimelic acid

The mother liquor from the above preparation was concentrated to give a residue (580 mg). It was found to be TLC pure.
IR $\nu_{\max}$ 2670, 1722 cm^{-1} .

Methyl ester

The free acid was converted into its methyl ester by treating it with excess of diazomethane in dry ether. This was found to be

single on TLC and VPC and distilled b.p. 140-150° bath/2 mm.

$$[\alpha]_D^{25} = -5.25^\circ.$$

Found: C 62.84, H 9.9. $C_{12}H_{22}O_4$ requires C 62.58, H 9.63%.

IR (Fig. 16c) ν max 2945, 1740, 1440, 1200, 1170, 1020 cm^{-1} . NMR indicated the compound to be β -isopropyl pimelic acid methyl ester.

Aqueous broth

The aqueous layer was centrifuged to remove the suspended cell material. The supernatant was treated with 200 ml of 1% 2,4 dinitrophenyl hydrazine solution (in 2N HCl) and allowed to remain overnight at room temperature giving a yellow orange precipitate which was filtered off. The residue which showed 3 spots on TLC among which one was in major amount. The major component was separated by preparative layer chromatography and crystallized from aqueous methanol giving orange-yellow needles m.p. 118-120°C, mixed m.p. 119-120° - with dnp of authentic methyl isopropyl ketone; reported m.p. 120°C.

IR (Fig. 18a) spectrum was found to be superimposable with that of 2,4 dnp derivative of methyl isopropyl ketone.

One of the minor derivatives was found to be 2,4 dnp itself. The other one could not be characterized due to paucity of the material.

CHAPTER 6

GROWTH, OXIDATION AND ADAPTIVE ENZYME STUDIES

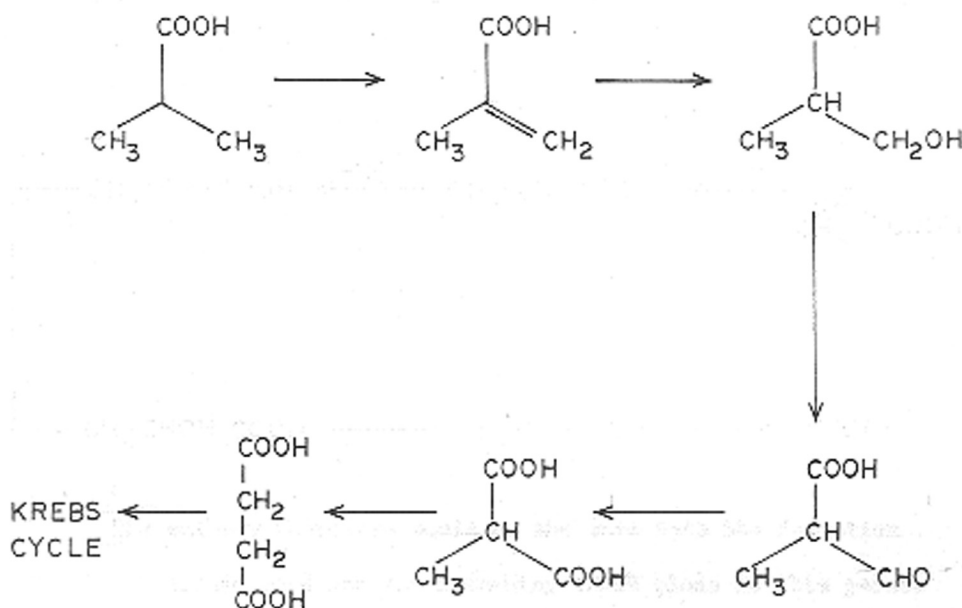
GROWTH AND ADAPTIVE ENZYME STUDIES

Long chain hydrocarbons have been shown to be utilized by different types of microorganisms and an extended study of the degradation and utilization of hydrocarbons has indicated the conversion of hydrocarbons to the carboxylic acid through the intermediate formation of alcohols and aldehyde (Harris, 1957; Thijsse et al. 1958). This seems to be the major pathway by which most of the organisms utilize hydrocarbons although there are a few which tend to oxidize the hydrocarbon at both the ends leading to the formation of a dicarboxylic acid. Another mechanism proposed is the conversion of the saturated hydrocarbons to an olefin which undergoes oxidation to a carboxylic acid (Chatanuet et al. 1962). Ultimately these carboxylic acids are further metabolized mostly by the ubiquitous β -oxidation mechanism shown to be present in all living tissues. A recent comprehensive and well documented survey has been compiled by McKenna and Kallio (1965).

A detailed study of the metabolism of isoprenoids by bacteria has been reported by Seubert and his co-workers (1960, 1963, 1964). They have studied isoprenoid acids with more than four carbon atoms. In such cases it has been possible to show that a carboxylation occurs obligatorily at the branched carbon methyl leading to the formation of a $-\text{CH}_2-\text{COOH}$ group. During the further breakdown, this group is eliminated as an acetic acid molecule and the chain is thus shortened giving rise to a β -ketoacid. The mechanism is outlined below in brief.

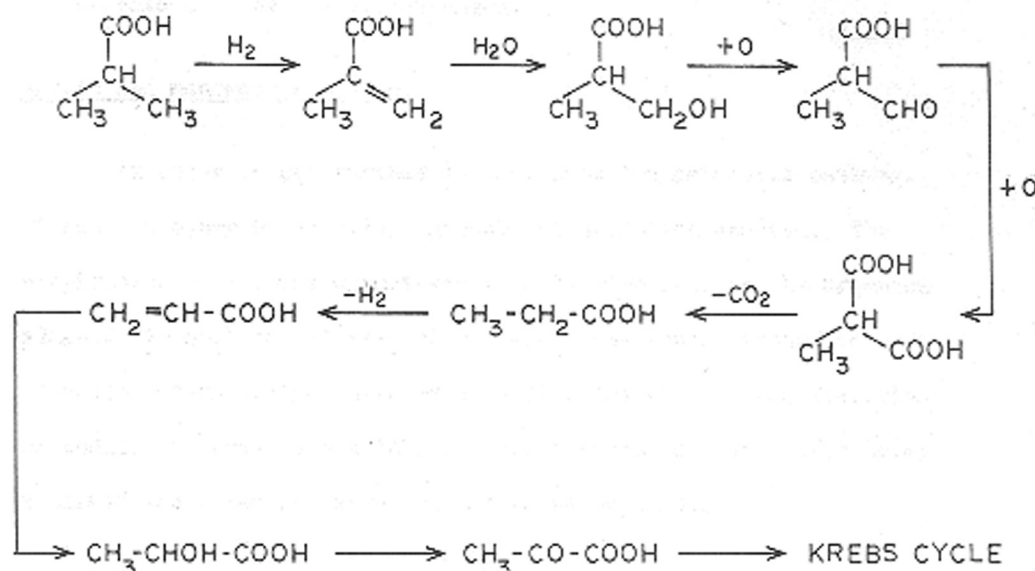
that

A survey of the literature indicated that the degradation of C_4 -branched chain fatty acids by microorganisms had not been studied except for the well documented fact that valine is degraded to isobutyric acid (Meister, 1963). The detailed study of the degradation of isobutyric acid in animal tissues has been made by Coon and his co-workers (1957, 1959, 1963) and the mechanism of breakdown of isobutyric acid is given below.



Regarding the breakdown of isobutyric acid by microorganisms, very little information is available especially in regard to the different steps in the pathway. Isobutyric acid could be utilized besides the mechanism given above and shown to be operative in animal

tissues by another possible mechanism as outlined below. Part of this mechanism viz., the utilization of propionate has been shown to be through a degradative method which is outlined below in a number of microorganisms, algae etc.



The pathway therefore would be the same upto the formation of methyl malonic acid and the branching takes place at this point. Microorganisms are known to utilize malonic acid and its derivatives and in many instances the metabolism follows the decarboxylation. Therefore the second mechanism outlined above in which methyl malonic acid by decarboxylation will give rise to propionic acid is also plausible in the present case as especially the metabolism of propionic acid by the second mechanism is known to occur in a number of microorganisms (Ayl, 1965; Kornberg, 1967).

In order to investigate mechanisms further, a study of the metabolism of isobutyric acid by the present PL-strain was also carried out. Isobutyric acid which is well utilized and metabolized by Δ^1 -p-menthene grown cells. This would indicate that isobutyric acid is very likely to be an intermediate in the degradation of Δ^1 -p-menthene or α or β -pinenes.

Growth and manometric studies

In order to get further insight into the catabolic pathway, it was necessary to undertake growth and oxidation studies. The respirometric work was undertaken to determine whether the organism adapted to utilize Δ^1 -p-menthene could degrade suspected or probable intermediates, with or without a lag phase. The formation of induced enzymes to metabolize various compounds was indicated by a marked lag phase in oxygen uptake in these tests.

A study of growth and oxidation patterns of the intermediates that lie in the catabolic pathway by the organism grown on the parent hydrocarbon however, could only indicate, but not conclusively prove, the mediation of the intermediates in the pathway for the degradation.

GROWTH STUDIES

Growth studies were recorded either visually or with a Klett-Summerson colorimeter using a flask developed by Shukla and Bhattacharyya, 1965).

Washed suspension of PL-strain grown on Δ^1 -p-menthene was found to grow without any lag period on isobutyrate, n-butyrate,

methyl isopropyl ketone, acetate, malate and succinate. The ready growth of this organism on the Krebs cycle acids such as succinate, malate and acetate could indicate that the Krebs cycle along with the glyoxalate bypass may be present in this bacterium.

Cross adaptation studies indicated that isobutyrate grown cells grew on Δ^1 -p-menthene after a lag period of 8 hr. Tables 3, 4 and 5 (Fig.22) show the growth pattern of different substrates with PL-strain.

The ready growth of the organism on methyl isopropylketone and isobutyrate suggests that they must be probable intermediates formed during the fermentation of β -isopropylpimelic acid. Confirmation of this hypothesis was sought through oxidation and other studies.

Growth studies on β -isopropyl pimelic acid

Growth experiments were conducted with synthetic dicarboxylic acid i.e., β -isopropyl pimelic acid (12) following the usual methods.

Initially the growth studies were undertaken using usual mineral salt medium in the shake culture flasks with the substrate concentration ranging from 0.2 to 0.4%. Even after an incubation period of several days no growth was observed.

Two reasons could be assigned for this failure (a) the substrate was not absorbed and therefore not oxidized probably due to a lack of permeability, and (b) it is possible that the dicarboxylic acid can be metabolized after activation as the Co-A derivative. In order to test some of these possibilities the

TABLE 3

GROWTH AND CROSS-ADAPTATION STUDIES

Inoculum *	Time (hr)	Growth pattern on**					
		Methyl isopropyl ketone	Δ^1 -p- menthene	Acetate	Isobutyrate	DCA Compd.(12)	Lactate
KLETT READING							
Δ^1 -p- menthene grown cells	0	85	67	65	56	46	60
	12	164	242	212	206	98	165
	24	160	325	210	340	105	150
	48	150	312	198	335	96	-

* An aliquot of 2-4 ml of cell suspension was taken from a 24 hr-grown culture with a turbidity reading of 260-280 m μ .

