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TERPENE METABOLISM

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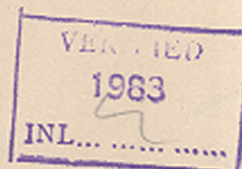

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ACKNOWLEDGMENT

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He wishes to express his indebtedness to the Director, National Chemical Laboratory, for allowing him to work as a honorary worker to complete the work undertaken and for his permission to submit it in the form of a thesis.

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C O N T E N T S

CHAPTER I	INTRODUCTION	...	1
CHAPTER II	MATERIALS AND METHODS	...	14
CHAPTER III	EXPERIMENTAL (Growth Studies)	...	22
CHAPTER IV	EXPERIMENTAL (Adaptive enzyme studies)	. ...	42
CHAPTER V	DISCUSSION	...	50
CHAPTER VI	SUMMARY	...	64
	BIBLIOGRAPHY	...	68

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List of abbreviations

ATP	-	Adenosine triphosphate
CMP	-	Chloramphenicol
FAD	-	Flavin adeninedinucleotide
FMN	-	Flavin mononucleotide
NAD	-	Nicotinamide-adeninedinucleotide
NADH	-	Reduced form of NAD
NADP	-	Nicotinamide-adeninedinucleotide phosphate
NADPH	-	Reduced form of NADP
Co-A-SH	-	Co-enzyme A (reduced form)

*

CHAPTER I

INTRODUCTION

INTRODUCTION

About four years ago a Pseudomonad, capable of growing on α-pinene (1) as the sole source of carbon was isolated from soil in this laboratory by enrichment culture techniques. (Bhattacharyya et.al., 1964 a, Shukla 1965) It was observed that this organism could also grow equally well on β-pinene (2) limonene (3), Δ^1p-menthene (4) and p-cymene (5).

Fermentation of limonene

Dhavalikar (1964) and Bhattacharyya (et.al.) have studied the fermentation of limonene by a variant of this pseudomonad and isolated and identified several products of fermentation. The neutral extractives of the fermentation were found to contain carvone (6), dihydrocarvone (7), carveol (8), p-menth-8-ene-1,2,trans-diol (9), p-menth 8-ene-1-ol-2-one (10) and p-menth-1-ene-6,9 diol (11). The acidic transofmration products contained mainly perillic acid (12), 2 hydroxy p-menth-8-ene-7-oic acid (13), β-isopropenyl pimelic acid (14) and 4,9 dihydroxy-p-menth-1-ene-7-oic acid (15) and several other unidentified compounds.

Growth and adaptive enzyme studies with intact cells indicated that none of the neutral compounds (6 to 11) supported growth or respiration of

limonene-grown cells, and are probably derived from limonene 1,2-oxide (16) in the manner shown in Chart I. (Pathway 1)

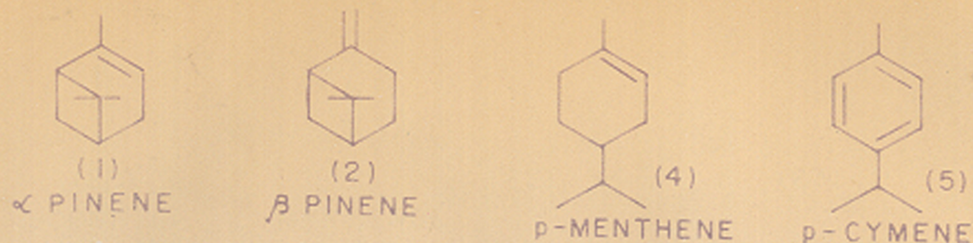
The acidic compounds (12 to 15) were on the other hand oxidised freely by limonene-grown cells with or without added chloramphenicol.

Based on the above observation, pathway No.2 (Chart 1) was proposed for the degradation of limonene. Limonene gets hydroxylated to perillic alcohol (17) which undergoes dehydrogenation in two successive stages through perillic aldehyde (18) to perillic acid (12). Hydration of the α - β-unsaturated acid (12) leads to the formation of the hydroxy acid (13) which is presumably oxidised to the -keto acid (19) ultimately yielding the open chain dicarboxylic acid (14) by a base-catalysed cleavage mechanism.

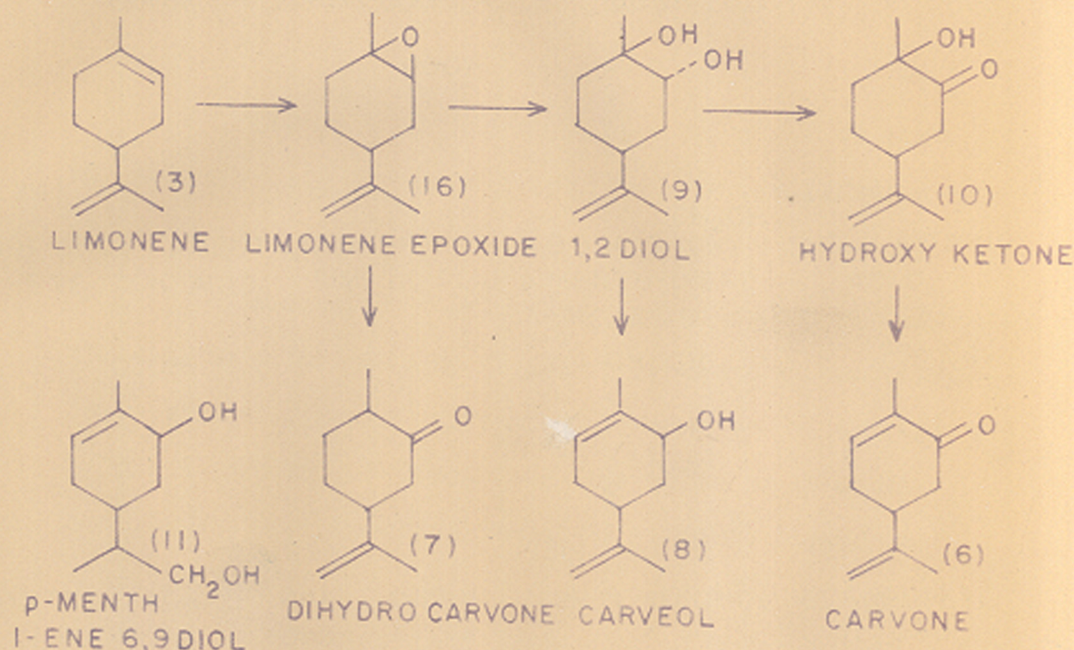
The mode of dissimilation of the dicarboxylic acid (14) ultimately to yield carbon dioxide, water and cell-material could not be established further, and the exact position of the dihydroxy acid (15) with respect to the proposed pathway was not elucidated.

Nevertheless, an indirect evidence regarding the ultimate degradation of limonene and perillic acid to two carbon fragments was deduced from the accumulation of a C-20 straight-chain fatty acid in the fermentation mixture.

CHART 1



PATHWAY 1 FOR LIMONENE DEGRADATION



PATHWAY 2 FOR LIMONENE DEGRADATION

(3) → CH_2OH (17) → CHO (18) → COOH (12)

LIMONENE   PERILLYL ALCOHOL   PERILL ALDEHYDE   PERILLIC ACID

COOH, OH (13) HYDROXY ACID → [COOH, O (19)] β-KETO ACID → COOH, COOH (14) DICARBOXYLIC ACID → ? CO_2 + H_2O + (CELL MAT.)

COOH, OH, CH_2OH (15)
4,9 DIHYDROXY
p-MENTH-1-ENE-
7-OIC ACID

limonene-grown cells, and are probably derived from limonene 1,2-oxide (16) in the manner shown in Chart I. (Pathway 1)

The acidic compounds (12 to 15) were on the other hand oxidised freely by limonene-grown cells with or without added chloramphenicol.

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The mode of dissimilation of the dicarboxylic acid (14) ultimately to yield carbon dioxide, water and cell-material could not be established further, and the exact position of the dihydroxy acid (15) with respect to the proposed pathway was not elucidated.

Nevertheless, an indirect evidence regarding the ultimate degradation of limonene and perillic acid to two carbon fragments was deduced from the accumulation of a C-20 straight-chain fatty acid in the fermentation mixture.

Further evidence supporting the pathway No.2 was obtained from the observation, that the fermentation of limonene by glucose- or sucrose-grown cells resulted in the accumulation of perillic acid (12) in substantial yields along with traces of perillyl alcohol (17).

Moreover, the fermentation of perillic acid (12) by perillic acid grown cells led to the accumulation of the dicarboxylic acid (14).

Studies with cell-free enzymes also corroborated the existence of pathway No.2 for limonene degradation. The hydroxylation of limonene was shown to be carried out by the sonicates of limonene-grown cells as well as the particulate fraction obtained by centrifugation of sonicates at 100,000 g. in presence of NADPH and oxygen. The supernatant from the 100,000 g. precipitate were shown to convert perillic alcohol (17) and perillic aldehyde (18) to perillic acid (12) in presence of NAD and a mixture of added FAD and FMN, respectively.

These preparations also converted perillic acid (12) to β-isopropenyl pimelic acid (14) in presence of NAD, ATP, Co-A-SH and Mg^{++}, An indirect pointer to the involvement of Co-A-SH at the perillic acid stage was obtained by the observed failure of the hydration reaction (12 → 13) under anaerobic condition with intact cells.

Fermentation of α-pinene and β-pinene

The product pattern from α- and β-pinene although almost identical in themselves exhibited considerable difference from that of limonene (Shukla 1965, Bhattacharyya et.al., 1964, Bhattacharyya et.al. 1965). From the neutral extractives of the fermentation mixture borneol (20) could be isolated. The presence of myrtenol, (21), in this fraction was demonstrated by vapour phase chromatography and thin layer chromatography.

From the acidic components the following compounds were isolated. Oleuropeic acid (22), 4-hydroxy-p-menthane-7-oic acid (23), (from α-pinene), 4-hydroxy-p-menth-l-ene-7- oic acid (24) (from β-pinene), β-isopropylpimelic acid (25) and 4,9 dihydroxy p-menth-l-ene-7-oic acid (26), Besides these acids the presence of three others viz. perillic acid (12) phellandric acid (27) and myrtenic acid (28) was established by comparative vapour phase chromatography with authentic samples. It is noteworthy that d-α-pinene gave rise to d-borneol and (+) oleuropeic acid while β-pinene gave the optical antipodes, l-borneol and (-) oleucropeic acid. The specific rotation of the β-isopropyl pimellic acid, irrespective of its origin from α - or β-pinene, was

identical ($[\alpha]_D$ -5°). The only products which were common from the fermentations of limonene α- and β- pinenes were perillic acid (12) and the dihydroxy acid (15). Besides these well defined compounds the fermentations mixture contained many others which could not be isolated and identified.

Growth and respiration studies with α- and β-pinene-grown cells indicated that borneol (20) myrtenol (21), myrtenic acid (28), oleuropeic acid (22) were not in the main pathway of the degradation of the pinenes, since none of these compounds supported growth or respiration of the organism.

All the compounds on the limonene pathway viz. (perillic alcohol (17), perillic aldehyde (18), perillic acid (12)) as well as phellandric acid (27) were oxidised by these cells with or without added chloramphenicol. The unsaturated dicarboxylic acid (14) from limonene, as well as the saturated dicarboxylic acid (25) from the pinenes were oxidized very slowly by pinene-grown cells.

Cell-free extracts from the pinene-grown cells exhibited enzymatic activities very similar to those from limonene-grown cells. The perillic alcohol dehydrogenase from these cells exhibited almost identical rates for NAD reduction with perillic alcohol (17), phellandrol (29) and cumyl alcohol (30).

The observed rate of oxidation of oleuropenyl alcohol (31) was higher. However, this enzyme was inactive towards myrtenol (21). It is noteworthy that the rate pattern of the dehydrogenase in the crude extract from α- or β-pinene grown cells was identical with that of the purified enzyme from the limonene - bacterium (Ballal et.al. 1965).

The aldehyde dehydrogenase in the crude extract showed almost identical rates with perillic aldehyde (18), phellandral (32) and, surprisingly enough, myrtenal (33). The oxidation of oleuropeyl aldehyde (34) again proceeded at a rate substantially higher than that of perillic aldehyde (18).

Based on the above observations, an overall scheme for degradation of α- and β-pinenes has been formulated (Chart 2) comprising of several parallel pathways. In one of these pathways the 7-methyl group (or the methylene group) of α- and β-pinene is hydroxylated and the resulting myrtenol (21) is dehydrogenated to myrtenal (33) by a specific enzyme. Myrtenal (33) can be dehydrogenated to myrtenic acid (28) by a common aldehyde dehydrogenase. In this pathway myrtenic acid (28) is the ultimate product, since this compound is not further oxidized by the cells.

CHART- 2

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

Pathways No.II, III, IV, V and VI have as a common initial step a protonation on the double bond of the pinenes, to lead to a common carbonium ion (35). In pathway No.II this carbonium ion (35) undergoes, Wagner-Meerwein rearrangement to borneol (20) which is an end product.

In the pathway No.III, IV, V and VI the carbonium ion (35) rearranges to the carbonium ion (36) by cleavage of the cyclobutane ring. In the pathway III, it is neutralised to yield α-terpineol (37) which gives rise to oleuropeic acid (22) as the end product by progressive oxidation at the 7-methyl position through the intermediates (31) and (34).

In pathway IV the open chain carbonium ion (36) is postulated to undergo elimination at 8-9 position to yield limonene (3) which can undergo all the transformation in the limonene pathway (Chart I, pathway 2).

It should be noted that another type of elimination in the carbonium ion (36) is possible leading to terpinolene (38) which may undergo the same fate as limonene by a parallel pathway. However, since none of the compounds isolated so far has the Δ^{4}-8-double bond, this pathway has not been shown in the Chart.

A hydride shift from position 4 to 7 in the carbonium ion (36) may give rise to another carbonium ion (39) from which pathway V and VI start. In pathway No. V the carbonium ion (39) is stereospecifically reduced by a suitable hydride donor (such as NADPH) to give rise to Δ^1-p-menthene (4) which ultimately gives rise to β-isopropyl pimellic acid (25) through phellandrol (29) phellandral (32) phellandric acid (27) and the hydroxy acid (41) by a pathway parallel to that of limonene.

Alternately, the neutralisation of the carbonium ion (39) may give rise to 4 terpineol (42), the precursor of hydroxy acids (23) and (24) by pathway No.VI.