** Growth on different substrates was measured turbidimetrically in a Klett-Summerson colorimeter at 660 m μ .

TABLE 4
GROWTH AND CROSS-ADAPTATION STUDIES

Inoculum*	Time (hr)	Growth pattern on**		
		Δ^1 -p-menthene	Isobutyrate	Lactate
<u>KLETT READING</u>				
Isobutyrate grown cells	0	52	46	40
	12	148	206	145
	24	252	236	125
	48	298	325	-

* An aliquot of 2-4 ml of cell suspension was taken from a 24 hr-grown culture with a turbidity reading of 260-280 mμ.

** Growth on different substrates was measured turbidimetrically in a Klett-Summerson colorimeter at 660 mμ.

TABLE 5
GROWTH AND CROSS ADAPTATION STUDIES

Inoculum*	Time (hr)	Growth pattern on**	
		KLETT READING	
		Acetate	DCA -Compl.(12)
Acetate- grown cells	0	69	63
	12	211	105
	24	253	106
	48	206	105

*An aliquot of 2-4 ml of cell suspension was taken from
a 24 hr-grown culture with a turbidity reading of 260-280 mμ.

**Growth on different substrates was measured turbidimetrically
in a Klett-Summerson colorimeter at 660 mμ.

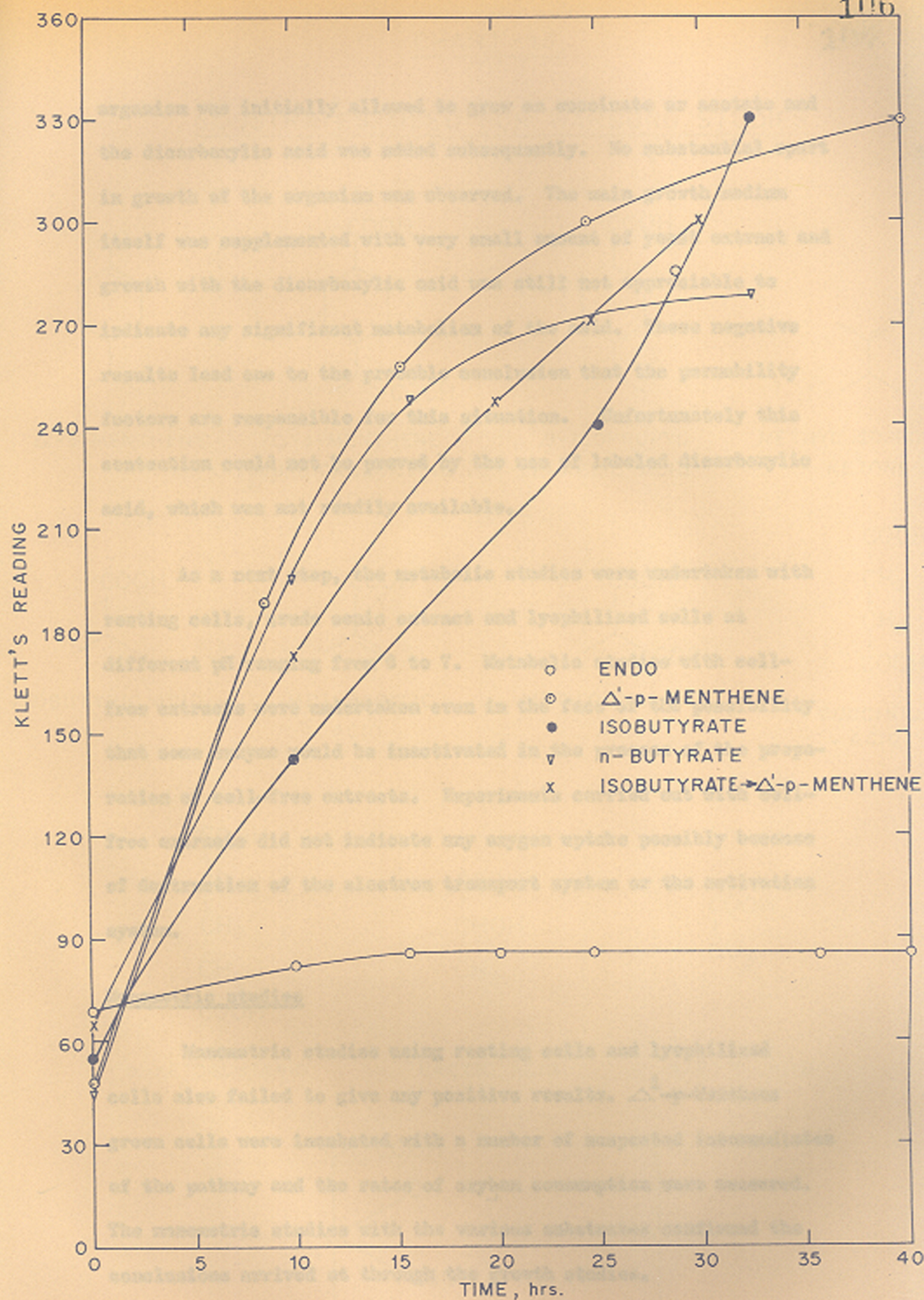


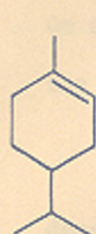
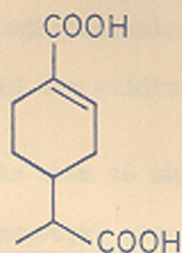
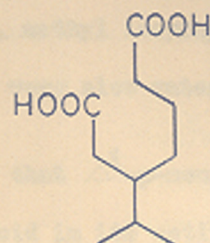
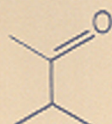
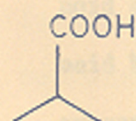
FIG. 22. GROWTH PATTERN OF DIFFERANT SUBSTRATES ON "PL STRAIN.

organism was initially allowed to grow on succinate or acetate and the dicarboxylic acid was added subsequently. No substantial spurt in growth of the organism was observed. The main growth medium itself was supplemented with very small amount of yeast extract and growth with the dicarboxylic acid was still not appreciable to indicate any significant metabolism of the acid. These negative results lead one to the probable conclusion that the permeability factors are responsible for this situation. Unfortunately this contention could not be proved by the use of labeled dicarboxylic acid, which was not readily available.

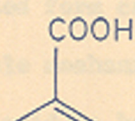
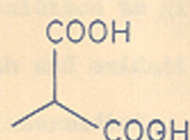
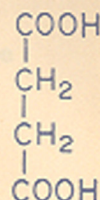
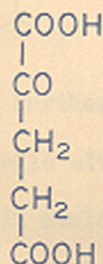
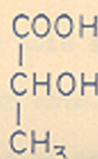
As a next step, the metabolic studies were undertaken with resting cells, crude sonic extract and lyophilized cells at different pH ranging from 6 to 7. Metabolic studies with cell-free extracts were undertaken even in the face of the possibility that some enzyme would be inactivated in the process of the preparation of cell-free extracts. Experiments carried out with cell-free extracts did not indicate any oxygen uptake possibly because of destruction of the electron transport system or the activation system.

Manometric studies

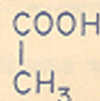
Manometric studies using resting cells and lyophilized cells also failed to give any positive results. Δ^1 -p-Menthene grown cells were incubated with a number of suspected intermediates of the pathway and the rates of oxygen consumption were measured. The manometric studies with the various substrates confirmed the conclusions arrived at through the growth studies.

SUBSTRATES USED FOR WARBURG STUDIES Δ' -p-MENTHENE Δ' -p-MENTHENE
7,9-DICARBOXYLIC
ACID β -ISOPROPYL
PIMELIC ACIDMETHYL ISOPROPYL
KETONE

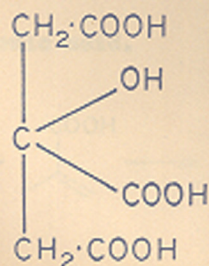
ISOBUTYRIC ACID

METHACRYLIC
ACIDMETHYL MALONIC
ACIDSUCCINIC
ACID α -KETOGLUTARIC
ACID

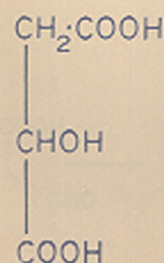
LACTIC ACID



ACETIC ACID



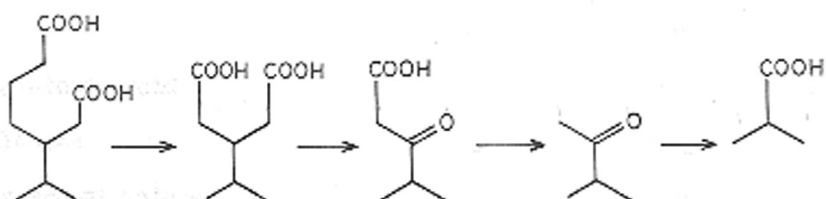
CITRIC ACID



MALIC ACID

It is apparent from the Q_{O_2} values listed in Tables 6 and 7 (Fig. 23 to 28) that Δ^1 -p-menthene grown cells oxidize readily isobutyrate, acetate, succinate, α -KGA, malate and methacrylate. On the other hand, methylmalonic acid, methyl isopropyl ketone and the dicarboxylic acid are oxidized at very slow rates.

These results seem to suggest that Δ^1 -p-menthene after degradation to β -isopropyl pimelic acid in its activated form may be further cleaved to give probably an unstable keto acid (isobutyrylacetic acid), which itself undergoes decarboxylation to give methyl-isopropyl ketone, which may be an artifact. Isobutyrylacetic acid or its activated form can be further oxidized to give isobutyric acid by an acceptable mechanism. The growth and oxidation studies support isobutyric acid to be a metabolic intermediate.



Further, methyl malonic acid is oxidized by Δ^1 -p-menthene grown cells although at a slower rate. This would indicate that this acid could be formed according to the mechanism operating in animal tissues through the hydroxylation of one of the methyl groups of isopropyl side chain of isobutyric acid to form β -hydroxyisobutyric acid, which may be oxidized to methyl malonic semialdehyde then to methyl malonic acid.

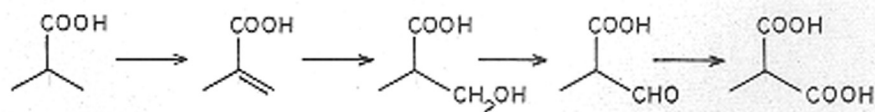


TABLE 6
OXYGEN UPTAKE BY Δ^1 -p-MENTHENE-GROWN CELLS

Substrate	Q_{O_2} μ l/mg dry wt/hr after deduction of endogenous
Δ^1 -p-Menthene	257
Lactate	163
Acetate	143
Isobutyrate	142
Succinate	134
Methacrylate	132
Malate	116
α -Ketoglutaric acid	110
Methylmalonate	43
Methyl isopropyl ketone	24
Saturated dicarboxylic acid (Compd. 12)	15

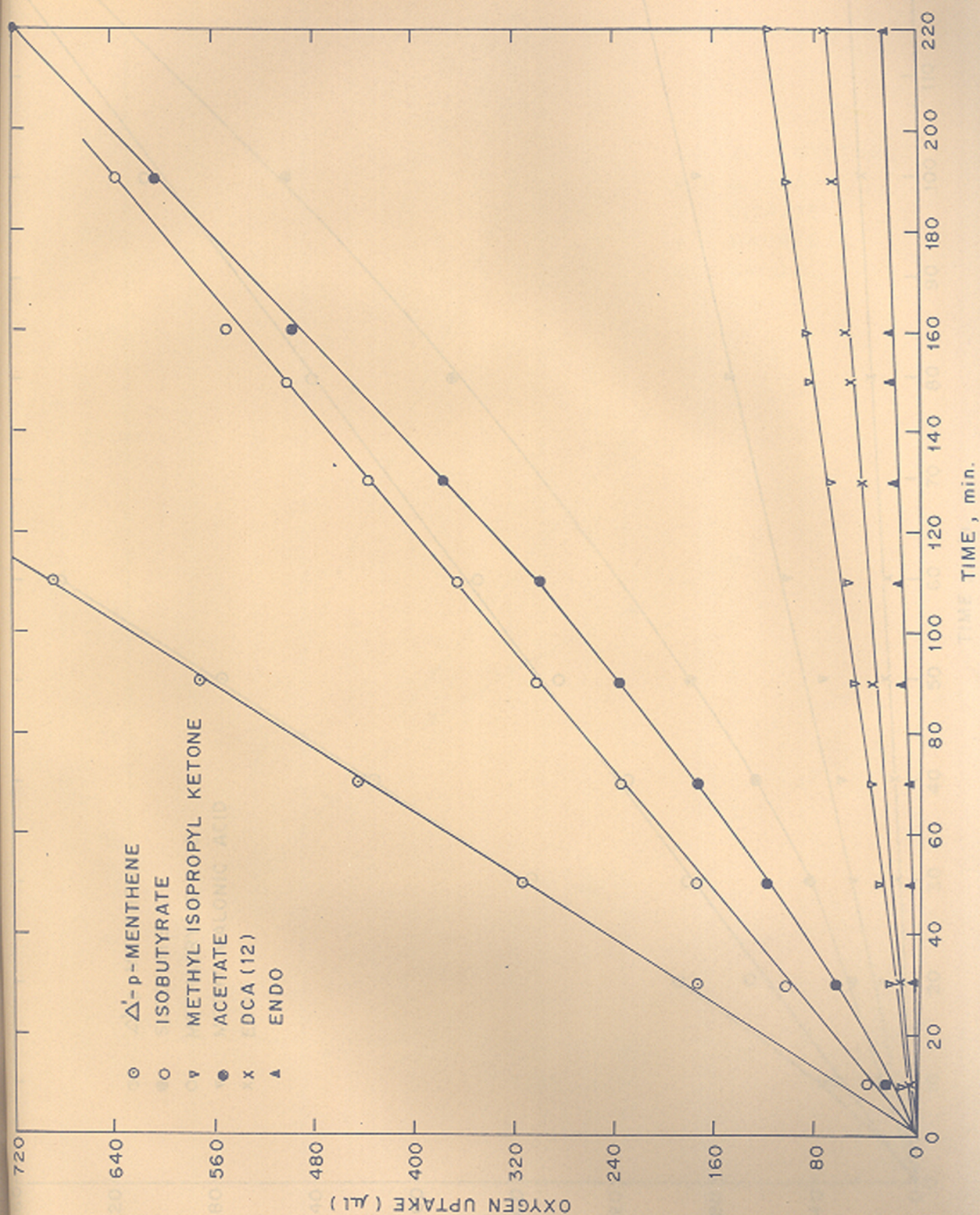
Each flask contained 1.5 ml of cell suspension containing (1.48 gm of dry wt. of cells), 10-12 μ M of substrates (side arm), 1 ml of phosphate buffer (pH 7) and 0.2 ml KOH (20%) in the central well. The total volume was made to 3 ml.

TABLE 7

OXYGEN UPTAKE BY ISOBUTYRATE-GROWN CELLS

Substrates	Q_{O_2} μ l/mg dry wt/hr after deduction of endogenous
Succinate	190
Isobutyrate	165
Acetate	138
Malate	126
Methacrylate	98
Δ^1 -p-Menthene	90 (after a lag period of 2 hr)
α -Ketoglutaric acid	77
Citrate	63 (after a con- siderable lag)
Methyl malonate	53
Methyl isopropyl ketone	18

Each flask contained 1.5 ml of cell suspension containing (1.48 gm of dry wt. of cells), 10-12 μ M of substrates (side arm), 1 ml of phosphate buffer (pH 7) and 0.2 ml KOH (20%) in the central well. The total volume was made to 3 ml.

FIG. 23. OXIDATION OF VARIOUS PROBABLE INTERMEDIATES BY Δ^1 -p-MENTHENE GROWN CELLS.

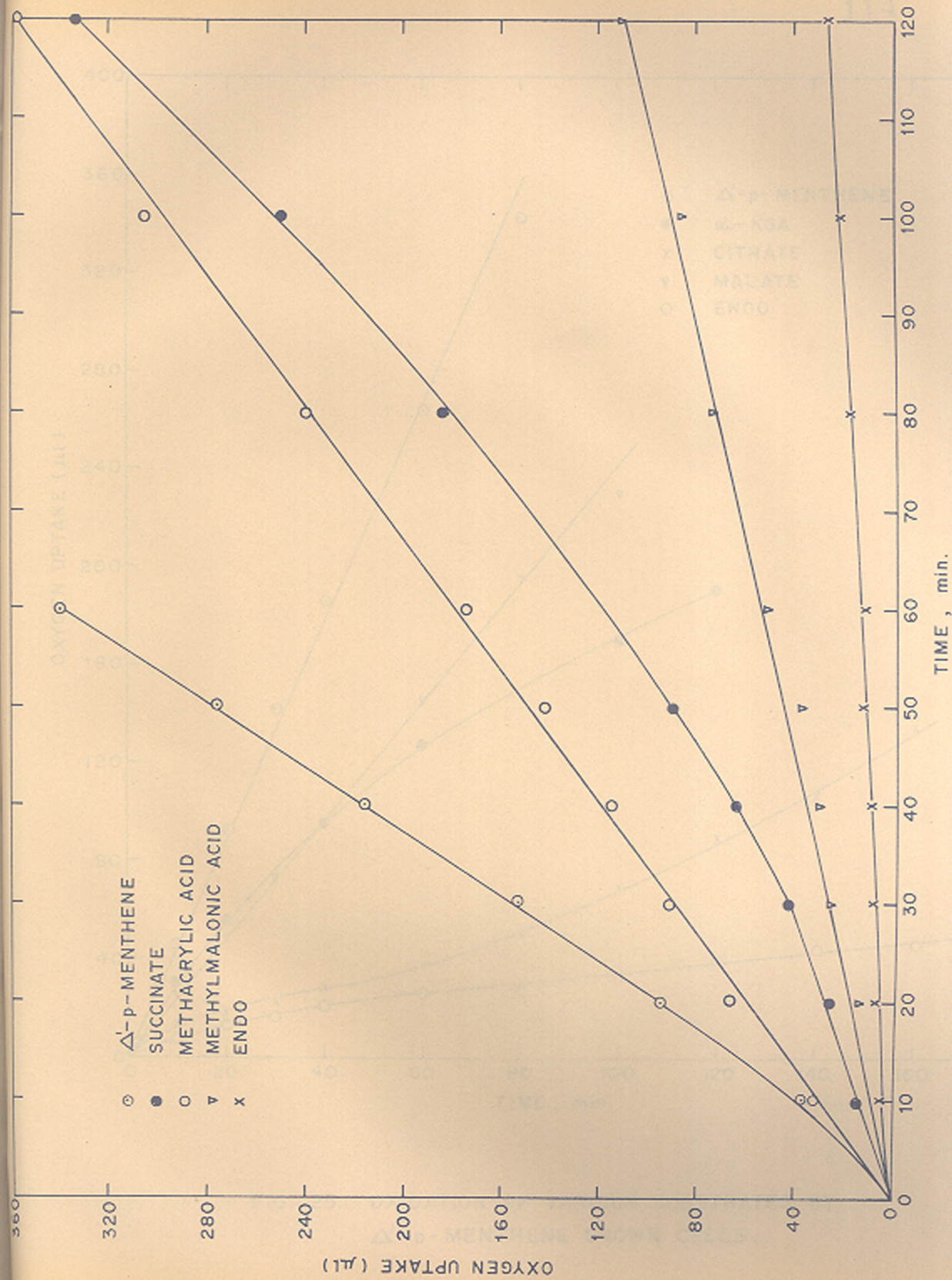


FIG. 24. OXIDATION OF VARIOUS SUBSTRATES BY Δ^1 -p-MENTHENE GROWN CELLS.

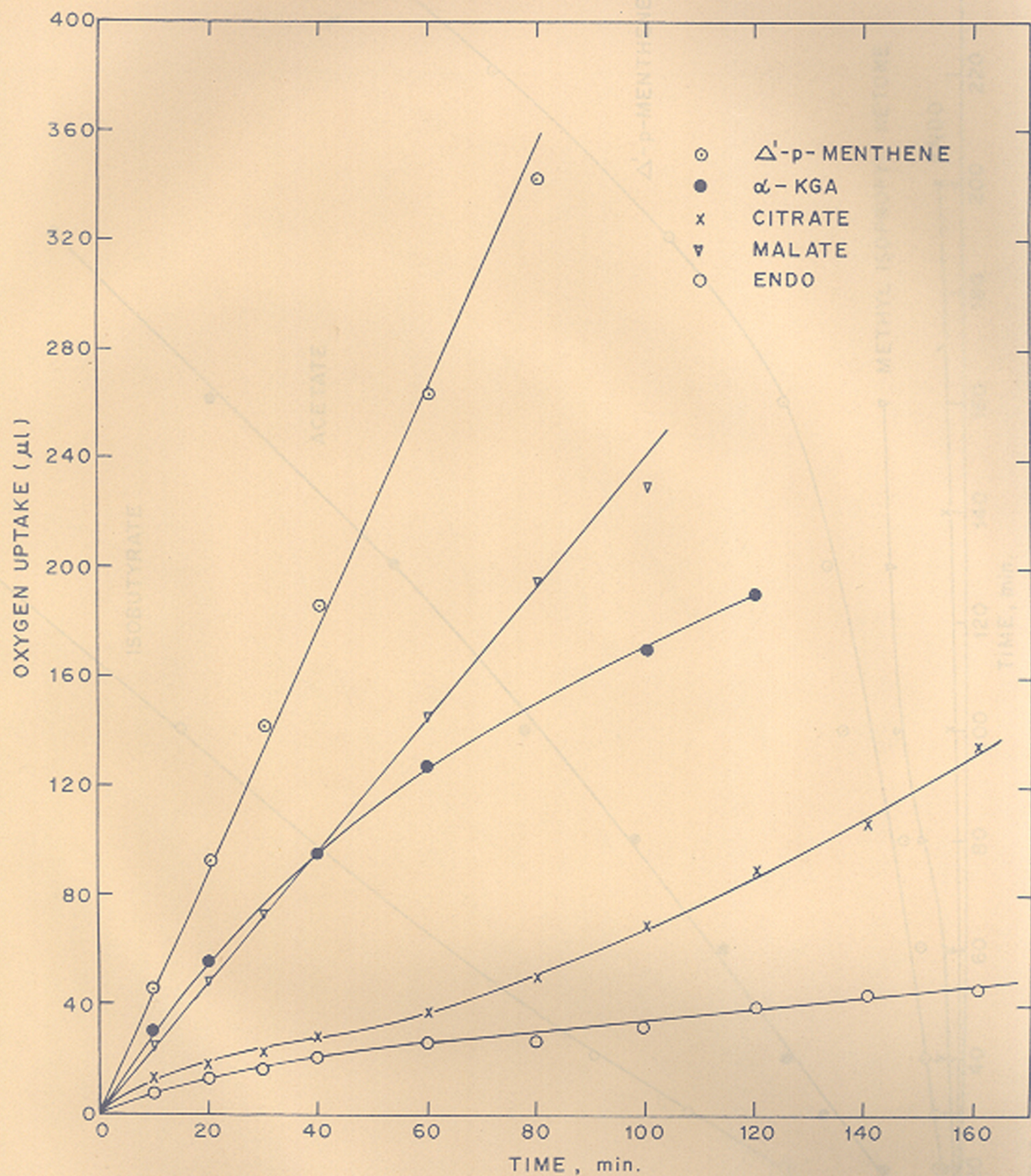


FIG. 25. OXIDATION OF VARIOUS SUBSTRATES BY Δ^1 -p-menthene GROWN CELLS.