Attempts to demonstrate the protonation reaction leading to pathways II to VI under anaerobic conditions were not successful. Nor could this enzyme be demonstrated in cell-free extracts. An explanation for the observed failure of the reaction has been forwarded by Shukla (1965) by assuming that the protonation in pinenes and the rearrangement of the carbonium ion (35) to (36) and (36) to (39) as well as the reduction of (39) to Δ^1-p-menthene (4) take place on the enzyme surface with NADPH as the hydride donor and in the process small amounts of the ions (35), (36) and (39) leak out to give rise to various products in the pathways II, III, IV and VI, pathway V being the main pathway for pinene

degradation. The reaction does not proceed under anaerobic conditions due to the restricted supply of the hydride donor (NADPH).

Fermentation of p-cymene

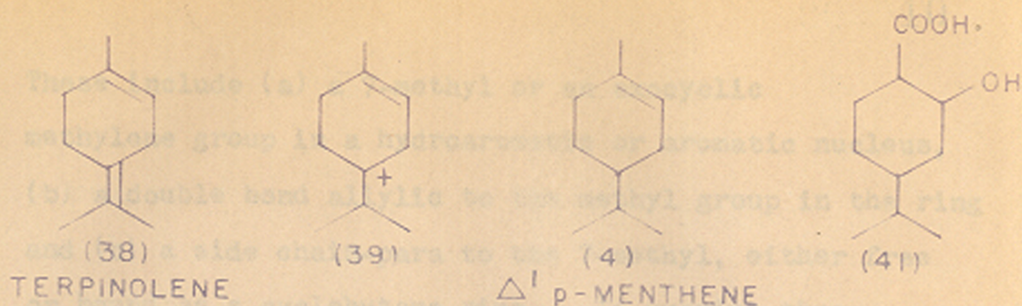
Madhyastha and Bhattacharyya (1965) have isolated cumic acid (43), 2,3 dihydroxy cumic acid (44) and 9-hydroxy-p-cymene (45) among the products of p-cymene (5) fermentation by the same organism. They have rationalised the formation of these products on the basis of two pathways (Chart 3, pathways 1 and 2).

In pathway No. 1, p-cymene (5) undergoes hydroxylation to cumyl alcohol (38) which undergoes progressive dehydrogenation to cumyl aldehyde (46) and cumic acid (43). Cumic acid (43) then undergoes dihydroxylation at positions 2 and 3 to compound (44) from which the ring repture takes place. In the pathway No. 2, 9 hydroxy-p-cymene (45) is obtained by hydroxylation at position 9. The modes of further degradation of these compounds (44) and (45) have not been established yet.

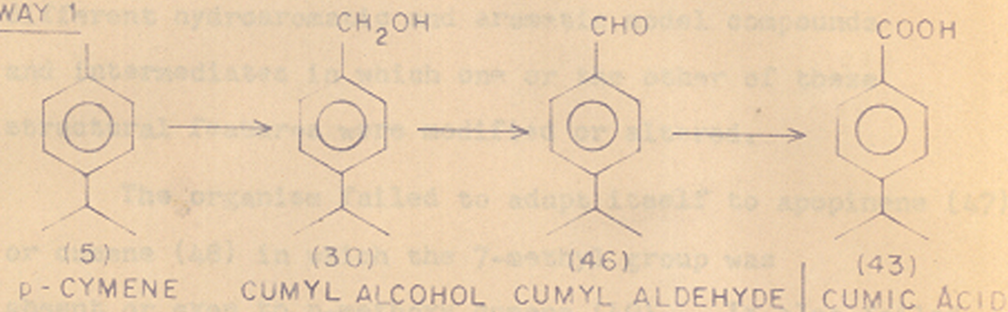
Substrate specificity studies

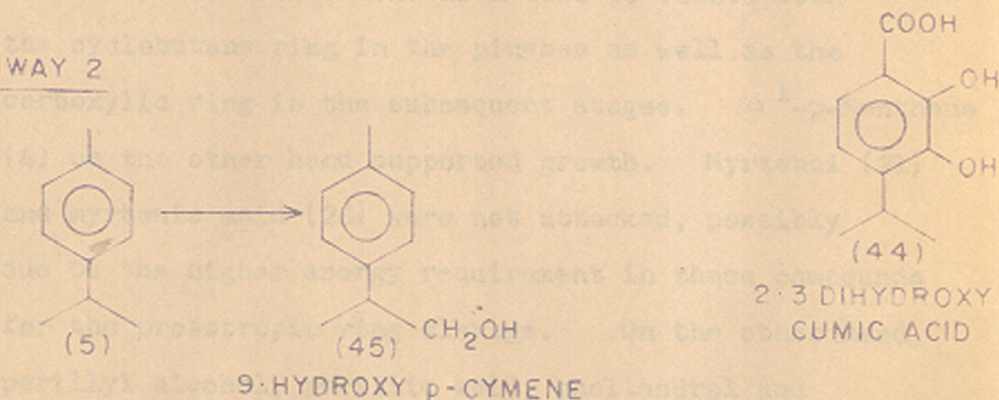
From the modes of degradation of limonene α-pinene, β-pinene, and p-cymene it was observed that certain structural and stereochemical features, the targets of chemical activity, were necessary in the molecule, before it can be attacked by the bacterium.

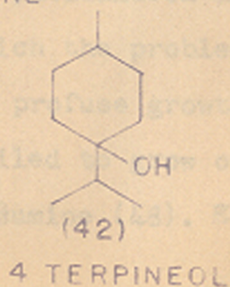
CHART-3



PATHWAYS FOR p-CYMENE DEGRADATION







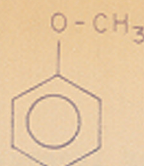
These include (a) a 7-methyl or an exocyclic methylene group in a hydroaromatic or aromatic nucleus, (b) a double bond allylic to the methyl group in the ring and (c) a side chain-para to the 7-methyl, either free or bound as a cyclobutane ring. To examine these features, further, growth studies were extended to different hydroaromatic and aromatic model compounds and intermediates in which one or the other of these structural features were modified or altered.

The organism failed to adapt itself to apopinene (47) or cumene (48) in which the 7-methyl group was absent or even to p-methoxy cumene (49). It also failed to grow on limonane (50) Δ^{8}-p-menthene (51) or pinane (52). Apparently it needed the endocyclic or exo-cyclic double bond as a tool to cleeve both the cyclobutane ring in the pinenes as well as the carboxylic ring in the subsequent stages. Δ^{1}-p-Menthene (4) on the other hand supported growth. Myrtenol (21) and myrtenic acid (28) were not attacked, possibly due to the higher energy requirement in these compounds for the prototropic ring clevage. On the other hand, perillyl alcohol, perillic acid, phellandrol and phellandric acid compounds in which the problem of ring rupture does not exist supported profuse growth. In the aromatic series, the organism failed to grow on benzene (53) toluene (54), p-xylene (55) and Cumine (48). Ethyl toluene (56),

CHART-4

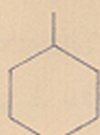
(47)
APOPINENE

(48)
CUMENE

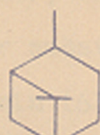


(49)
p-METHOXY CUMENE

(50)
LIMONANE



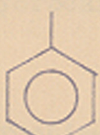
(51)
Δ^8 p – MENTHENE



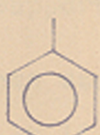
(52)
PINANE

(53)
BENZENE

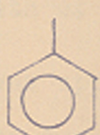
(54)
TOLUENE



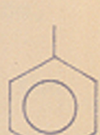
(55)
p XYLENE



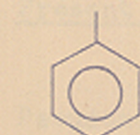
(56)
ETHYL TOLUNE



(57)
p-ISO
PROPENYL TOLUENE

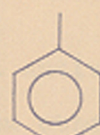


(58)
p-n -PROPYLTOLUENE

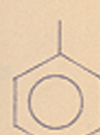


(59)
p-ISO BUTYL TOLUENE

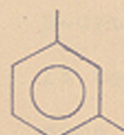
(60)
p-ETHYL-ISO
PROPYL-BENZENE



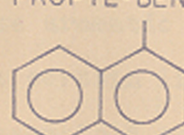
(61)
p-3 PENTYL
TOLUENE



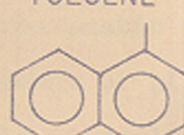
(62)
3-4 DIMETHYL CUMENE



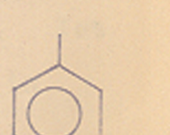
(63)
2-4 DIMETHYL CUMENE



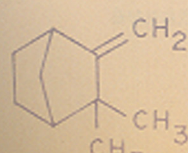
(64)
APOCADALENE



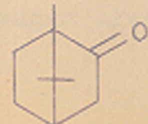
(65)
I-METHYL 4-ISO
PROPENYL-NAPTHALENE



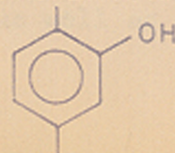
(66)
THYMOL



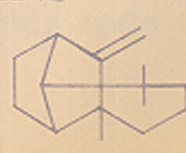
(68)
CAMPHENE



(69)
CAMPHOR



(67)
CARVACROL



(70)
LONGIFOLENE

p-isopropenyl toluene (57), p-n-propyl toluene (58), p-isobutyl toluene (59) supported growth after a lag phase. p.Ethyl-iso-propyl benzene (60) and p-3-pentyl toluene (61), on the other hand, failed to support growth. Further substitution on the ring such as in 3, 4 dimethyl cumin (62) 2,4 dimethyl cumin (63), apocadalene (64) and 1,-methyl-4- isopropenyl-naphthalene (65) thymol (66) and carvacrol (67) resulted in a complete loss of ability of these hydrocarbons to support the growth of the organism.

From these studies it was apparent that the organism behaved as if it has its own chemical logic for degrading the terpenoid substrates. It refuses to act on substrates in the degradation of which this logic cannot be easily applied.

Present work

The pattern of chemical activity of this Pseudomonad distinguishes it from other known Pseudomonads. It is characterised by a remarkable degree of substrate specificity particularly towards p-cymene-like aromatic and hydroaromatic compounds. Yet it tolerates certain limited amount of alterations in some "non essential" part of the molecule. It was, therefore, necessary to undertake growth and adaptation studies with some common microbial substrates such as sugars, sugar alcohols as well as the Krebs cycle acids to

ascertain whether there is any uniqueness or abnormality in this bacterium in its behaviour with respect to these substrates. Furthermore, it was anticipated that such studies may throw some light on the terminal oxidation pathways for fragments obtained by terpene degradation and or whether or not there is any interrelationship among the pathways of degradation of terpenes and carbohydrates in this organism.

Chapter II of this thesis deals with the materials and methods used in this investigation.

The growth experiments on different substrates are recorded in Chapter III. The conclusion has been drawn that the organism is likely to have the enzymes of tricarboxylic acid cycle and can grow on most of the common carbohydrates employed with or without a lag phase.

Adaptation and respiration studies are reported in Chapter IV. The organism showed some interesting pattern of cross adaptation on terpenes and sugars. It was concluded that the activity to grow on limonene seems to be constitutive as far as this organism is concerned whereas the ability to grow on sugars in most cases is an adaptation phenomenon. The other observation that was made indicated that when grown on sorbose the organism converts it to mannitol or sorbitol which in turn is oxidized to fructose to enter the glycolytic pathway.

The results have been discussed in Chapter V.

Chapter VI gives a brief summary of the results.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

The micro-organism used for the present investigations was a soil bacterium identified as a Pseudomonad, isolated by enrichment culture techniques, using α-pinene (I) as a sole source of carbon. It was found to grow equally well on β-pinene (2), limonene (3), Δ^1-p-menthene (4) and p-cymene (5) (Shukla & Bhattacharyya 1965).

Purification of the culture

The organism was purified by dilution and streak plate method.

Propogation of the culture

The culture was propogated on a nutrient agar slant of the following composition.

Peptone - (Armour)	1.0 g.
Sodium chloride (B.D.H.)	0.5 g.
Yeast extract (Difco)	0.5 g.
Beaf extract (Difco)	0.5 g.
Agar	2.5%
pH	7

diluted to 100 ml. with distilled water and adjusted with 4 N sodium hydroxide to pH 7.

For making agar slants 2.5% of agar washed thoroughly with distilled water was added to the above basal medium. The flask containing medium was steamed for one hour.

Aliquots (5-8 ml.) of the above medium were taken in 19 x 180 cm. Pyrex or Borosil tubes and autoclaved at 15 psi (121°) for 20 minutes. The tubes containing agar were slanted and the solidified slants were inoculated from a single bacterial colony separated from the broth by streak plate technique, and incubated at 26°-30° for 24 to 48 hours depending upon the growth of the organism.

The growth media

For growth of the organism, a mineral salt medium of the following composition was used:

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

diluted to 10 l.

Source of carbon used:

α-Pinene - B.D.H. Redistilled over metallic sodium b.p.154°/710 mm. (α_D+ 21°)

Limonene - obtained by fractional distillation from orange oil redistilled over metallic sodium b.p.173°C/710 mm.

(d + l) Camphor - (B.D.H.)

Camphene - Light & Co.

Longifolene - B.D.H.

Borneol - Purified α-pinene fermentation product

D-Glucose - A.R. (B.D.H.)

Sucrose - A.R. (B.D.H.)

L-Sorbose - (B.D.H.)

D-Fructose - (B.D.H.)

Maltose - (B.D.H.)

Lactose - (B.D.H.)

Sorbitol - (Merck)

Mannitol - (Difco)

Na gluconate - } Prepared according to method
5 Keto-Na gluconate } discussed (vide supra)

DL Malic acid - (City Chemical Corporation)

Succinic acid - (B.D.H.)

Sodium acetate - (Merck Proanalysis)

Sodium citrate - A.R. (B.D.H.)

Antibiotic used in Manometric Study

Chloramphenicol - Parke Davis

Preparation of 10% carbohydrate solution

Aliquots of 10 g. of sugars and sugar alcohols were dissolved in 100 ml. distilled water. The solutions were then steamed for one hour a day. This process was repeated for three days. Aliquots of 10 ml. of this solution were used with 90 ml. growth medium.

Preparation of sodium and ammonium salts of organic acids

Aliquots of 10 g. of the organic acids were dissolved in distilled water. The pH was adjusted to 6.5 to 7 by adding sodium or ammonium hydroxide (4N) dropwise and then the volume was made upto 100 ml. The aqueous solution of the sodium salts were autoclaved at 15 lbs. for 20 mins. Aliquots of 10 ml. of this solution were used with 90 ml. of growth medium to give a strength of 1 per cent.

Preparation of sodium salts of Sugar Acids

Sodium gluconate and 5 keto-sodium gluconate were prepared from calcium gluconate (B.D.H.) and 5-keto-calcium gluconate (obtained from the pilot plant of this Laboratory) by ion exchange resins.