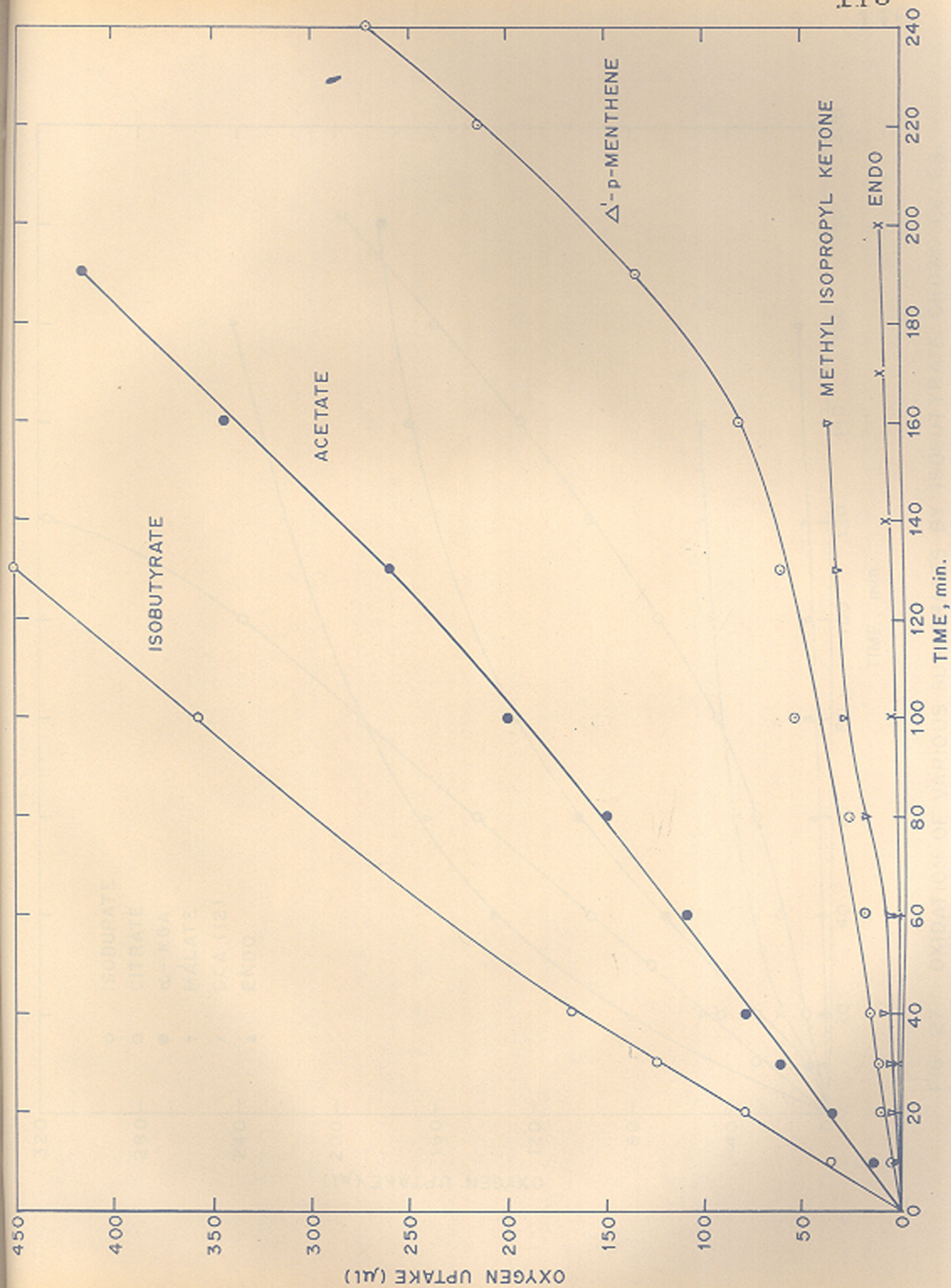
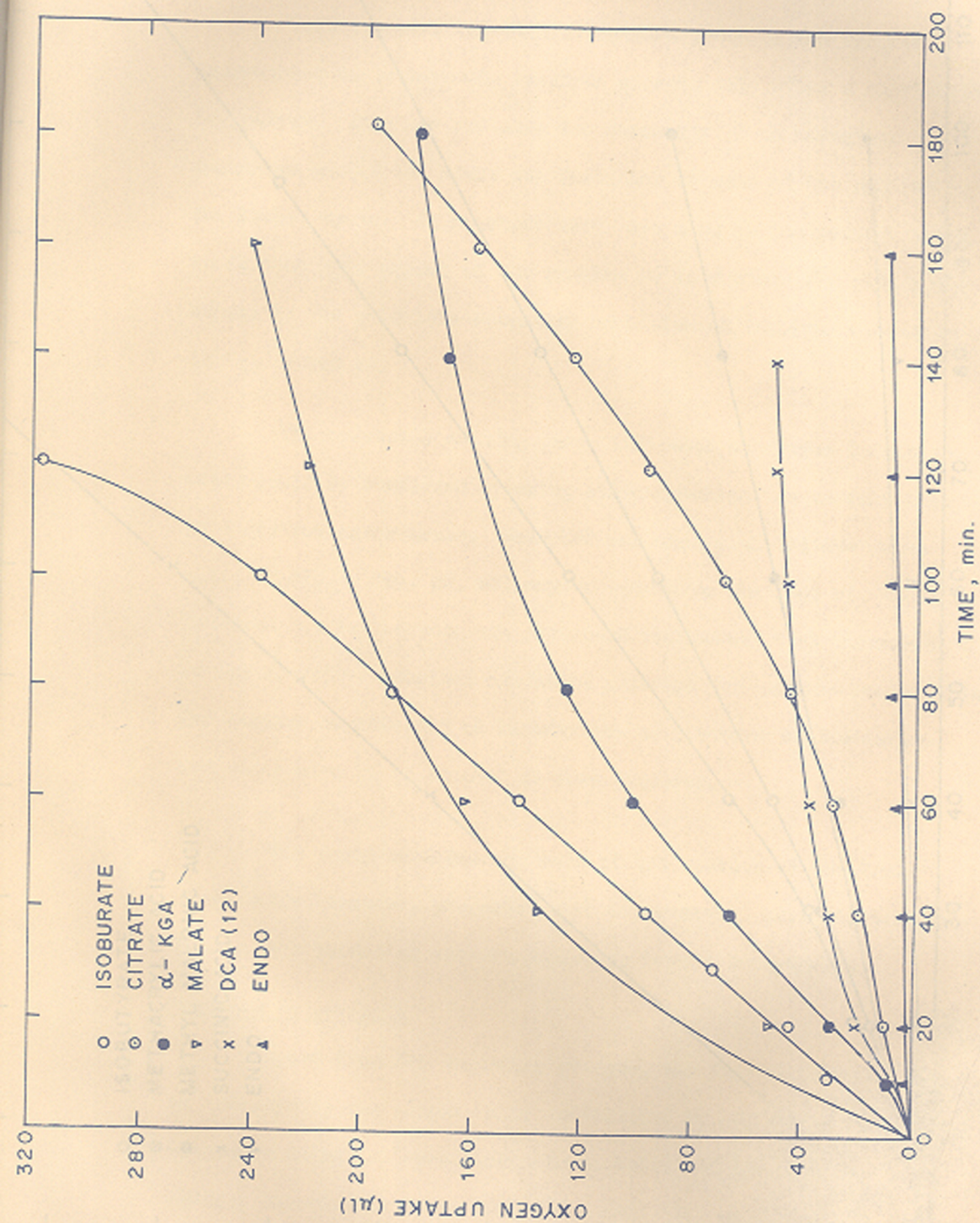


FIG. 26. OXIDATION OF VARIOUS SUBSTRATES BY ISOBUTYRATE GROWN CELLS.

FIG. 27. OXIDATION OF VARIOUS SUBSTRATES BY ISOBUTYRATE GROWN CELLS.



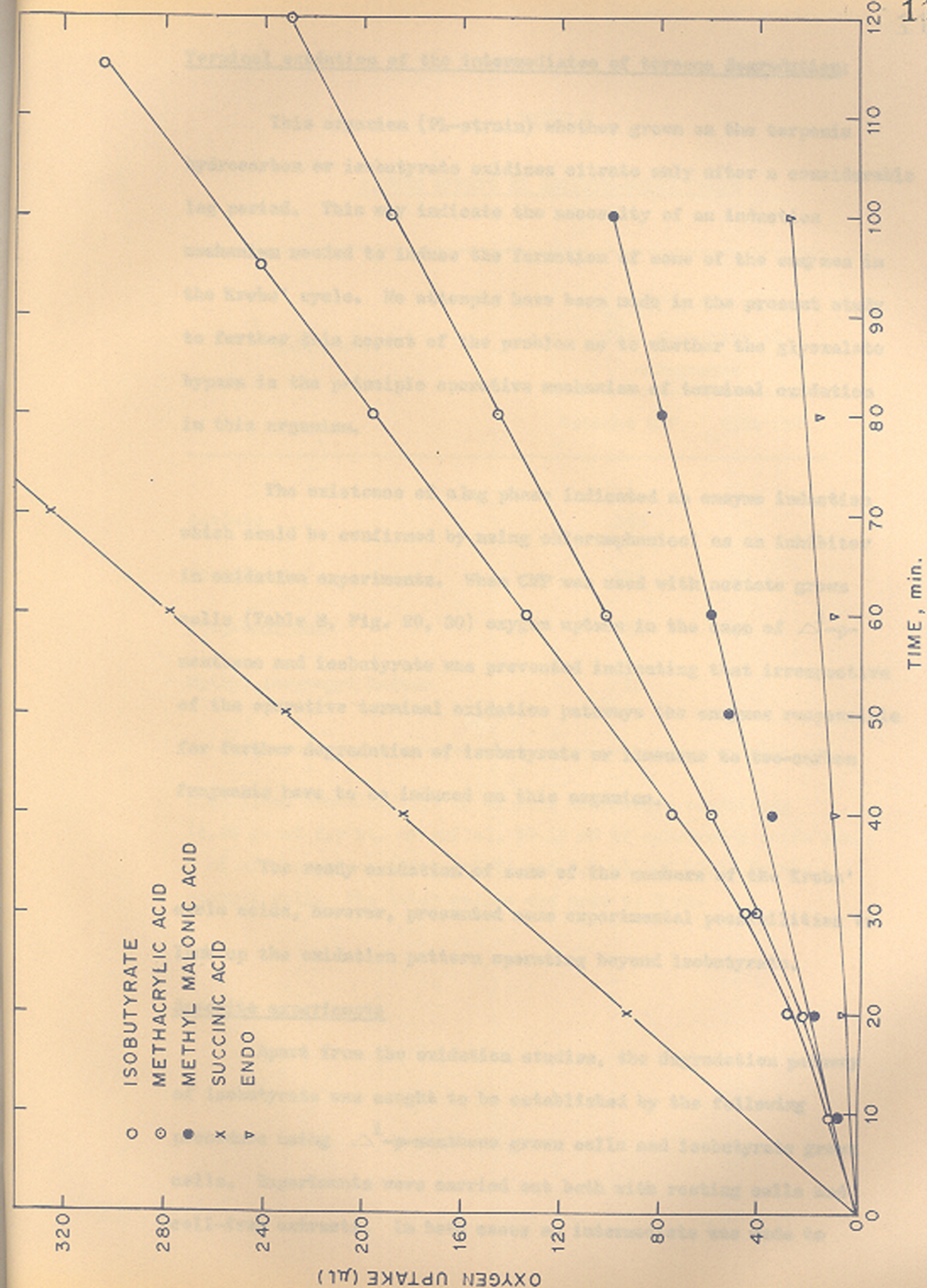


FIG. 28. OXIDATION OF VARIOUS SUBSTRATES BY ISOBUTYRATE GROWN CELLS.

Terminal oxidation of the intermediates of terpene degradation:

This organism (PL-strain) whether grown on the terpenic hydrocarbon or isobutyrate oxidizes citrate only after a considerable lag period. This may indicate the necessity of an induction mechanism needed to induce the formation of some of the enzymes in the Krebs' cycle. No attempts have been made in the present study to further this aspect of the problem as to whether the glyoxalate bypass is the principle operative mechanism of terminal oxidation in this organism.

The existence of a lag phase indicated an enzyme induction which could be confirmed by using chloramphenicol as an inhibitor in oxidation experiments. When CMP was used with acetate grown cells (Table 8, Fig. 29, 30) oxygen uptake in the case of Δ^1 -p-menthene and isobutyrate was prevented indicating that irrespective of the operative terminal oxidative pathways the enzymes responsible for further degradation of isobutyrate or limonene to two-carbon fragments have to be induced on this organism.

The ready oxidation of some of the members of the Krebs' cycle acids, however, presented some experimental possibilities to link up the oxidation pattern operating beyond isobutyrate.

Arsenite experiments

Apart from the oxidation studies, the degradation pathway of isobutyrate was sought to be established by the following procedure using Δ^1 -p-menthene grown cells and isobutyrate grown cells. Experiments were carried out both with resting cells and cell-free extracts. In both cases an intermediate was made to

TABLE 8

OXYGEN UPTAKE BY ACETATE-GROWN CELLS

Substrate	Q _{O₂} μ l/mg dry wt/hr after deduction of endogenous	
	Without CMP	With CMP
Acetate	342	-
Isobutyrate	207	33
Δ^1 -p-Menthene	175	25
Methyl isopropyl ketone	75	-

Each flask contained 1.5 ml of cell suspension containing (1.48 gm of dry wt. of cells), 10-12 μ M of substrates (side arm), 1 ml of phosphate buffer (pH 7) and 0.2 ml KOH (20%) in the central well. The total volume was made to 3 ml.

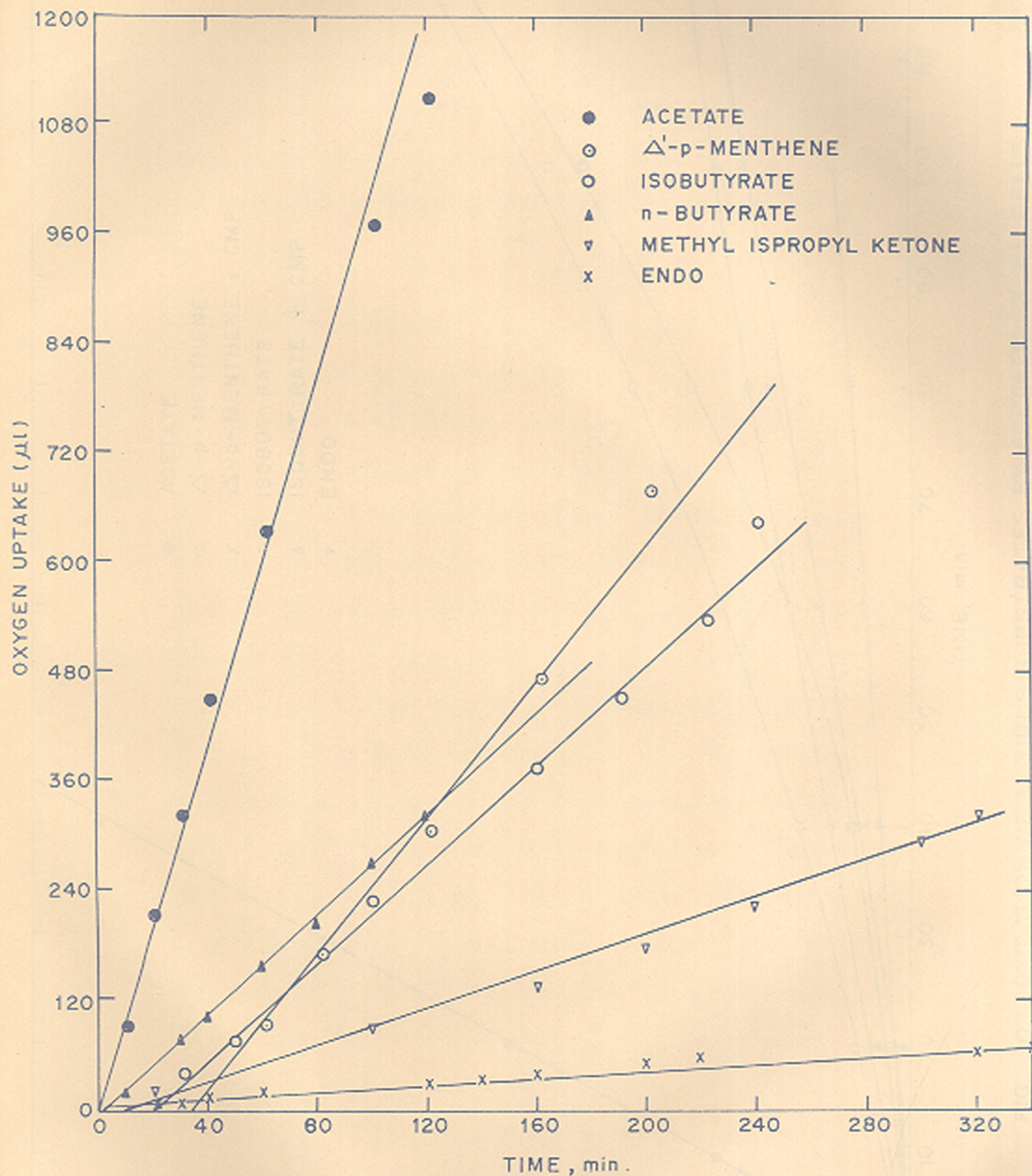


FIG. 29. OXIDATION OF VARIOUS SUBSTRATES BY ACETATE GROWN CELLS.

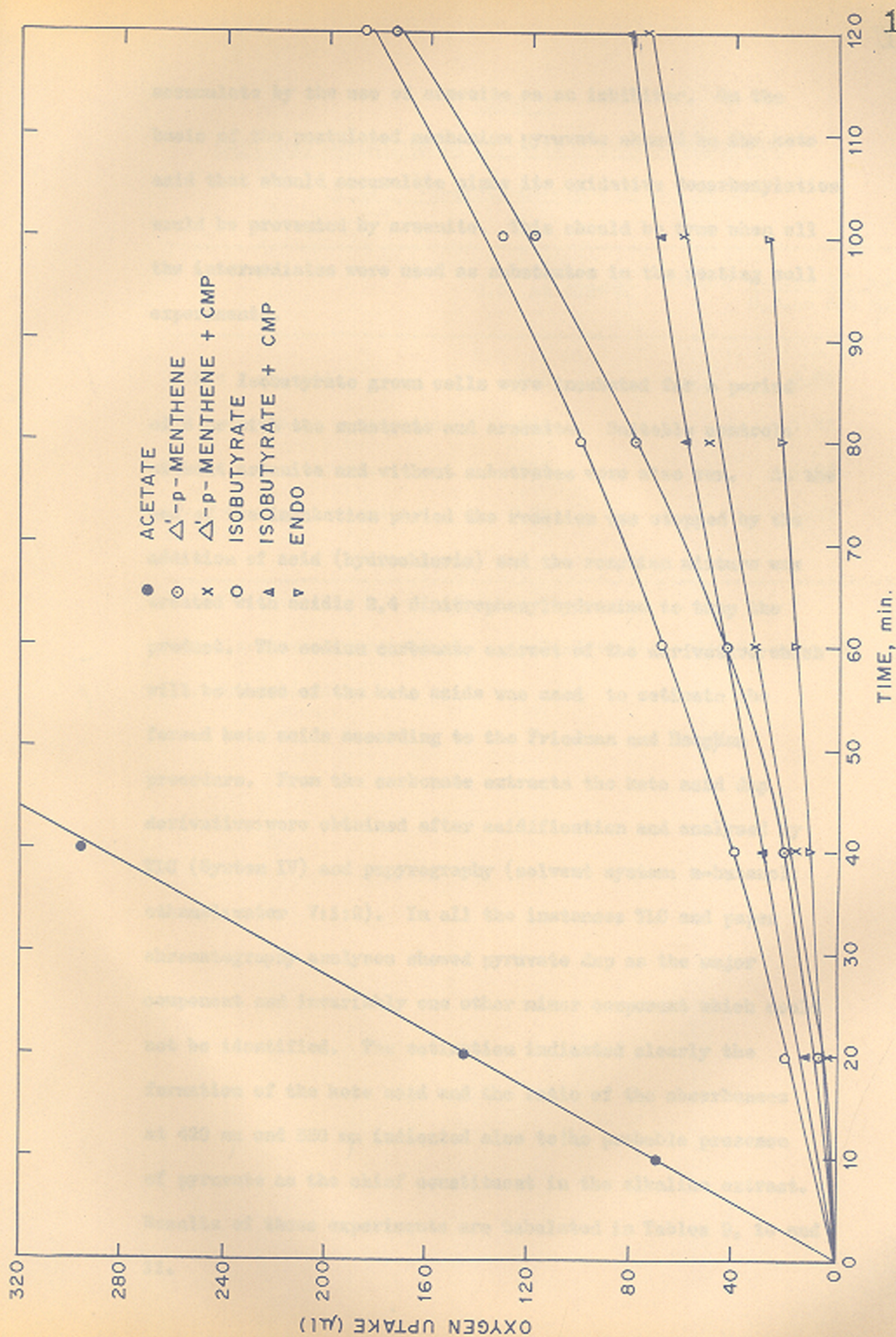


FIG. 30. OXIDATION OF VARIOUS SUBSTRATES BY ACETATE GROWN CELLS.

accumulate by the use of arsenite as an inhibitor. On the basis of the postulated mechanism pyruvate should be the keto acid that should accumulate since its oxidative decarboxylation would be prevented by arsenite. This should be true when all the intermediates were used as substrates in the resting cell experiments.