The resin, Dowex 50, (3 ml. wet vol. /m equivalent) in the H^+ phase was stirred with a thick slurry of the calcium salts with mechanical stirring

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for 4 hrs. until the clear supernatant was free of calcium (oxalate test). The supernatant was decanted and the resin bed washed several times with distilled water with stirring followed by decantation until the pH of the washings was above 5. The combined supernatants were neutralised (pH 7.0) with 4 N sodium hydroxide, filtered, made upto a 10% solution (based on the free acid) and sterilized by autoclaving (2 x 10 lb./15 mins.). Aliquots of 10 ml. of this solution were diluted with 90 ml. of the medium to make a total concentration of 1%.

Preparation of inoculum for growth studies:

The growth studies were conducted in 500 ml. Pyrex Erlenmeyer flasks containing 100 ml. of the growth medium.

Each flask was directly inoculated, with a loop of 24 hours old bacterial colony from the slants under aseptic conditions with α-pinene (0.3 ml.) or limonene (.5 ml.) as a sole source of carbon.

The incubation was carried out at $28^{\circ} \pm 1^{\circ}$ for 24 hours to 48 hours on a rotatory shaker at 220 r.p.m.

The cells were centrifuged, washed thoroughly with M/20 phosphate buffer (pH 7) and recentrifuged. Finally the cells were resuspended in phosphate buffer to give a Klett reaging at 250(O.D) at 660 mµ using Red Filter.

These cells were used to inoculate fresh flasks containing different substrates. These flasks were again incubated on a rotary shaker at 220 r.p.m. at $28^{\circ} \pm 1^{\circ}C$.

The contents of the flasks were carefully shaken before determining the optical density of aliquots at 660 mμ red filter on a Klett Summerson Colorimeter after different intervals.

Maintenance of the culture

As a routine the culture was maintained on a nutrient agar slants for one month. It was also maintained simultaneously in shake cultures in the growth medium containing both α-pinene (0.3%) and limonene (0.3%) as carbon sources.

A third stock of organism was maintained on nutrient agar slants under paraffin oil. It appeared to retain its viability for periods exceeding six months under paraffin oil.

The viability of the culture was determined by following its growth in the growth media as well as on nutrient agar.

Manometric Techniques

Oxygen uptake by the intact cells grown on different carbon sources, was studied by using the conventional Warburg's manometric apparatus. (Umbreit, Burris, Stauffer 1959).

Preparation of cell suspensions for Manometric studies

Cells grown on α-pinene for 24 hours were harvested by centrifugation, washed with the basal medium and resuspended in the growth media to give an optical density of 250 at 660 mμ red filter on a Klett-Summerson colorimeter. Aliquots of 5 ml. of this cell suspension were used as an inoculum for 100 ml. of media, containing different carbon sources. The cells were subcultured in the same media containing the same carbon source once more. After 24 hours, the cells were centrifuged, washed twice with M/20 phosphate buffer pH 7.1; and resuspended in the same buffer to give a cell density at 250 (Klett reading) at 660 mμ (red filter).

Each Warburg flask contained the following:

In the centre well	- 20% potassium hydroxide (0.2 ml.) with a piece of folded filter paper.
In the side arms	- substrate solution or suspension (0.04 ml.) 40 ug. in 0.04 ml.
In the reaction chamber	- Cell suspension (2.5 ml.) O.D.250 and M/20 phosphate buffer (0.2 ml.) ± 50 ug chloramphenicol

and buffer (pH 7.1) to make the total volume to 3 ml.

The flasks were then incubated at 28.5°C, in a Warburg apparatus with a continuous shaking arrangement at 110 strokes/min.

Dry Weight Determination

Cells grown for 24 hrs. on different carbon sources were harvested by centrifugation, washed twice with glass distilled water and resuspended in glass distilled water to give an optical density of 250 (Klett reading) at 660 mμ red filter on a colorimeter. Aliquots of 2.5 ml. of this suspension were dried in tared weighing bottles, in an oven at 110°C. to constant weights (3-4 hrs.) Usually these aliquots weighed approximately 2 mg.

CHAPTER III

EXPERIMENTAL
(Growth Studies)

CHAPTER III

E X P E R I M E N T A L

Optimum concentration of α-pinene and limonene for the growth of the Pseudomonad

Preliminary experiments were undertaken to study the optimum concentration of α-pinene and limonene for the growth of the organism. Cells adapted to α-pinene were harvested after 24 hours by centrifugation and washed with the basal medium under aseptic conditions. The cells were resuspended in the growth medium to give an optical density of 250 at 660 mμ (red filter) as inoculum for these experiments.

Each flask containing 100 ml. sterile mineral salt medium was inoculated by 5 ml of the above inoculum with the simultanious addition of α-pinene in different concentration (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0) using three flasks for each concentration. Controls were run (a) with α-pinene 0.3% and limonene 0.3% without cells and (b) cells on mineral salt medium without the hydrocarbons.

The incubation was carried out as usual on a rotary shaker for 24 hours at 220 r.p.m. at 28°. The growth was then determined by turbidity measurements.

The results are presented in Fig. 1.

Similarly, the optimum concentration of limonene was studied with the cells adapted to limonene for 24 hours. (Fig. 1).

It will be observed that with -pinene, the growth went down beyond a level of 0.7% presumably due to toxicity, whereas limonene showed no toxic effects even upto a level of 1%.

Nutritional requirements:

Attempts were made to substitute α-pinene and limonene by various other terpenes, carbohydrates and other organic acid salts as growth substrates.

Terpenoid compounds used in the present study were (d + l) camphor, camphene (B.D.H.), borneol, Δ^3-carene, longifolene, along with α-pinene, β-pinene and limonene. Cells adapted to α-pinene and limonene for 24 hrs. were centrifuged, washed, and inoculated into 100 ml. growth medium with different concentrations of the above terpenes. Table 1 indicates the utilization of different terpenes by the cells adapted to α-pinene and limonene.

<u>Comparison of rates of growth of α-pinene- and limonene-grown cells on limonene and α-pinene as substrates</u>

Cells adapted to α-pinene could utilize α-pinene and limonene very easily but cells when adapted to limonene usually failed to grow on α-pinene readily. This was however a variable behaviour sometimes growth on limonene-adapted cells on α-pinene was observed without any lag phase.

It was necessary to compare the rates of growth of the cells adapted to α-pinene on α-pinene and limonene, with that of the limonene-adapted cells on limonene.

In the experiments 0.3 ml. α-pinene was added to two sets of flasks (A & B) each containing 100 ml. growth medium. In another two sets of flasks (C & D) 0.3% limonene was added as the substrate. Cells adapted to α-pinene were used as inoculum (O.D. 250 in growth medium) to inoculate sets A & C. Whereas sets B & D received limonene-grown cells (5 ml./flask O.D. 250). The flasks were then incubated on rotary shaker at $28^{\circ} \pm 1^{\circ}$ at 220 r.p.m. Every 4 hrs. 5 ml. aliquots were withdrawn aseptically for turbidity determination from each flask.

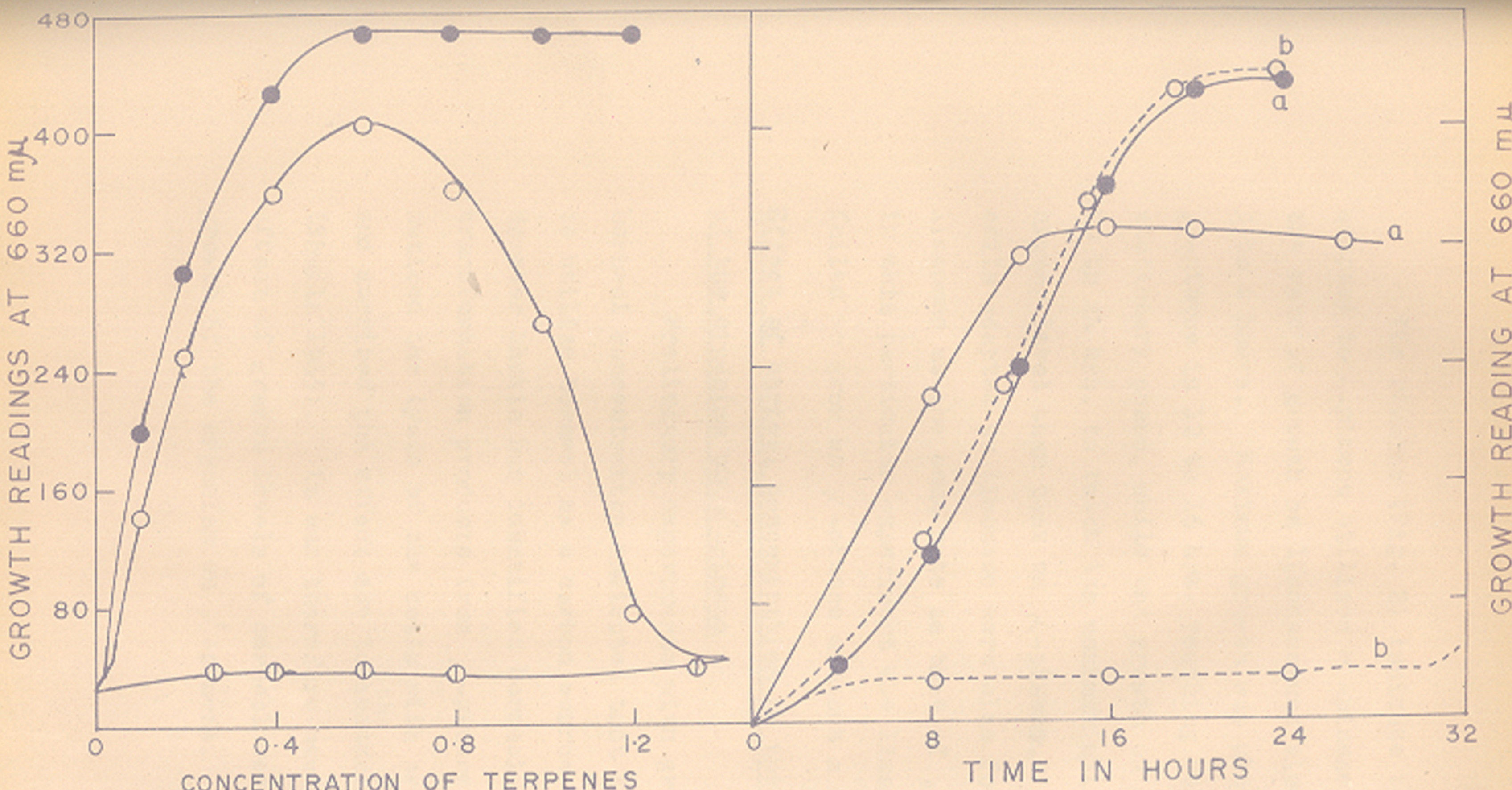


FIG. 1 EFFECT OF CONCENTRATION OF TERPENE ON CELLS ADAPTED TO α-PINENE INOCULUM 5% O.D. 250

●——● LIMONENE ○——○ α-PINENE

⦶——⦶ BORNEOL, CAMPHOR, LONGIFOLINE

FIG. 2 (a) GROWTH OF PINENE ADAPTED CELLS IN LIMONENE (·3 ml) INOCULUM 5% O.D. 250

(----b----) GROWTH OF LIMONENE ADAPTED CELLS IN LIMONENE & α-PINENE.

●——a——● LIMONENE ○——a——○ α-PINENE

●----b----● LIMONENE ○----b----○ α-PINENE

The results (Fig. 2) indicate that the cells adapted to α-pinene utilized α-pinene rapidly while the rate of growth on limonene was slower for the first 3 to 4 hours. Maximum growth was observed with α-pinene in 12 to 16 hrs. reaching subsequently to a stationary phase, while the growth on limonene required 20 to 24 hrs. to reach its maximum which was at a higher level than that on α-pinene. However the cells adapted to limonene were also found to grow on limonene at the same rate as that of α-pinene grown cells. In this particular experiment the limonene-grown cells failed to grow on α-pinene without a lag phase.

<u>Effect of borneol concentration on the growth of the organism on α-pinene</u>

Preliminary experiments with graded levels of borneol concentration indicated that the organism failed to utilize borneol as a carbon source for its growth. However Shukla has identified borneol as one of the transformation products from α-pinene but he also noticed the lysis of the cells with prolonged incubation and ascribed the effect to the accumulation of borneol (Shukla 1965). It was, therefore, necessary to study the effect of graded levels of borneol concentration on the growth of the organism on α-pinene.

Flasks containing graded levels of borneol (5, 10, 20, 30, 40, 50, 100 mg./100 ml.) with 0.3% α-pinene were inoculated with 2% of suspension of α-pinene grown cells (O.D. 250) and incubated as usual. One flask containing 0.3 ml. α-pinene alone was also incubated as control. The turbidimetric measurements after 24 hrs. indicated that high concentrations of borneol have an inhibitory effect on the growth of the bacterium. The results are presented in Table 2.

<u>Utilization of different carbohydrates as carbon source for the growth of the organism</u>

Different carbohydrates were used in the growth medium as sole source of carbon. Among the sugars used were D-glucose, sucrose, sorbose and D-fructose. Among the sugar alcohols, D-sorbitol and D-mannitol were chosen for the present study.

Sodium salts of gluconic acid and 5-keto-D-gluconic acid were prepared from calcium salts of gluconic acid and 5-keto-D-gluconic acid in the manner described in the Chapter on Materials and Methods.

Twenty-four hour culture on α-pinene was harvested by centrifugation, washed with the basal medium under aseptic condition to remove the adhering material (recentrifuged), and resuspended in the growth medium to give an optical density 250 at 660 mµ (red filter).

Aliquots of 5 ml. cell suspension were used as inocula in the growth medium containing 1% of the test compounds as sole source of carbon. Two sets of flasks containing 0.3 ml. of α-pinene and limonene respectively were also incubated. The experiments were run in triplicates. Turbidimetric measurements were made every 4 hrs. in 5 ml. aliquots.

Fig. 3 indicates the results obtained.

From the Fig. 3, it can be noticed that the growth on glucose is rapid and reaches a plateau after 20 hrs. Fructose is utilized with a slight lag period of 4 hrs. and the rate of growth is slower than that on glucose, reaching a stationary phase after 32 hrs. But in case of sorbose a prolonged lag period of 40 hrs. was observed. These cells grow on sucrose with only a small lag period of 3 to 4 hrs. and the growth in this substrate shows a diphasic pattern with a plateau from 8 - 12 hrs. After this period the growth rate improves and reaches a maximum after 36 hrs.

The two polyols are utilized only after a lag period of 20 to 24 hours. After this period growth on these substrate become comparable to that on glucose.