Isobutyrate grown cells were incubated for a period of 6 hr with the substrate and arsenite. Suitable controls without arsenite and without substrates were also run. At the end of the incubation period the reaction was stopped by the addition of acid (hydrochloric) and the reaction mixture was treated with acidic 2,4 dinitrophenylhydrazine to trap the product. The sodium carbonate extract of the derivative which will be those of the keto acids was used to estimate the formed keto acids according to the Friedman and Haugken procedure. From the carbonate extracts the keto acid dnp derivatives were obtained after acidification and analysed by TLC (System IV) and papyrography (solvent system: n-butanol: ethanol:water 7:1:2). In all the instances TLC and paper chromatography analyses showed pyruvate dnp as the major component and invariably one other minor component which could not be identified. The estimation indicated clearly the formation of the keto acid and the ratio of the absorbances at 420 m μ and 520 m μ indicated also to the probable presence of pyruvate as the chief constituent in the alkaline extract. Results of these experiments are tabulated in Tables 9, 10 and 11.

TABLE 9

KETO ACID ESTIMATION BY FRIEDMAN & HAUGHEM PROCEDURE

(with Δ^1 -p-menthene-grown cells, 24 hr)

	O.D. at 435m μ	Ratio: $\frac{\text{O.D. at 420 m}\mu}{\text{O.D. at 520 m}\mu}$
Cells + arsenite	.026	2.00
Δ^1 -p-Menthene + arsenite	.086	1.63
Cells + isobutyrate	.015	1.40
Isobutyrate + arsenite	2.34	1.29

Total sodium carbonate 3 ml. 0.3 ml of the extract was used
for the estimation.

TABLE 10

KETO ACID ESTIMATION BY FRIEDMAN & HAUGEN PROCEDURE

(with isobutyrate-grown cells, 24 hr)

	O.D. at 435 mμ	Ratio: $\frac{\text{O.D. at 420 mμ}}{\text{O.D. at 520 mμ}}$
Cells + arsenite	0.019	2.0
Cells + methacrylate	0.014	2.2
Cells + methacrylate + arsenite	1.000	1.34
Cells + methylmalonate	0.015	2.3
Cells + methylmalonate + arsenite	0.060	1.3
Cells + succinate	0.067	1.8
Cells + succinate + arsenite	0.852	1.76

Total sodium carbonate 3 ml. 0.3 ml of the extract was used for the estimation.

TABLE 11

KETO ACID ESTIMATION BY FRIEDEMANN & HAUGEN PROCEDURE

(with isobutyrate-grown cells, 40 hr)

	O.D. at 435 m μ	Ratio: $\frac{\text{O.D. at 420 m}\mu}{\text{O.D. at 520 m}\mu}$
Cells + arsenite	0.006	2.8
Cells + isobutyrate	0.004	2.1
Cells + isobutyrate + arsenite	2.535	1.3
Cells + methacrylate	0.018	2.00
Cells + methacrylate + arsenite	0.420	1.39
Cells + methylmalonate	0.016	2.3
Cells + methylmalonate + arsenite	0.750	1.32
Cells + succinate	0.031	1.70
Cells + succinate + arsenite	0.234	1.39

Total sodium carbonate extract 3 ml. 0.3 ml of the extract was used for the estimation.

In most of the cases when the cells were incubated with arsenite small amounts of other keto acids could be detected both by quantitative and by TLC analyses. This observation indicates only the presence of small amounts of intermediate products within the cell.

In some of the instances, as in the case of isobutyrate and methacrylate as substrates, sufficient amounts of dnp derivatives were obtained. It was possible to isolate the major component by preparative layer chromatography. The material so obtained was compared for its physical properties like m.p. and I.R. spectrum with those of an authentic sample of pyruvate dnp derivative (Fig. 21 a, b).

	<u>R_f values</u>	<u>cm</u>
Major component: m.p. 216-218°C	pyruvate, authentic	0.84
reported 218°	pyruvate from expts.	0.84
mixed 216-217°	pyruvate mixed	0.84
Minor component: R _f value 0.77 cm.		

No attempt was made to identify the minor dnp derivative as the material available was very small in all cases. It had a mobility pattern very close to that of the dnp derivative of methyl malonic semialdehyde.

Experiments with cell-free extracts

A similar series of experiments using the arsenite inhibition procedure was carried out employing isobutyrate and methylmalonate as substrates with crude cell-free sonicates of isobutyrate grown cells. Initial experiments without the addition of cofactors failed to show any accumulation of a keto acid. Hence experiments were designed in which DPN, FAD, ATP, CoA and vitamin B₁₂ were all added as cofactors.

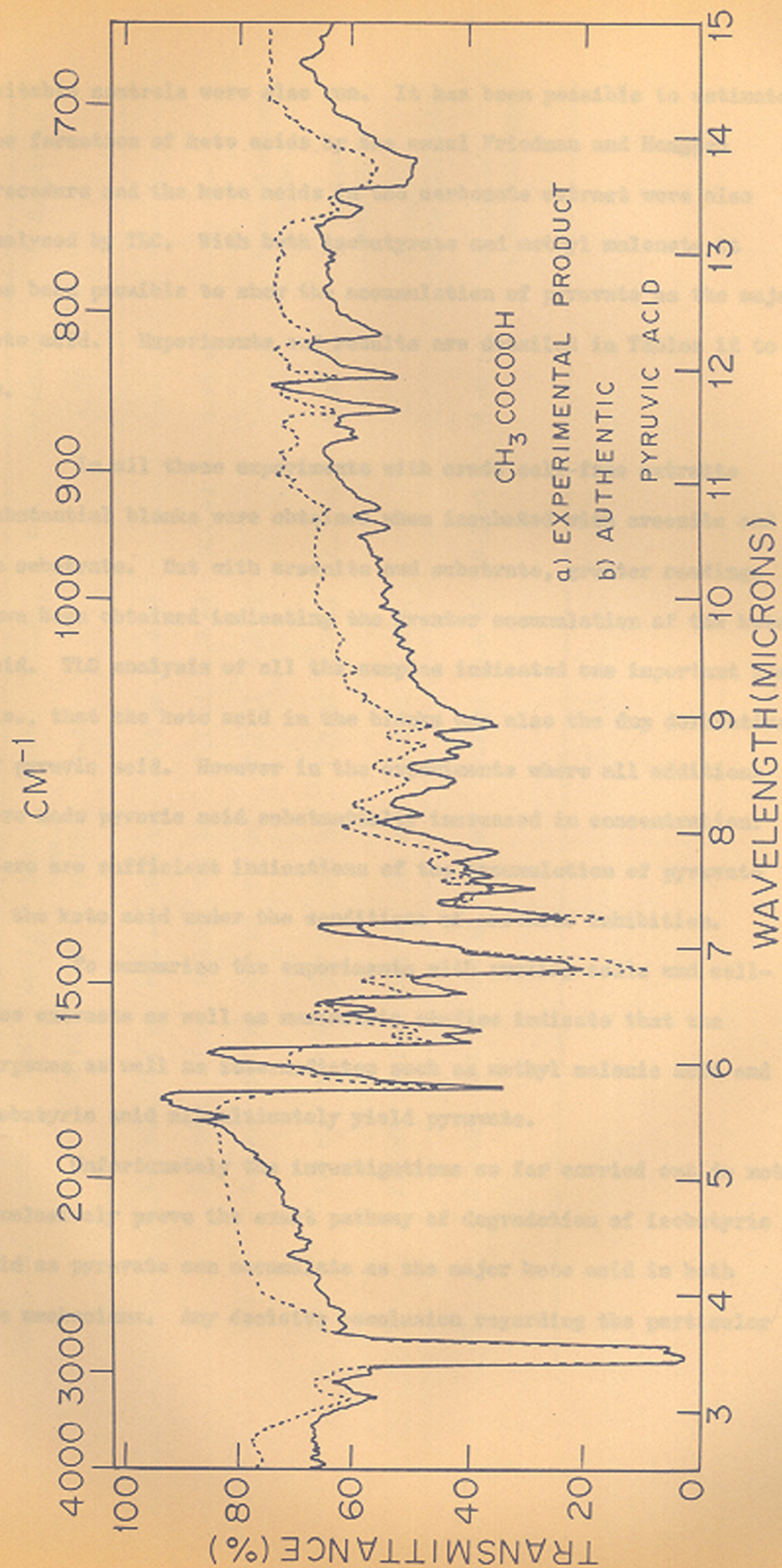


Fig. 21

Suitable controls were also run. It has been possible to estimate the formation of keto acids by the usual Friedman and Haugken procedure and the keto acids in the carbonate extract were also analysed by TLC. With both isobutyrate and methyl malonate it has been possible to show the accumulation of pyruvate as the major keto acid. Experiments and results are detailed in Tables 12 to 18.

In all these experiments with crude cell-free extracts substantial blanks were obtained when incubated with arsenite and no substrate. But with arsenite and substrate, greater readings have been obtained indicating the greater accumulation of the keto acid. TLC analysis of all the samples indicated one important fact viz., that the keto acid in the blanks was also the dmp derivative of pyruvic acid. However in the experiments where all additions were made pyruvic acid substantially increased in concentration. There are sufficient indications of the accumulation of pyruvate as the keto acid under the conditions of arsenite inhibition.

To summarise the experiments with resting cells and cell-free extracts as well as manometric studies indicate that the terpenes as well as intermediates such as methyl malonic acid and isobutyric acid all ultimately yield pyruvate.

Unfortunately the investigations so far carried out do not conclusively prove the exact pathway of degradation of isobutyric acid as pyruvate can accumulate as the major keto acid in both the mechanisms. Any decisive conclusion regarding the particular

TABLE 12

EXPERIMENTAL CONDITIONS WITH CELL-FREE EXTRACT

(with isobutyrate cells, 24 hr)

Expt. No.	CPE	DPN	MgCl ₂	Liv. conc.	FAD	Vit. B ₁₂	CoA	Arsenite	Substrate
		0.1M	0.1M	0.1M	0.1M			0.2M	
I	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	-	-	-
II	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	-
III	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	-	-	30 μ moles iso-butyrate
IV	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	30 μ moles isobutyrate
V	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	-	-	30 μ moles methylmalonate
VI	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	30 μ moles methylmalonate

TABLE 13KETOACID ESTIMATION BY FRIEDMAN AND HAUGEN PROCEDURE

(Results of experiments in Table 12 using isobutyrate-grown cells - 24 hr)

Expt. No.	O.D. at 435 m μ	Ratio:	<u>O.D. at 420 mμ</u>
			<u>O.D. at 520 mμ</u>
I	0.438		1.08
II	0.708		1.10
III	0.500		1.10
IV	0.700		1.27
V	0.522		1.13
VI	0.884		1.20

Sodium carbonate extract (3 ml) was used for the estimation.

TABLE 14

EXPERIMENTAL CONDITIONS WITH CELL-FREE EXTRACT

(with isobutyrate cells, 24 hr)

Expt. No.	CPE	DPN	MgCl ₂	Liv. conc.	FAD	ATP	Vit. B ₁₂	CoA	Arsenite	Substrate
		0.1M	0.1M	0.1M	0.1M	0.1M			0.2M	
I	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	-	-	-
II	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	-
III	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	-	-	50 μ moles isobutyrate
IV	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	50 μ moles isobutyrate
V	1.5 ml	-	0.1 ml	-	0.1 ml	0.1 ml	-	-	0.02 ml	50 μ moles isobutyrate
VI	1.5 ml	-	-	-	-	-	-	-	0.02 ml	50 μ moles isobutyrate

TABLE 15

KETO ACID ESTIMATION BY FRIEDMAN AND HAUGEN PROCEDURE

(Isobutyrate grown cells 24 hr. Results of the experiments described in Table 14)

Expt.No.	O.D.at 435 mμ	Ratio: $\frac{\text{O.D.at 420 mμ}}{\text{O.D.at 520 mμ}}$
I	0.345	1.07
II	0.975	1.23
III	0.450	1.22
IV	0.765	1.21
V	1.120	1.27
VI	0.690	1.40

Sodium carbonate extract (3 ml) was used for estimation.

TABLE 16

KETOACID ESTIMATION BY FRIEDMAN AND HAUGEN PROCEDURE

(Isobutyrate grown cells 40 hr*)

Expt.No.	O.D.at 435 $m\mu$	Ratio:
		$\frac{\text{O.D. at 420 } m\mu}{\text{O.D. at 520 } m\mu}$
I	0.252	1.28
II	0.600	1.32
III	0.520	1.40
IV	0.711	1.23
V	0.522	1.39
VI	0.345	1.30

Sodium carbonate extract (3 ml) was used for estimation.

*Similar set of experiments as given in Table 12
was run with isobutyrate (40 hr) grown cells.

TABLE 17

EXPERIMENTAL CONDITIONS WITH CELL-FREE EXTRACTS

(with 40 hr isobutyrate-grown cells)

Expt. No.	CPE	MgCl ₂ 0.1M ²	ATP 0.1M	CoA	Vitamin B ₁₂	DPN	Arsenite 0.2 M	Substrate (Methyl malonate)
I	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	-
II	1.5 ml	0.1 ml	-	-	-	-	0.02 ml	50 μ moles
III	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	0.02 ml	50 μ moles
IV	1.5 ml	0.1 ml	0.1 ml	0.1 ml	0.5 ml	0.1 ml	-	50 μ moles
V	1.5 ml	-	-	-	-	-	0.02 ml	-

Protein concentration was calculated to be 22.8 mg/ml.

Concentration of Vitamin B₁₂ used was 10 γ /ml

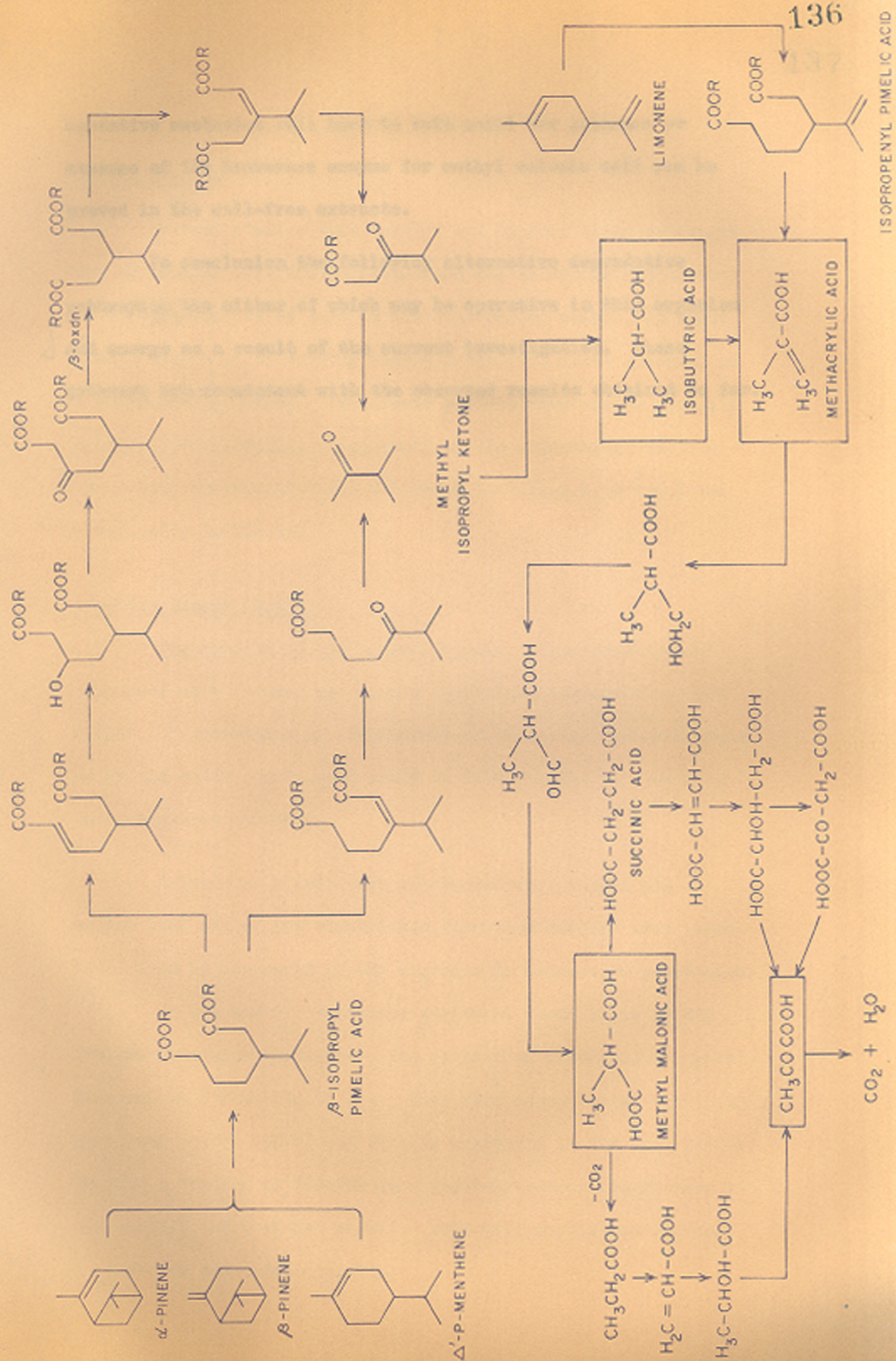
CoA concentration 10 mg/ml

TABLE 18KETO ACID ESTIMATION BY FRIEDMAN AND HAUGEN PROCEDURE

(Results of experiments described in Table 17)

Expt.No.	O.D.at 435 mμ	Ratio:	O.D. at 420 mμ
			O.D. at 520 mμ
I	0.420		1.2
II	0.468		1.48
III	0.600		1.33
IV	0.381		1.3
V	0.390		1.4

Sodium carbonate extract (3 ml) was used for estimation.



operative mechanism will have to wait until the presence or absence of the isomerase enzyme for methyl malonic acid can be proved in the cell-free extracts.

In conclusion the following alternative degradative pathways ~~as~~ either of which may be operative in this organism ~~and~~ emerge as a result of the current investigation. These pathways are consistent with the observed results obtained so far.

EXPERIMENTAL

Growth Studies

Growth studies were carried out by incubating cells with 100 ml mineral salt medium containing the different substrates at a concentration of 0.1% as the sole carbon source, in shake flasks with 2 ml of freshly washed cells (300 Klett reading) of a 24 hr grown culture. The growth was recorded either visually or turbidimetrically. The growth of the organism on various suspected intermediates are presented in Tables 3, 4 and 5 and graphically in Fig.22.

Adaptive Enzyme Studies

Preparation of cells for manometry: Experiments were conducted with neutral and acidic suspected intermediates with either Δ^1 -p-menthene or isobutyrate-grown cells. In most cases 24 hr old cells and in some cases 40 hr old cells were used in the manometric studies.

Erlenmeyer flasks (500 ml) containing mineral salt medium (100 ml) of the composition described earlier were inoculated from 24 hr-growth of the organism in respective substrates.

At the end of incubation period of 20-24 hr at $28 \pm 2^\circ\text{C}$ (unless otherwise mentioned), the cells were harvested by centrifugation at 5°C at $15,000 \times g$. They were washed twice with M/20 phosphate buffer (pH 7) and finally suspended in the same buffer to give a reading of 260-300 in a Klett-Summerson colorimeter at 660 m μ (1.3 - 1.5 mg dry wt/ml). This cell suspension was used as such for manometric studies.

Dry weight of washed cell suspension

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

Manometric methods

The oxidation of the various substrates was followed by usual manometric procedures using air as the gas phase (Umbreit *et al.* 1959). Cell suspension containing resting cells (1.5 ml of cell suspension, $R = 260-300$ at 660 m μ about 1.3 to 1.5 mg dry wt) and 1.0 ml of phosphate buffer (M/20, pH 7) were placed in the main compartment of the flask.

The substrates (10-20 μ M) were placed in the side arm. Central well contained 0.2 ml of 20% (w/v) KOH when used. The final concentration of chloramphenicol when used was 40 μ g/ml and the final volume was adjusted to 3.0 ml. The incubations were carried out at $28^{\circ} \pm 2^{\circ}$. The rates of oxidation are listed in Tables 6, 7 and 8 and graphically represented in Fig. 23 to 30.