Of the two sugar acids, gluconate is utilized with a slight lag phase of 4 hrs. and the growth reaches a maximum in 8 to 12 hours. 5-Keto-D-gluconate is not used as a carbon source by the organism.

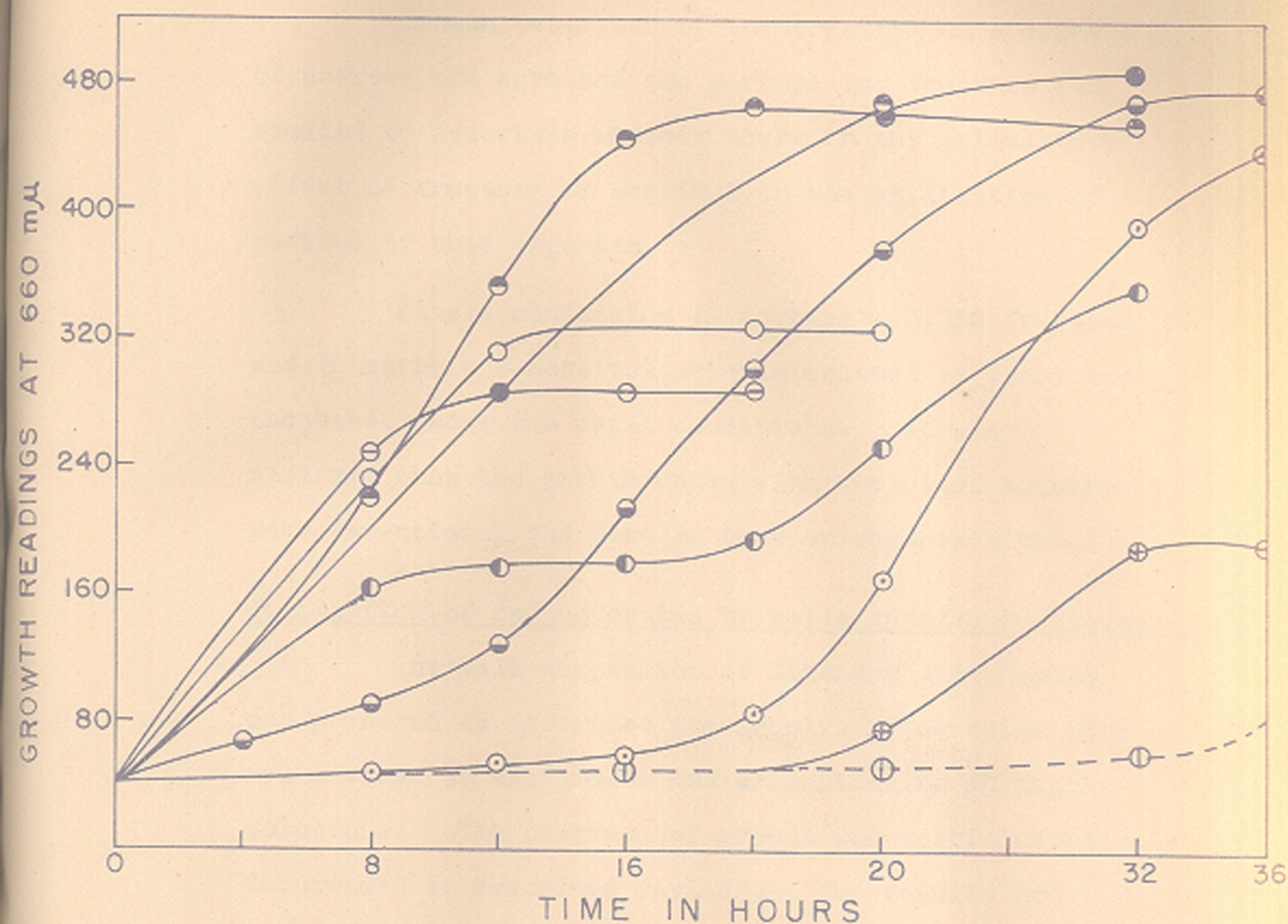


FIG. 3 UTILIZATION OF 1% CARBOHYDRATES BY CELLS ADAPTED TO α-PINENE
INOCULUM 5% O.D. 250

LIMONENE (·3 ml) — FRUCTOSE — GLUCOSE

SORBITOL — SUCROSE — α-PINENE (·3 ml.)

GLUCONIC ACID — SORBOSE + 0·1% SORBITOL

SORBOSE (GROWTH AFTER 2 DAYS)

Growth behaviour of the organism on a mixture of sorbose and sorbitol and sorbose and fructose was studied to ascertain whether there is any stimulatory effect of fructose or sorbitol on the utilization of sorbose by this organism.

Flasks containing 1% sorbose + 0.01% fructose and 1% sorbose + 0.1% sorbitol were inoculated as usual and incubated under the usual conditions. It was observed that the growth rates with the mixed substrates were essentially the same as that on sorbose alone.

Utilization of carbohydrates by cells adapted to limonene

The cell suspension of limonene grown cells was prepared as described previously. The cells were transferred to the growth medium containing 1% sugar solutions. The progress of growth was turbidimetrically determined as described earlier. The results are presented in Fig. 4 A.

It is apparent that the growth pattern was similar to that with α-pinene grown cells with the notable exception that sucrose was utilized by the limonene grown cells with difficulty after a prolonged lag-phase of 28 hrs.

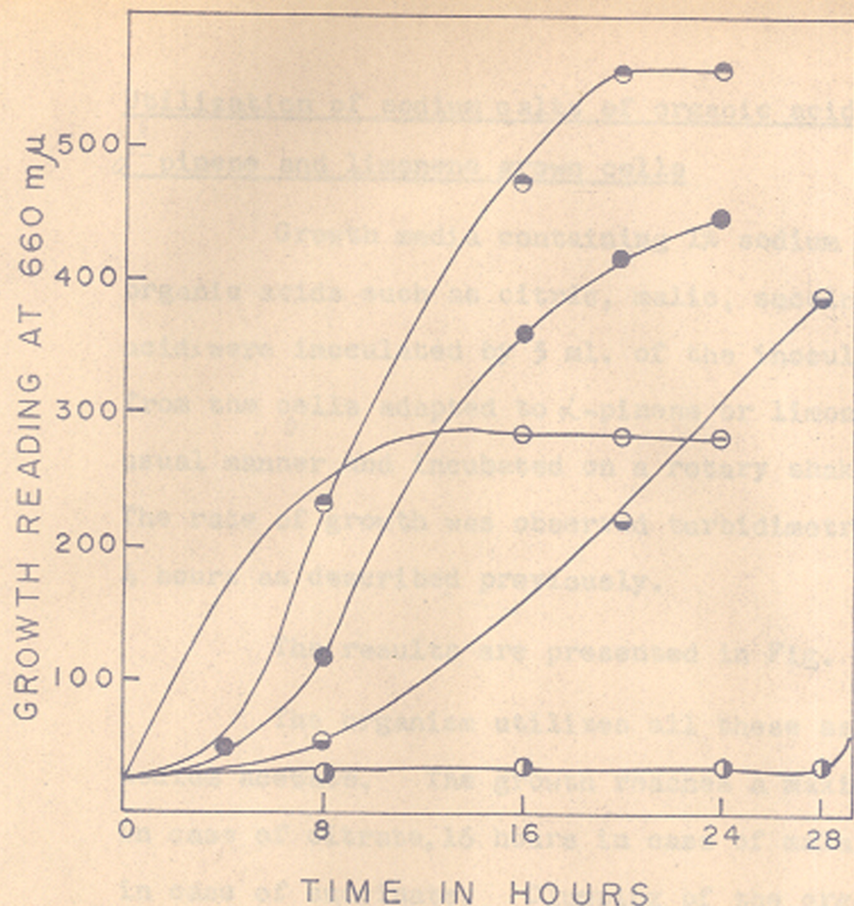
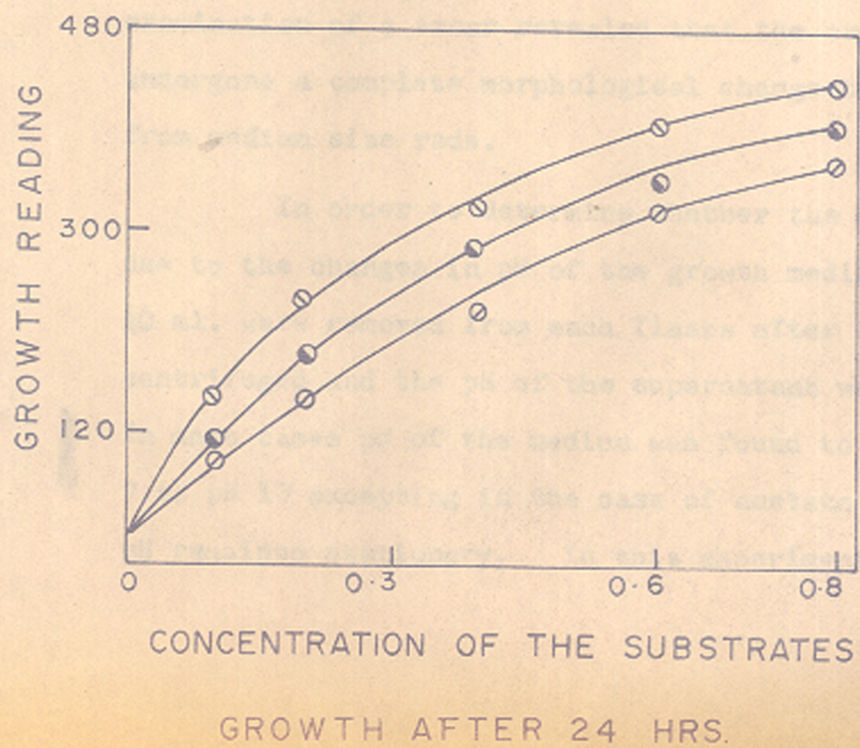


FIG. 4 A

UTILIZATION OF CARBOHYDRATES BY CELLS ADAPTED TO LIMONENE

- GLUCOSE
- LIMONENE
- FRUCTOSE
- GLUCONIC ACID
- SORBOSE, SUCROSE



GROWTH AFTER 24 HRS.

FIG. 4 B

EFFECTS OF GRADED LEVELS OF SUBSTRATES ON THE GROWTH OF α-PINENE ADAPTED CELLS.

INOCULUM 2% O.D. 250
Reading represent O.D after 24 hours.

- SODIUM SUCCINATE
- SODIUM MALATE
- SODIUM CITRATE

Utilization of sodium salts of organic acids by α-pinene and limonene grown cells

Growth media containing 1% sodium salts of organic acids such as citric, malic, succinic and acetic acids were inoculated by 5 ml. of the inoculum prepared from the cells adapted to α-pinene or limonene in the usual manner and incubated on a rotary shaker as usual. The rate of growth was observed turbidimetrically every 4 hours as described previously.

The results are presented in Fig. 5 A.

The organism utilizes all these salts except sodium acetate. The growth reaches a maximum in 12 hours in case of citrate, 16 hours in case of malate and 20 hours in case of succinate. Clumping of the organism was observed with these salts as substrates. A microscopic examination of a smear revealed that the organism had undergone a complete morphological change to the coccal form from medium size rods.

In order to determine whether the clumping was due to the changes in pH of the growth media, aliquots of 10 ml. were removed from each flasks after 24 hours, centrifuged and the pH of the supernatant was determined. In most cases pH of the medium was found to rise from pH 7 to pH 10 excepting in the case of acetate where the pH remained stationary. In this experiment the organism

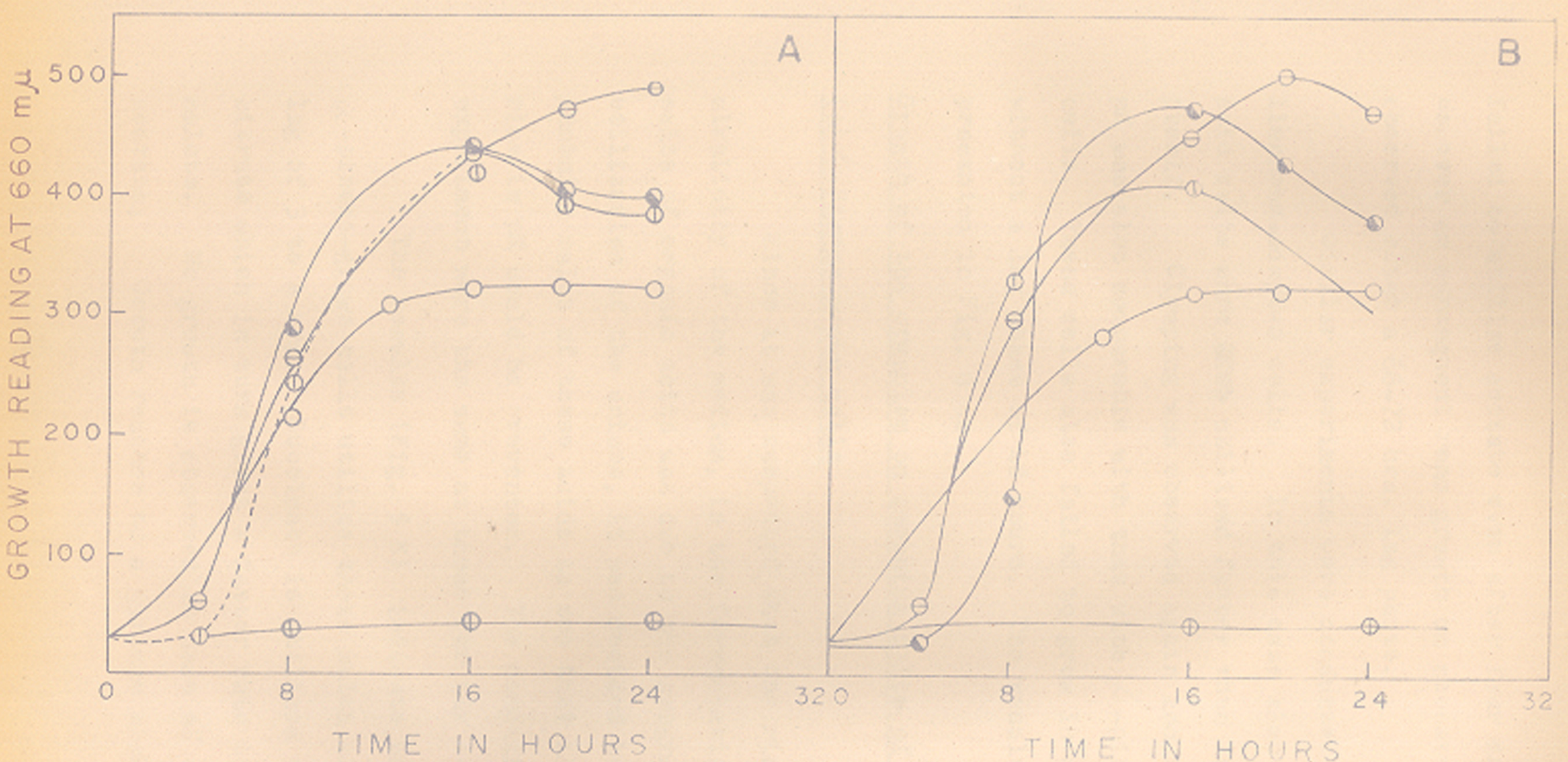


FIG 5 GROWTH OF α-PINENE ADAPTED CELLS ON 1% SODIUM SALTS OF DI & TRI CARBOXYLIC ACIDS (FIG. A) AND ON 1% AMMONIUM SALTS (FIG. B) (INOCULUM 5% O.D. 250)

22242

failed to utilize acetate even after four days. However on subsequent runs growth on acetate has been observed after a 66-72 hrs. lag phase.