Sonication of cells

Six flasks containing 100 ml basal medium were sterilized at 15 psi for 20 min. Aliquots of 2 ml of 24 hr Δ^1 -p-menthene or isobutyrate grown cell suspension in M/20 buffer were added to each flask along with the respective substrate and the flasks incubated on a rotary shaker for 24-40 hr at $28 \pm 2^{\circ}\text{C}$. After the incubation period the cells were centrifuged for 1 hr

at 20,000 x g. The subsequent operations were carried out at 0°C. The cells were washed with phosphate buffer (M/20), recentrifuged and were suspended in 25 ml M/20 phosphate, buffer, pH 7, (3 gm wet weight in 25 ml). The suspension was subjected to sonication (Raytheon magnetostriction sonic oscillator, 10 kc, 250 W) for 15 min. The sonicate was centrifuged for 45 min at 20,000 x g and the supernatant was used for the experiment. The protein concentration in the extracts was estimated according to the method of Warburg and Christian (1942).

Enzymatic reactions

The reactions were carried out in boiling tubes. After the addition of substrates, cofactors and the enzyme suspension and arsenite solution 0.2 M to the final concentration of 2.5×10^{-2} along with suitable blanks, the tubes were kept on a rotary shaker and the reaction mixture was incubated for 4 hr at $28^{\circ} \pm 2^{\circ}\text{C}$. The reaction was stopped by the addition of 3N HCl (1 ml each tube) and the contents in each tube (experimental and control) were mixed with 3 ml of 2,4-dinitrophenyl hydrazine in 3N HCl and left for 3 hr at room temperature with frequent shaking and then extracted with (2 x 3 ml) of ethylacetate (sodium carbonate washed). It was separated into acid and neutral fractions by extracting the organic layer with 3 x 2 ml of sodium carbonate solution (10%). After neutralization of the carbonate layer, it was extracted with 3 x 2 ml of ethyl acetate and finally concentrated to a minimum amount for testing on TLC and isolated into the individual component by preparative layer chromatography. System THF 60 ml, Benzene 30 ml, HAc 2 ml.

Pyruvate formation

An aliquot of 2.5 ml of sonicate extract of resting cells was suspended in 0.05 ml buffer in 5 different flasks. Isobutyrate (50 μ mole) was added to each of flasks along with suitable enzyme blanks were incubated on a rotary shaker at 120 r.p.m. at $28^{\circ}\pm 2^{\circ}\text{C}$ for a period of 2 hr. After the required incubation period, the reaction was arrested by acidifying with HCl (5N) to congo red. The denatured protein was removed by centrifugation. To the clear supernatant 0.1% 2,4 dinitrophenyl hydrazine solution in 3N HCl was added in slight excess and was allowed to stand at 30°C for 4 hr. The precipitated 2,4 dnp derivatives were taken in ethyl-acetate and acidic and neutral components were separated by partitioning with 10% sodium carbonate solution. 1 ml aliquots of each flasks were used for the estimation of keto acids by Friedman-Haughen procedure. Similar experiments were carried out with methyl malonic acid and succinate.

The acidic 2,4 dnp fraction was found to contain three components on TLC examination out of which one was found to be in major quantity. This fraction was further subjected to preparative layer chromatography and the component which was in major quantity was recovered. It was further crystallized from ethanol to orange colored needles m.p. 218°C . When examined on TLC (R_f 0.84) it was found to have the mobility pattern identical with that of 2,4 dnp of authentic pyruvic acid. No depression in the melting point was observed when

the preparation was admixed with an authentic sample. The presence of pyruvic acid in the reaction mixture was then conclusively proved by the spectral comparison of the hydrazone of the authentic sample with that of the compound isolated from the experiment.

Chromatography on paper also indicated identical mobility pattern of the sample with the authentic compound in n-butanol-ethanol-water 7:1:2.

Two other acidic hydrazones were present in the mixture which could not be isolated in sufficient amounts for further characterization.

2,4-DNP derivative of neutral carbonyl compounds

The neutral 2,4-dnp on TLC examination was found to contain two compounds out of which one was in major quantity. On TLC the major component had an identical mobility pattern as that of 2,4 dnp of authentic acetone. The formation of acetone may be traced to the possible presence of acetoacetic acid which, when treated with acidic 2,4 dnp solution, might have undergone decarboxylation.

Corresponding enzyme blanks and substrates were worked out in the similar way and the results have been detailed and discussed in the previous chapter.

CHAPTER 7

S U M M A R Y

SUMMARY

It has been known since long that microorganisms can be utilized extensively for the production of various compounds of economic value, but less is known about the detailed nature of dissimination of the organic compounds and their entry into the carbon cycle. Recently, several groups of workers have shown widespread interest in the biodegradation of various hydrocarbons. Their studies by way of working with the whole cells or with the isolated enzymes have yielded some definite ideas about the way in which these compounds are metabolised by these microorganisms.

A soil pseudomonad "PL-strain" isolated by enrichment culture technique on α -pinene was found to grow not only on hydroaromatic terpenoid substrates such as α -pinene, β -pinene, limonene and Δ^1 -p-menthene but also on the aromatic compound hydrocarbon, p-cymene.

Fermentation of limonene by this organism resulted in the formation of a large number of neutral and acidic transformation products. The neutral compounds had been isolated and identified as (+) Dihydrocarvone, (+) Carvone, (+) Carveol, (+)-p-mentha-8-ene 1-ol, 2-one, (+) p-mentha-8-ene trans, 1,2 diol, (+) p-mentha-8-ene-cis-1,2 diol and (+) p-mentha-1-ene-6,9-diol.

An epoxide intermediate has been assumed in the biodegradation of limonene and Δ^1 -p-menthene and the epoxide is subsequently expected to undergo various transformations leading to the formation of several of these neutral compounds. In the

present study a similar epoxide is formed chemically from limonene and transformed also by chemical synthesis to find out the nature and structure of the compounds formed. This method of attack of the problem is expected also to throw light on the stereochemistry of the compounds under examination. Another direct method employed in this study has been the synthesis of some of these compounds which would again lead to a clearer understanding of the absolute configuration.

1) Synthesis of these neutral intermediates was essential in order to confirm the structures and the stereochemistry of these products. Following were the synthetic methods developed for preparing these neutral components.

Prolonged peracetic acid oxidation of (+) limonene yielded (+) dihydrocarvone, (-) carvone, (+) carveol, (+)-p-mentha-8-ene 1-ol, 2-one and (+) p-mentha-8-ene trans-1,2 diol. Parallelism between chemical degradation of (+) limonene with fermentation of (+) limonene is discussed.

Synthesis of p-menth-8-ene-cis-1,2 diol was furnished by following Woodward's hydroxylation method namely, (+) limonene was hydroxylated using silver acetate, iodine and wet-acetic acid to yield the cis diol.

p-Mantha-1-ene, 6,9 diol was prepared by selective hydroboration of (+) carveol using bis-3-methyl-2 butyl borane.

2) The structure of β -isopropyl-pimelic acid (12), a fermentation product of α -pinene, β -pinene and Δ^1 -p-menthene and of β -isopropenyl pimelic acid (8) from limonene $\int$ as

comp.(8) gives comp.(12) on catalytic reduction] are established by the total synthesis of (12).

a) Starting from β -isopropyl glutaric acid, which was converted to its monomethyl ester acid chloride, and condensed with malonic ester to give a ketotricarboxylic ester. This ketotriester after Clemenson reduction yielded β -isopropyl pimelic acid (12).

The absolute configuration of comp.(12) [as well as that of comp.(8)] has been established by another synthesis starting from (+) Δ^1 -p-menthene.

b) Ozonolysis of (+) Δ^1 -p-menthene gave a keto acid which was subjected to Willgerodt reaction (Kindler modification) to give β -isopropyl pimelic acid (12) having a specific rotation $[\alpha]_D^{25} = -6.2^\circ$ which is identical with that obtained in the bacterial fermentation.

3) The earlier workers had failed to isolate and identify any other intermediate with lesser number of carbon atoms than those found in β -isopropyl pimelic acid or β -isopropenyl pimelic acid. A careful examination of the fermented broth from Δ^1 -p-menthene showed the presence of a water soluble keto compound which was isolated as its dnp derivative and identified unequivocally as methyl isopropyl ketone. The isolation of this compound combined with some of the growth studies gave sufficient clue to the probable mechanism of the breakdown of β -isopropyl pimelic acid to carbon dioxide and water.

a) Growth and oxidation

The organism grown on Δ^1 -p-menthene was found to grow without any lag on methyl isopropyl ketone, isobutyrate, n-butyrate, succinate, malate and acetate. The ready growth of this organism on some of the members of the Krebs' cycle acids such as malate, succinate and acetate could indicate the probable participation of the Krebs' cycle along with the glyoxalate by-pass.

Isobutyrate grown cells grew on Δ^1 -p-menthene after a lag period of 8 hr. Different attempts to grow this organism on the dicarboxylic acid, β -isopropyl pimelic acid did not meet with success and inability to grow has been discussed.

b) The ready growth of the organism on methyl isopropyl ketone and isobutyrate suggested these as probable intermediates during the breakdown of β -isopropyl pimelic acid and this was sought to be confirmed through the oxidation studies.

Δ^1 -p-Menthene grown cells oxidized readily isobutyrate, acetate, succinate, malate, α -ketoglutarate and methacrylate. Methyl malonate, methyl isopropyl ketone and the saturated dicarboxylic acid, β -isopropyl pimelic acid were oxidized at rather very slow rates.

Isobutyrate grown cells, on the other hand, showed from good to very fair oxidation rates with succinate, isobutyrate, acetate, malate, methacrylate, α -ketoglutarate. Methyl malonate was oxidized much better and methyl isopropyl ketone was oxidized slowly. Δ^1 -p-Menthene was oxidized only after a lag period of 2 hr and citrate was oxidized after a considerable lag.

Acetate grown cells oxidized after a lag Δ^1 -p-menthene and isobutyrate and the induced nature of the enzymes for their oxidation could be demonstrated by the use of chloramphenicol. Methyl isopropyl ketone was oxidized by these cells at a comparatively higher rate.

The catabolic pattern emerging out of these growth and oxidation studies was sought to be utilized for evolving a mechanism by a few resting cell and cell-free extract studies using arsenite as an inhibitor.

Δ^1 -p-Menthene grown cells and isobutyrate grown cells were used under resting conditions with Δ^1 -p-menthene and isobutyrate as substrates. An analysis of the reaction products indicated the accumulation of a keto acid. Suitable blanks have also been run to obtain unequivocal results. The keto acid has been isolated and identified by different techniques as pyruvic acid. These experiments had been repeated using isobutyrate grown cells with methacrylic acid, methyl malonic acid and succinic acid as substrates. In all these cases pyruvic acid could be shown to be formed as the major keto acid.

The arsenite inhibition experiment had been repeated with cell free extracts obtained from isobutyrate grown cells and isobutyrate and methyl malonate as substrates. Various cofactors have been externally added and suitable blanks have been run. These experiments also showed the formation of pyruvic acid as the major keto acid accumulating under the conditions of experimentation.

Based on the growth, oxidation and resting cell experiments with isobutyrate, a probable mechanism of breakdown of this compound

to known intermediates of the oxidative pathway has been suggested. Two possible mechanisms of degradation of isobutyrate have been suggested and a clear-cut major pathway can be ascertained only after isolation and study of the properties of some of the enzymes involved in these mechanisms.

In conclusion a more plausible pathway has been outlined for the breakdown of β -isopropyl and β -isopropenyl pimelic acids before they are converted to carbon dioxide and water.

THE
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