Similar experiments were conducted with limonene-adapted cells. In this case also the organic acid salts were utilized faster than limonene itself. Clumping was observed again and the rise in pH was also comparable with that with α-pinene grown cells. These cells also failed to grow in sodium acetate without a lag phase of 48 hours. The results are presented in Fig. 6 A.

Growth of the organism on ammonium salts of organic acids

(a) α-Pinene-grown cells

Since it was suspected that the rise in alkalinity of the medium, in the fermentation of sodium salts of organic acids was due to the progressive utilization of the anions, it was decided to use ammonium salts of these acids to see that if the sharp rise in pH could be prevented. The conditions of the experiment were the same as those for the sodium salts.

The results (Fig. 5 B) indicate that the α-pinene-grown cells utilize these salts with a small lag of 3 to 4 hrs. Succinate is utilized faster than citrate which in turn gives a faster rate of growth than malate. No growth is observed in case of ammonium acetate; Growth reaches to a maximum within 12 to 16 hours

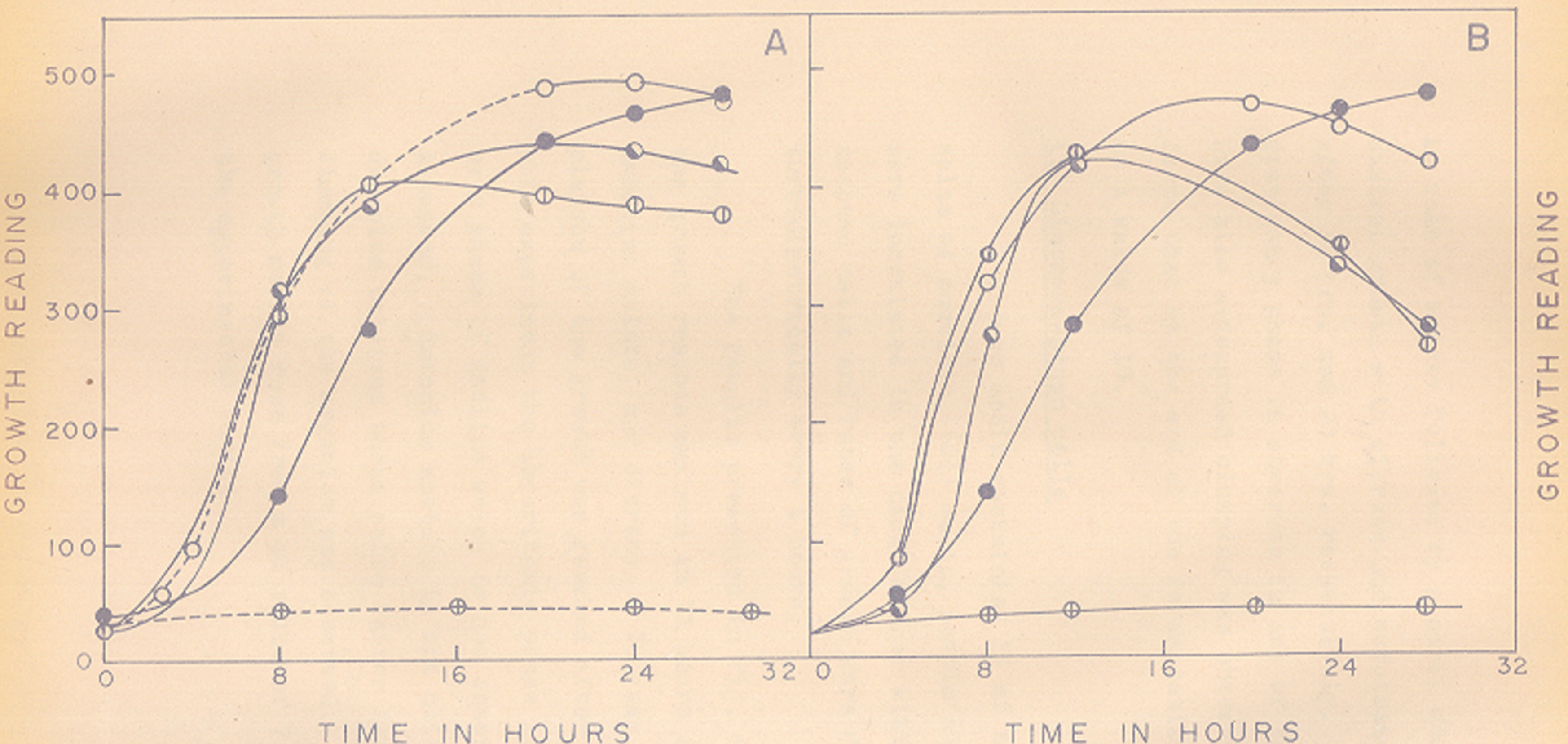


FIG. 6 GROWTH OF LIMONENE ADAPTED CELLS ON SODIUM SALTS OF DI AND TRICARBOXYLIC ACIDS. (A)

INOCULUM 5% O·D. 250.
ALL SUBSTRATES EMPLOYED AT 1% LEVEL EXCEPTING LIMONENE (·3 ml. / 100 ml.)

FIG. 6 GROWTH OF LIMONENE ADAPTED CELLS ON AMMONIUM SALTS OF DI AND TRICARBOXYLIC ACIDS. (B)

(INOCULUM 5% O.D. 250) SUBSTRATE CONC. 1%. LIMONENE (·3 ml./100 ml.)

●——● LIMONENE, ○——○ SUCCINATE, ◐——◐ MALATE, ⦶——⦶ CITRATE.
⊕——⊕ ACETATE

in case of malate followed by a small decline, while maximal growth with citrate and succinate are obtained after 16 hrs. and 20 hrs. respectively. No stationary phase is observed in any of this substrates. Here also widespread clumping was observed. It was found that at the end of the fermentation the pH rose to a value of 10.

(b) Limonene-grown cells

Growth media containing 1% of the ammonium salts of succinic, (dl) malic, acetic and citric acids were inoculated in the usual manner with limonene-adapted cells and rates of growth were determined turbidimetrically every 4 hours.

The results presented in Fig. 6 B indicate that the growth reaches maximum in 12 hours in case of ammonium malate, and citrate. In case of succinate the plateau of the growth was reached after 20 hours. In this experiment the logarithmie phase is soon followed by a phase of decline which is not observed with limonene. Ammonium acetate is not utilized. The organism utilizes these salts faster than limonene. Clumping of the organism was observed. The pH of the growth medium rose from a pH value of 7 to 10, during the experimental period.

Effect of pH on the morphology of the bacterium

Since the pH of the medium underwent a rise from pH 7 to pH 10 with the sodium and ammonium salts of Krebs cycle-acids accompanied by a clumping of the cells, attempts were made to correlate the rise in pH with the morphological changes in this organism.

Aliquots (5 ml.) were removed from the fermentation medium under aseptic conditions and centrifuged. The pH of the supernatant was recorded. It was observed that the pH rose slowly with the progress of the fermentation. After every 4 hr. a smear of the culture was prepared on a glass plate, and observed under microscope after staining with the usual Gram staining techniques. It was observed that the organism showed morphological changes from medium size rods to short rods and finally to the coccal form with increase in the pH. At pH 9 a complete change to the coccal form took place. When these coccal cells were transferred back to the growth media containing 0.3 ml. α-pinene, a reversal to the medium rod form was observed. This change was completed along with the onset of logarithmic phase of growth. These transformations from the rod form to the coccal form and back to the rod form

could be repeated at least ten times without any apparent loss in the viability of the organism.

Effects of the concentration of the growth substrates on the growth of the organism and the pH of the medium

It was observed in the previous experiments that the pH of the growth medium increases as the organic acid substrates were utilized from their sodium salts and the morphology of the organism changed with the rise in pH.

Experiments with graded levels of sodium salts of the above acids from 0.1 to 1.0% were carried out to ascertain whether the concentration of the substrates had any effect on the growth, the change in pH and the morphology of the bacteria. The results presented in Fig. 4 B would show that the growth is a linear function of the concentration of the substrates upto a certain concentration and that the rise in final pH occured irrespective of the concentration of the substrate. However, with the lower concentrations of the salts, the clumping was less pronounced.

Effect of molarity of phosphate buffer on the growth of the organism

In the above experiments it was observed that the pH was increasing even with low concentrations of the substrate. It was decided to study the effect of molarity

of phosphate buffer on the growth of the organism and the pH of the medium in order to ascertain whether any variation in buffer strength can be employed to control the pH during fermentation.

Experiments were conducted by using potassium phosphate buffer of different ionic strength.

The following concentrations of buffer were employed (0.01, 0.02, 0.03, .04, .07, .09, .1, .12, .15 M) along with the mineral salt medium with 0.3 ml. α-pinene. α-Pinene adapted cells were used to inoculate the media. The growth measurements were recorded after 24 hours. The results Table 3 indicate that 0.04 M of phosphate buffer was optimal for the growth of the organism.

In the subsequent experiments on the effect of initial pH on the growth of the organism a buffer strength of 0.04 M was used.

The flasks containing the basal media with the mineral salts and 0.3% α-pinene and the buffer of 0.04 M of different pH were inoculated with α-pinene adapted cells. After 24 hours the growth was determined turbidimetrically. The results (Table 4) indicate that the growth was maximum at pH 6 to pH 7.

Since it was observed that the lowering of pH upto 6 did not affect the bacterium, the organism was grown in 1% sodium salt of malate using phosphate buffer pH 6. The growth and the pH were determined after 24 hrs. In this experiment also the rise in pH could not be prevented although the maximum pH at the end of the fermentation was less than that with an initial pH of 7. (Table 5)

Even with higher buffer strengths (0.1M) the rise in pH as well as morphological alteration of the bacterium could not be prevented.

<u>The effect of different ammonium salts on the final pH of the medium</u>

Attempts were made to substitute ammonium nitrate in the growth media by various other ammonium salts with a view to ascertain whether any of the ammonium salts would keep a better control of pH during fermentations.

Among the salts tested were ammonium sulphate, ammonium chloride and ammonium nitrate. The results (Table 6) indicate that all these salts were utilized by the organism and the growth was as good as that on ammonium nitrate. However rise in the pH could not be controlled with any of the Krebs Cycle acids as substrates.

TABLE 1

Growth of the Pseudomonad on different substrates

Growth substrates	Cells adapted to α-Pinene	Cells adapted to Limonene
α-Pinene	+	* @
β-Pinene	+	* @
Limonene	+	+
Camphor (d+l)	-	-
Camphene	-	-
Longifolene	-	-
Borneol	-	-
δ-Carene	-	-

\+ Growth

\- No growth

* Growth was observed after a prolonged lag-phase of 24 hrs.

@ This adaptation to the pinenes was a variable behaviour. Sometimes the lag phase was not observed.

TABLE 2

Indicating the effect of borneol conc. on α-pinene fermentation

Conc. of α-pinene in cc./100 ml	.3	.3	.3	.3	.3	.3	.3	.3	.3	.3
Borneol in mg./100 ml	nil	5	10	15	20	30	40	50	100	150
Growth after 24 hrs. on colorimeter	290	290	280	260	250	170	160	130	90	60

Inoculum α-pinene-grown cells 2% O.D.250.

TABLE 3

The effect of Molarity of phosphate buffer (pH 7) on α-pinene fermentation

Molarity of phosphate buffer	0.01	0.03	0.04	.05	.06	.08	0.1	0.15
Growth after 24 hrs.	170	206	230	206	200	204	188	91

1) Potassium phosphate buffer pH 7.
2) Inoculum 2% α-pinene grown cells. O.D. 250
3) Substrate 0.3 ml. α-pinene
4) Growth after 24 hrs.

TABLE 4

The effect of different initial pH of the media on the growth of the organism on α-pinene.

pH of Buffer	5.9	6.2	6.8	7	7.2	7.6
Growth after 24 hrs.	102	238	251	260	244	238

1) Inoculum 2% α-pinene grown cells O.D. 250.

2) Potassium phosphate buffer 0.04M

3) Pinene 0.3 ml(100 ml.)

TABLE 5

Growth of the organism on 1% sodium malate with higher buffer strength

Molarity of buffer	.04	.05	.06	.08	0.1	0.15	.2
Growth after 24 hrs.	470	465	465	455	465	465	430
Final pH	8.7	8.7	8.7	8.6	8.6	8.3	7.5

1) Inoculum 2% pinene grown cells. O.D. 250.

2) Potassium phosphate buffer pH 6.

TABLE 6

Table indicating the utilization of different Ammonium Salts

Substrate	Growth	pH
Ammonium nitrate	465	8.5
Ammonium chloride	475	8.1
Ammonium sulphate	480	8.3
Ammonium phosphate	490	8.1

1. Nitrogen source used 0.1% in the growth medium containing 1% sodium malate.
2. K Phosphate buffer pH 6.[illegible]0.1M
3. Inoculum used α-pinene grown cells (O.D. 250)
4. Growth after 24 hours.

CHAPTER IV

EXPERIMENTAL

(Adaptive enzyme studies)

CHAPTER IV

EXPERIMENTAL

MANOMETRIC STUDIES

The manometric studies were conducted in a Warburg respirometer at 28° ± 1°C. The conditions for these experiments have been described in detail in Material and Methods.

Briefly, cells were grown on the respective substrates for 24 hours. Two passages were allowed for each substrate to ensure that the adaptation phenomenon is not due to traces of other substrates carried over during the inoculation. The cells were centrifuged, washed with buffer at least three times (recentrifuged each time) and finally suspended in buffer to an optical density of 250 (Klett reading) in each case.

α-Pinene grown cells

The oxygen uptake with α-pinene-grown cells was studied with α-pinene, limonene, fructose, glucose, sorbitol, mannitol, sorbose and sucrose. Besides, the oxidations of glucose and fructose were studied with added chloramphenicol.

The results (Fig. 7A) indicate that the oxidation of both α-pinene and limonene proceeds without a lag phase. Lag phases are observed with fructose and glucose and this phase is more prolonged in case of glucose. In presence of chloramphenicol neither of the two substrates is oxidised. The other carbohydrates and sugar alcohols are not oxidised even upto 3 hrs.

Limonene-grown cells

Limonene-grown cells were tested for their ability to oxidise the above substrates. The pattern of adaptation observed (Fig. 7B) was qualitatively the same as that with α-pinene-grown cells. The lag phases with fructose and glucose, however, were prolonged and addition of chloramphenicol, completely inhibited this adaptation. Again sorbitol, mannitol, sorbose and sucrose were not oxidised.

It should be mentioned that regarding the adaptation of limonene-grown cells on α-pinene a considerable amount of variation was observed from batch to batch, inspite of strict adherence to experimental conditions. In some preparations no adaptation was observed. In some others oxidation of α-pinene started after a lag phase ranging between 30 mins. and 3 hrs.

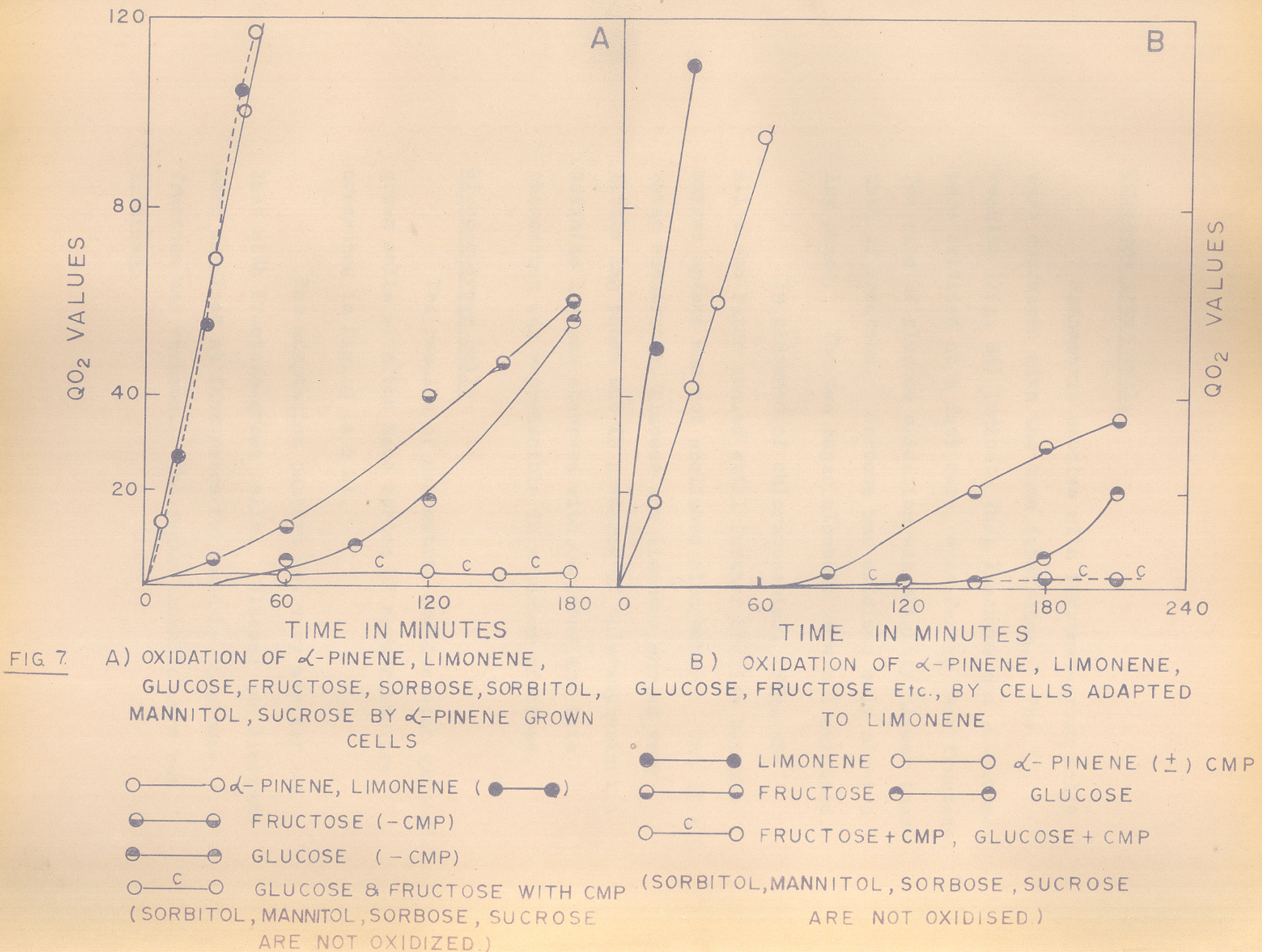


FIG. 7. A) OXIDATION OF α-PINENE, LIMONENE, GLUCOSE, FRUCTOSE, SORBOSE, SORBITOL, MANNITOL, SUCROSE BY α-PINENE GROWN CELLS

B) OXIDATION OF α-PINENE, LIMONENE, GLUCOSE, FRUCTOSE Etc., BY CELLS ADAPTED TO LIMONENE

Fructose grown cells

Manometric studies with fructose-grown cells were conducted with the same substrates. The results (Fig. 8A) indicate that limonene and α-pinene were oxidised at a fast rate, even faster than fructose. The rate of glucose oxidation was slightly slower than that of fructose. Sorbose was oxidised only after a lag phase. The two sugar alcohols were not oxidised.

In presence of chloramphenicol (Fig. 8B) all these rates showed still further decline but the oxygen uptake was not abolished altogether. Out of all these substrates, glucose and limonene showed very little lag phase but in presence of chloramphenicol a definite lag was observed with α-pinene and this phenomenon was reproducible in a number of times.

Glucose-grown cells

The results of manometric studies with glucose-grown cells with the same series of substrates are presented in Fig. 9 (A & B).

The adaptation pattern was very similar to that with fructose-grown cells. Glucose and limonene were oxidised at fast rates followed by α-pinene, fructose and sorbose. The sugar alcohols were not oxidised.

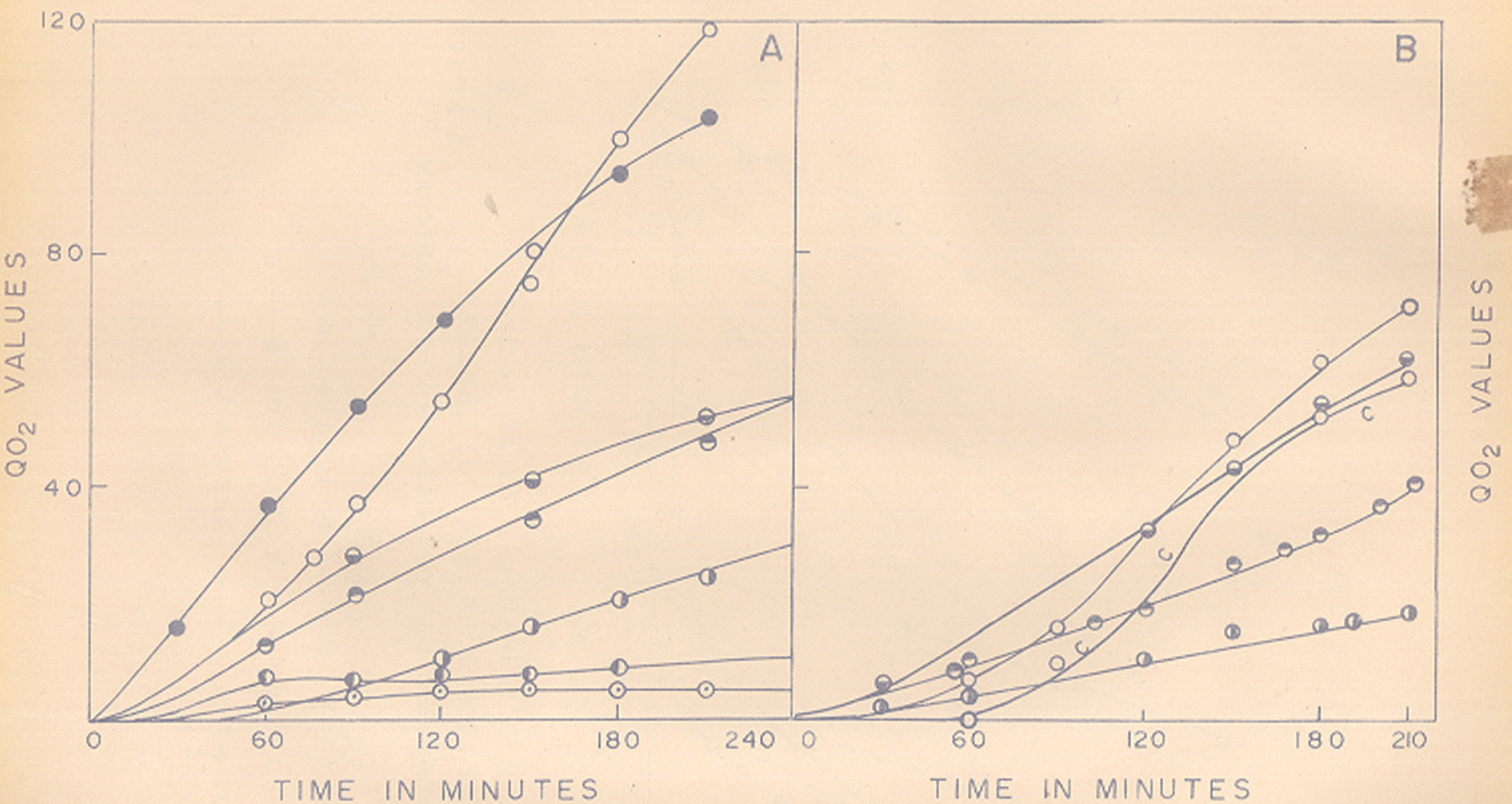


FIG. 8 (A+B) OXIDATION OF α-PINENE, LIMONENE, GLUCOSE, SORBOSE, MANNITOL, SORBITOL, SUCROSE, FRUCTOSE BY (± CMP) FRUCTOSE GROWN CELLS

PINENE, LIMONENE, FRUCTOSE, PINENE + CMP (c), GLUCOSE (± CMP), SORBOSE (± CMP), SUCROSE, SORBITOL & MANNITOL

ENDOGENOUS VALUES SUBSTRACTED

A

B

QO_2 VALUES

100
80
60
40
20

0 60 120 180 240

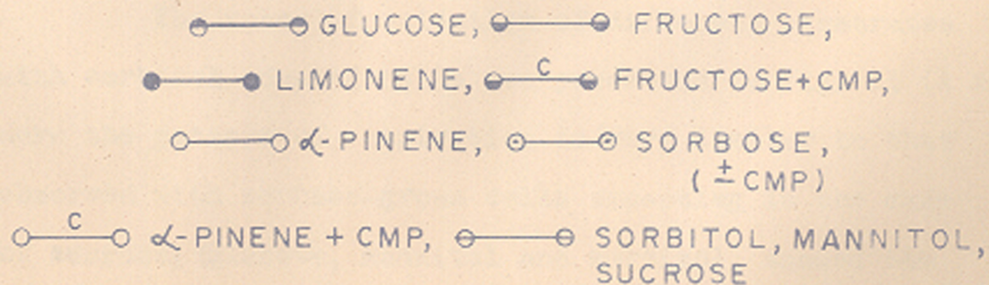
TIME IN MINUTES

0 60 120 180 240

TIME IN MINUTES

QO_2 VALUES

FIG. 9 (A & B) OXIDATION OF LIMONENE, PINENE SORBOSE, SUCROSE, FRUCTOSE, SORBITOL MANNITOL WITH (+ α-CMP) BY GLUCOSE CROWN CELLS



(C), CMP - CHLORAMPHENICOL

Addition of chloramphenicol (Fig. 9B) did not inhibit respiration, but a short lag phase was observed with fructose and a lowering of rate of respiration. In this case the rates of limonene and α-pinene oxidation are completely unaffected by chloramphenicol.

Sorbose-grown cells

The respiration rates observed with sorbose-grown cells on the same set of substrates are presented in Fig. 10 (A & B).

In this case the entire adaptation pattern seemed to have undergone a drastic change. Sorbitol, mannitol and fructose are metabolised at very fast rates followed by sorbose, glucose, limonene, sucrose and α-pinene. Here for the first time a slow oxidation of sucrose becomes apparent. Addition of chloramphenicol did not stop the oxidation of limonene, α-pinene or sucrose, although a lowering of oxidative rates is observed with limonene.

Sorbitol-grown cells

The rates of oxidation of different substrates with sorbitol-adapted cells are presented in Fig. 11 (A & B). Here the pattern of respiration is very similar to that observed with sorbose-grown cells excepting in the case of sucrose, glucose, mannitol and sorbitol, showed the

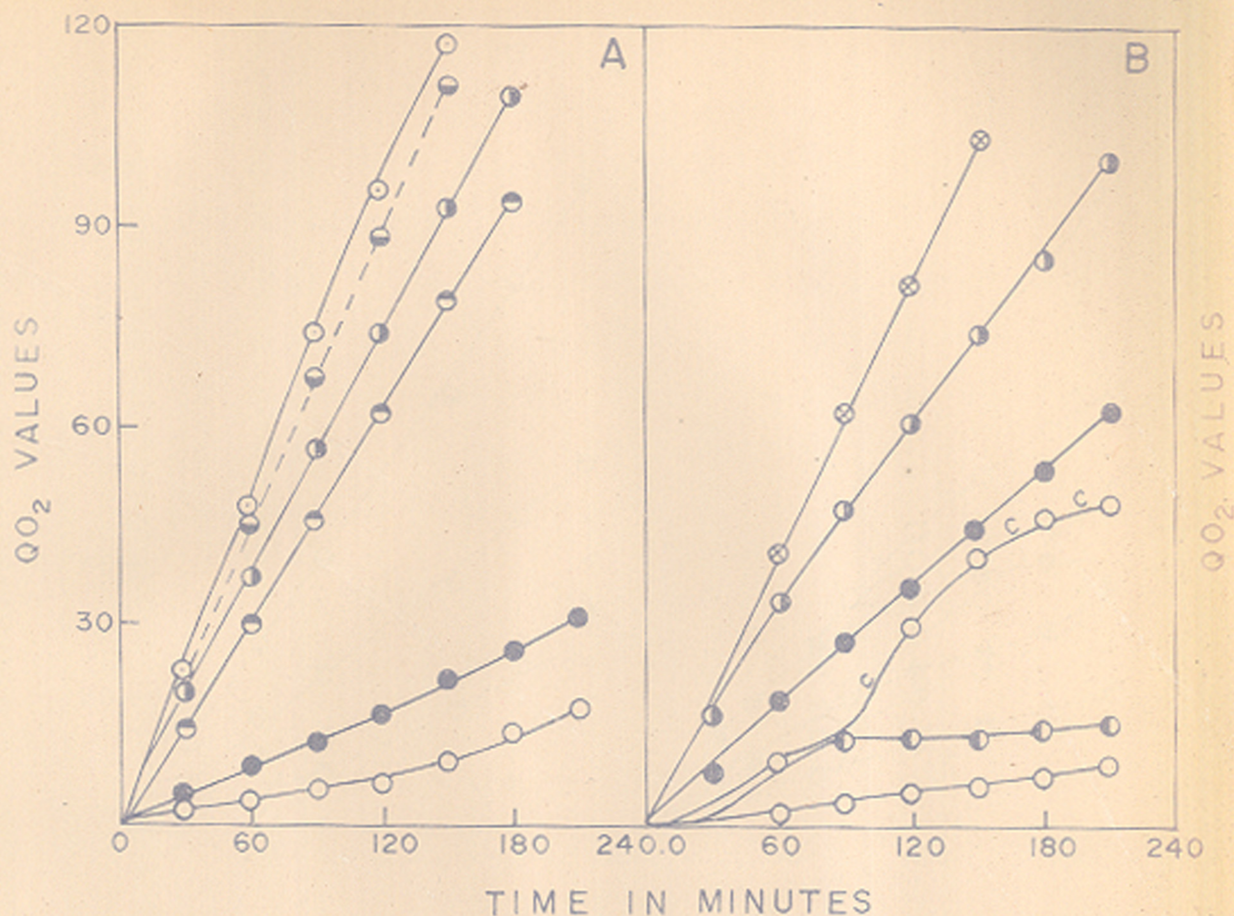


FIG. 10 A & B — OXIDATION OF SORBITOL, MANNITOL, FRUCTOSE, SORBOSE, α-PINENE, LIMONENE, GLUCOSE, SUCROSE (± CMP) BY SORBOSE GROWN CELLS

ENDOGENOUS VALUES SUBSTRACTED

⊙———⊙ SORBITOL ◒———◒ FRUCTOSE ⊗———⊗ MANNITOL

◑———◑ SORBOSE ⊖———⊖ GLUCOSE ●———● LIMONENE (± CMP)

◐———◐ SUCROSE ○———○ α-PINENE ○—c—○ α-PINENE + CMP

(C), CMP – CHLORAMPHENICOL

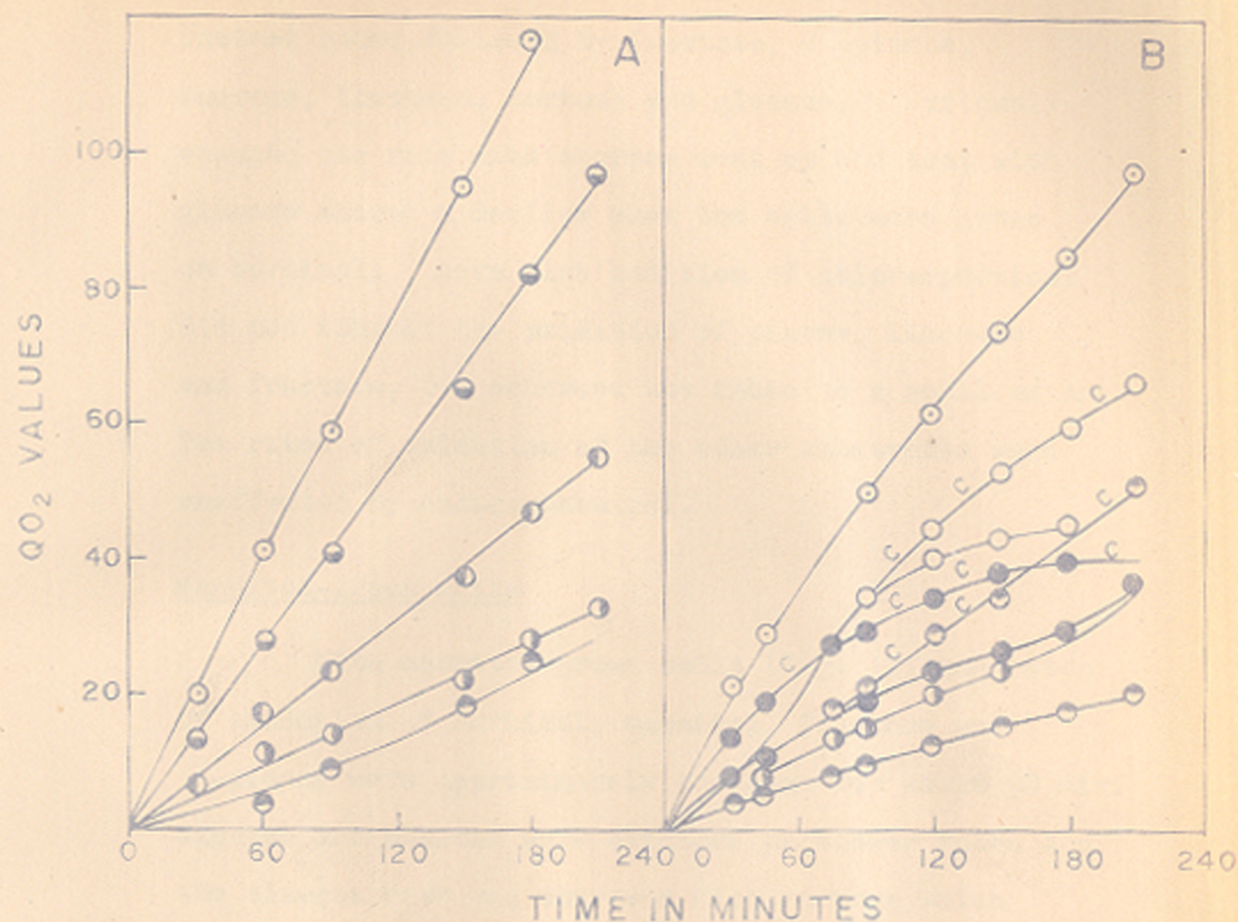


FIG. 11 A & B OXIDATION OF MANNITOL, SORBITOL, SORBOSE, GLUCOSE, LIMONENE, α-PINENE, SUCROSE, FRUCTOSE (± CMP) BY SORBITOL GROWN CELLS. ENDOGENOUS VALUES SUBSTRACTED

○——○ SORBITOL & MANNITOL ◓——◓ FRUCTOSE

◐——◐ SUCROSE ○——○ PINENE ○—c—○ PINENE + CMP

●——● LIMONENE ●—c—● LIMONENE + CMP

◑——◑ SORBOSE (± CMP), ⊖——⊖ GLUCOSE

⊖—c—⊖ GLUCOSE + CMP

fastest rates followed by fructose, α-pinene, sucrose, limonene, sorbose and glucose. Curiously enough, the rate with sucrose went up and that with glucose showed a decline when the cells were grown on sorbitol. Here also addition of chloramphenicol did not inhibit the oxidation of pinene, limonene and fructose, but enhanced the rates to a small extent. The rates of oxidation of the other substrates were unaffected by chloramphenicol.

Mannitol-grown cells

With mannitol-grown cells (Fig. 12) the rates of oxidation of sorbitol, mannitol, fructose and α-pinene were approximately the same for about 90 min. Glucose and sucrose were oxidised at slower rates and the slowest rate was observed with sorbose which showed a small lag phase. Addition of chloramphenicol enhanced the rate of α-pinene oxidation but not of limonene or sorbose.

Sucrose-grown cells

The rates of oxidation of the substrates by sucrose-adapted cells are presented in Fig. 13. Among the sugars and sugar alcohols, the oxidation of sorbitol is the fastest followed by sucrose, glucose, sorbose and fructose. The oxidation of mannitol however, is negligible. Addition of chloramphenicol showed no inhibitory effect on rates of oxidation of

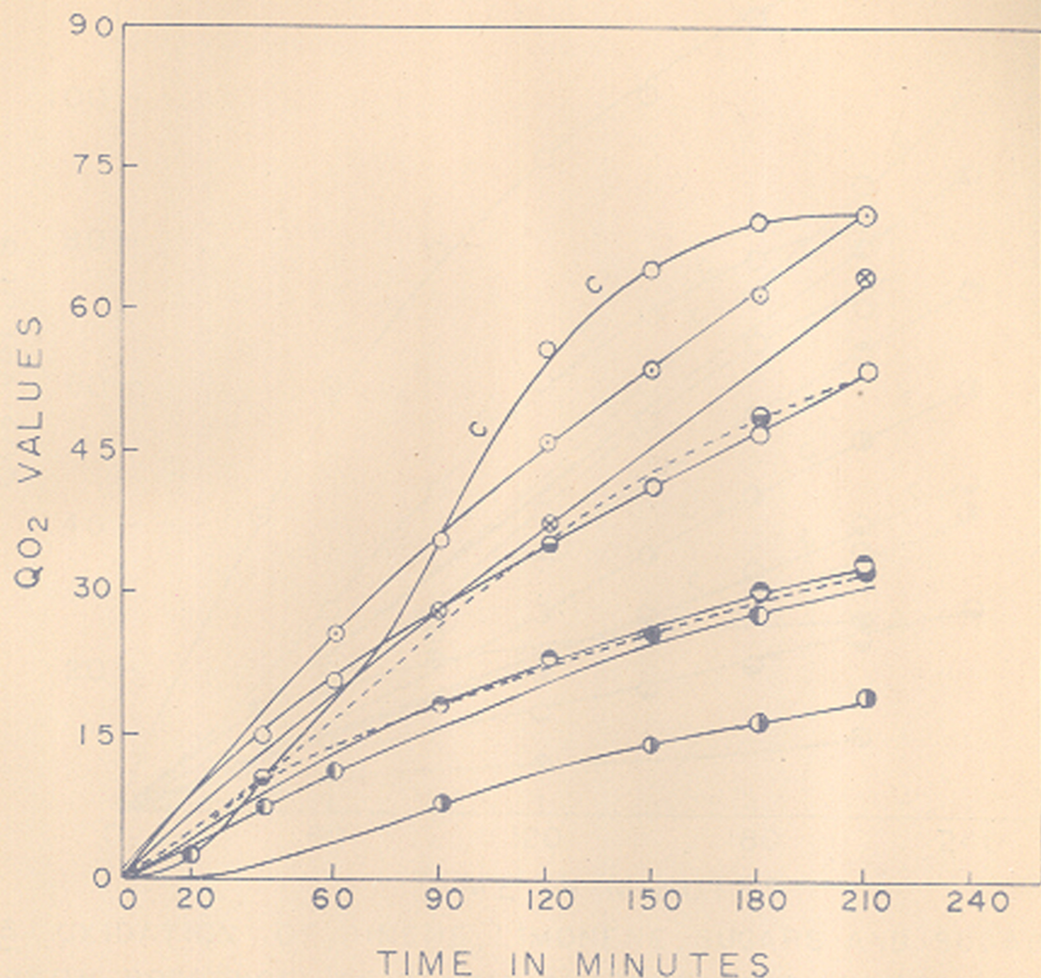


FIG. 12 OXIDATION OF SORBITOL, MANNITOL, FRUCTOSE, α-PINENE, GLUCOSE, LIMONENE, SUCROSE, AND SORBOSE ($\pm$ CMP) BY MANNITOL GROWN CELLS.

⊙——⊙ SORBITOL, ⊗——⊗ MANNITOL, ◒- - - -◒ FRUCTOSE,
○——○ α-PINENE, ○—C—○ PINENE + CMP, ⊖——⊖ GLUCOSE,
●- - - -● LIMONENE ($\pm$ CMP), ◐——◐ SUCROSE, ◑——◑ SORBOSE. ($\pm$ CMP)

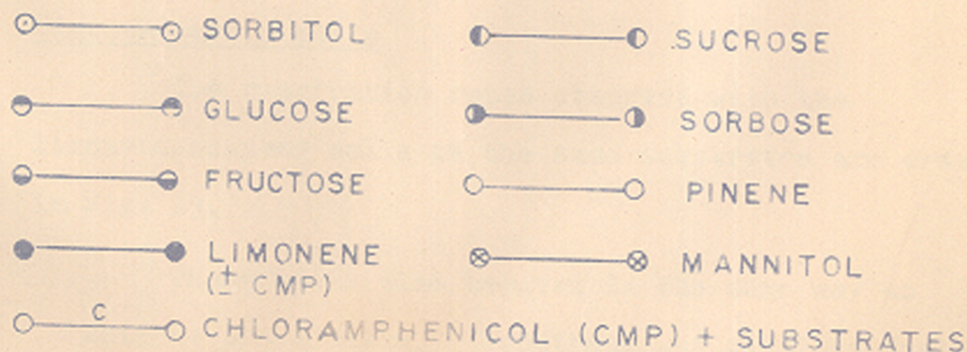
ENDOGENOUS VALUES SUBSTRACTED
C, CMP — CHLORAMPHENICOL

QO2 VALUES

120
100
80
60
40
20
0
60
120
180
240

TIME IN MINUTES

FIG. 13 OXIDATION OF PINENE, LIMONENE, SUGARS AND SUGAR ALCOHOLS BY (IN PRESENCE OF (±) CMP) SUCROSE GROWN CELLS.



ENDOGENOUS VALUES SUBSTRACTED

these sugars but enhanced them to a small extent. These cells oxidise α-pinene faster than glucose and fructose while limonene is oxidised very slowly. Addition of chloramphenicol showed no inhibitory effect on the oxidation of these terpenes but the oxidation of α-pinene was enhanced to a small extent.

Oxidation of Krebs Cycle Acids

α-Pinene-grown cells

Manometric studies were undertaken with sodium succinate malate, and citrate with or without added chloramphenicol with α-pinene-grown cells. The results (Fig. 14) indicated that succinate is utilised fast while malate is oxidised slower. Citrate-oxidation shows a definite lag phase. Addition of chloramphenicol does not affect the respiration rates of succinate or malate but inhibits the respiration on citrate.

Limonene-grown cells

The respiration rates observed with the limonene-adapted cells on the same substrates are presented in Fig. 15.

These cells also behaved in the same way as α-pinene grown cells. Succinate was oxidised faster than malate, followed by citrate. Addition of

FIG. 15 OXIDATION OF SUCCINATE, MALATE, CITRATE WITH (±) CHLORAMPHENICOL BY LIMO-NENE GROWN CELLS

FIG. 16 OXIDATION OF KREBS CYCLIC ACID SALTS WITH (±) CMP BY GLUCOSE GROWN CELLS.

ENDOGENOUS VALUES SUBSTRACTED

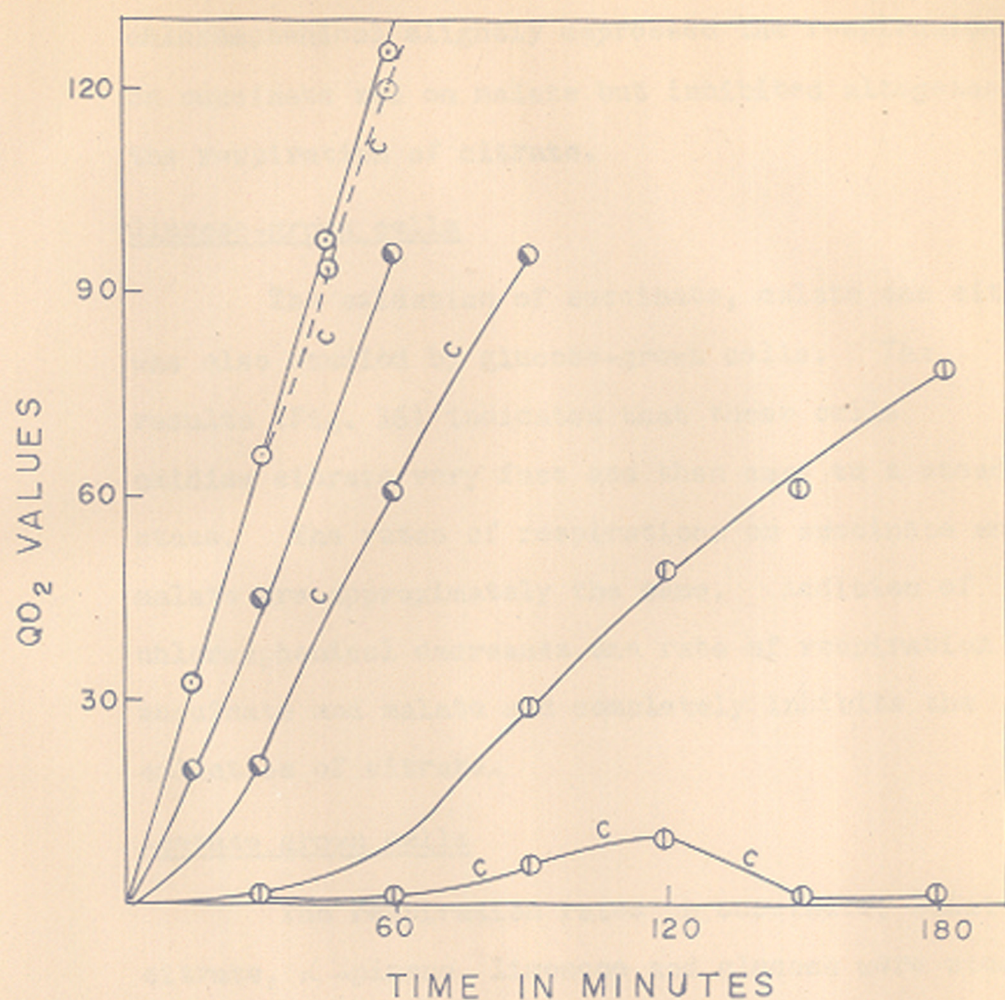


FIG. 14 OXIDATION OF SUCCINATE, MALATE AND CITRATE (± CMP) BY α-PINENE GROWN CELLS

⊙———⊙	SUCCINATE	⊙—c—⊙	SUCCINATE + CMP
◐———◐	MALATE	◐—c—◐	MALATE + CMP
⦶———⦶	CITRATE	⦶—c—⦶	CITRATE + CMP

ENDOGENOUS VALUES SUBSTRACTED

chloramphenicol slightly depressed the respiration on succinate and on malate but inhibited altogether the respiration of citrate.

<u>Glucose-grown cells</u>

The oxidation of succinate, malate and citrate was also studied by glucose-grown cells. The results (Fig. 16) indicates that these cells oxidise citrate very fast and then come to a steady state. The rates of respirations on succinate and malate are approximately the same. Addition of chloramphenicol decreases the rate of respiration on succinate and malate and completely inhibits the oxidation of citrate.

<u>Acetate grown cells</u>

The respiration rates on succinate, malate, citrate, α-pinene, limonene and glucose were studied with cells adapted to sodium acetate. The results (Fig. 17) indicate that the cells oxidised succinate and malate at the same rate. However, a lag phase is observed in the oxidation of citrate. The oxidation of limonene, α-pinene and glucose by these cells is note-worthy. Initially limonene is oxidised at the same rate as succinate and malate but soon is followed by a decline. A lag phase is observed in glucose oxidation by these cells and this

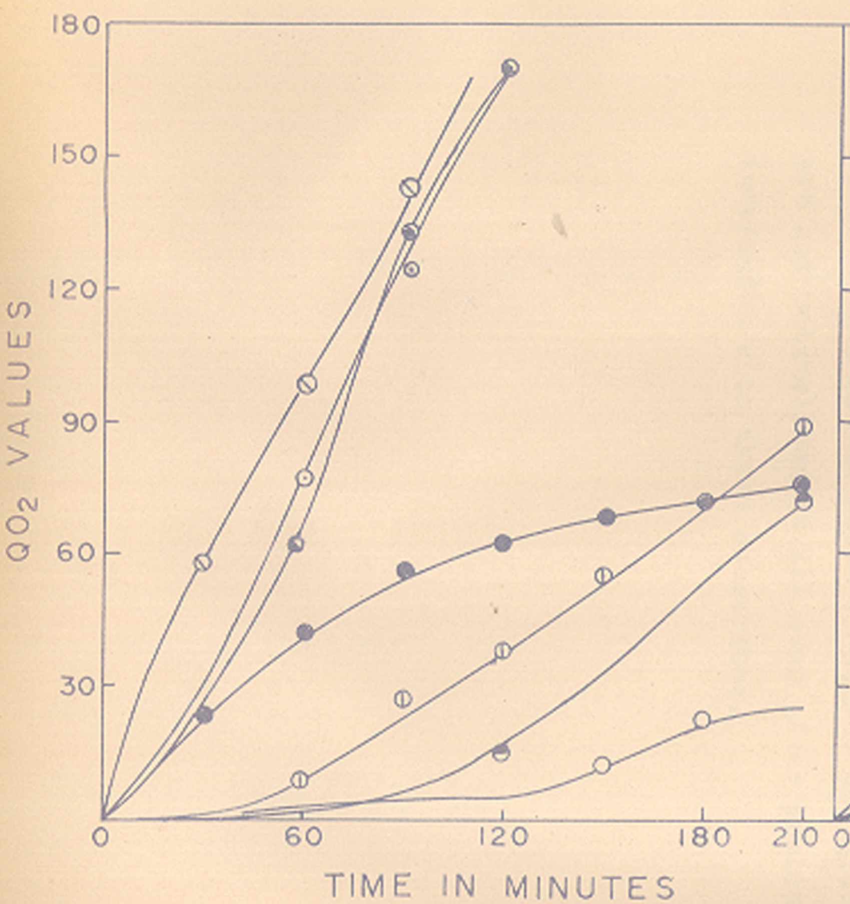


FIG. 17 OXIDATION OF PINENE, LIMONENE, GLUCOSE, & KREBS CYCLIC ACID SALTS BY ACETATE GROWN CELLS. ENDOGENOUS VALUES SUBSTRACTED.

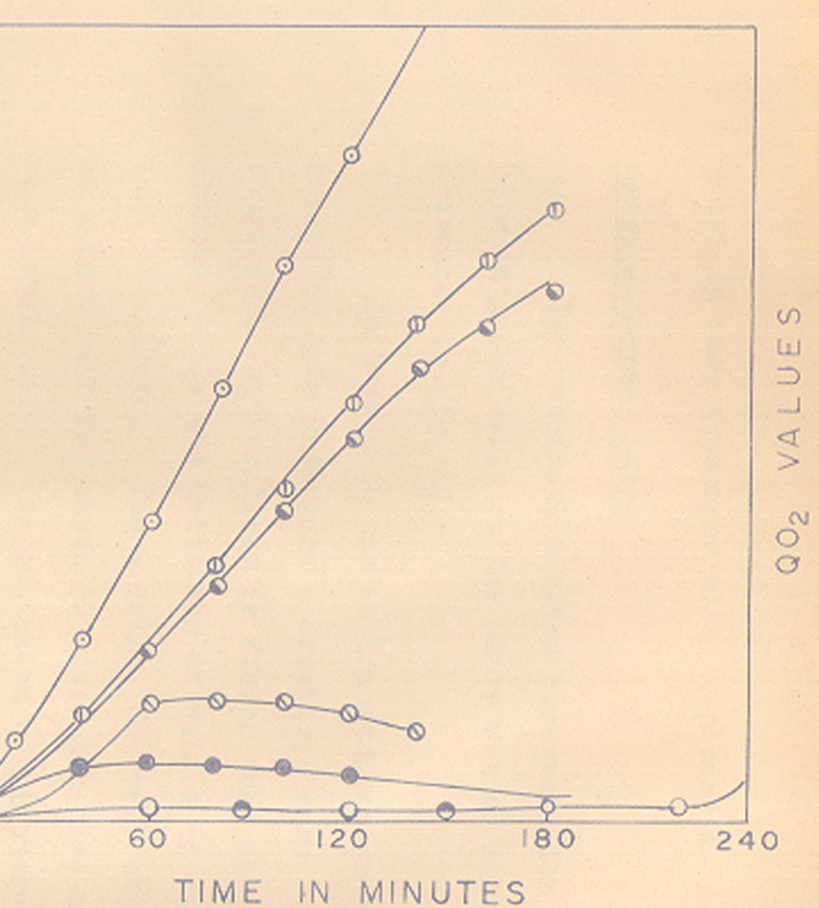


FIG. 18 OXIDATION OF PINENE LIMONENE, GLUCOSE & KREBS CYCLIC ACID SALTS BY CITRATE GROWN CELLS.

PINENE — LIMONENE — GLUCOSE — CITRATE — SUCCINATE — ACETATE — MALATE

phase is more prolonged with α-pinene as a substrate.

Citrate-adapted cells

The respiration rates observed with the citrate grown cells on the above set of substrates are presented in Fig. 18.

In this case the entire adaptation pattern seemed to have undergone a drastic change. The respiration on succinate is faster than that on citrate, while the oxidation of malate is slower than that of citrate. These cells, however, oxidise acetate at a fast rate for about 60 mins. and then this rate levels off. The oxidation of limonene by these cells is negligible while α-pinene and glucose are not metabolised at all even after prolonged incubation with these substrates.

CHAPTER V

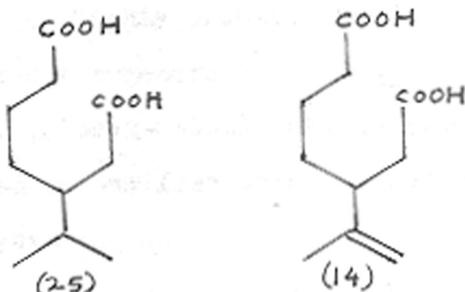
DISCUSSION

CHAPTER V

D I S C U S S I O N

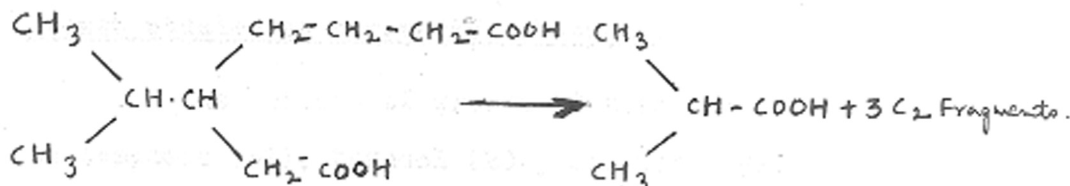
GROWTH STUDIES

It has been reported earlier in the introductory chapter that the degradation of the pinenes, limonene, and perhaps Δ^1-p-menthene takes place through the β-substituted pimelic acid intermediates:



The ultimate fate of these two acids are unknown. However, a C_{20} fatty acid has been isolated from the fermentation mixture of limonene as well as the pinenes. The appearence of these fatty acids may be construed as an indirect evidence that these dicarboxylic acids are ultimately converted to active two carbon-fragments presumably to acyl-Co-A and malonyl-Co-A.

Growth experiments on isobutyrate which may be visualised to be formed by β-oxidation of the dicarboxylic acid by the following sequence did not provide any clue to the pathways.



Growth was observed on this acid after a prolonged lag phase (24 to 48 hrs.). On acetate in most experiments also no growth was observed even upto 72 hrs. However, in occasional batches growth on acetate started after 66-72 hrs.

It is highly probable that these acids even if produced are generated in <u>situ</u> in the activated form e.g. as acyl- +CO-A intermediate and the activation of the free acids do not proceed very well in the system.

Dhavalikar found (1964) that intact cells under nitrogen do not carry out the hydration of perillic acid (12) to β-hydroxy-p-menth-8-ene-7-oic acid (13). However, the cell free extracts in presence of added CoA-SH converted perillic acid ultimately to the substituted pimellic acid.

Further, both Shukla (1965) and Dhavalikar (1964) observed that the dicarboxylic acids (13) and (25) are oxidised very slowly by pinene and limonene grown cells. It appears probable that even in these cases the activation of these acids may be the rate limiting steps.

Growth studies on terpenoid substrates

The failure of growth ~~of~~ this organism on camphene (68), borneol (20), camphor (69), longifolene (70) etc. indicated that the organism shows a sterfospecific affinity towards the p-menthenoid compounds or compound which can give rise to these compounds by prototropic rearrangements. It differs from the pseudomonad strains C_1 and C_2 which can grow on camphor (Conrad, H.E., 1961). et.al.(1959) It also differs from two other pseudomonad isolated in this laboratory by enrichment culture techniques on camphene (68) and longifolene (70).

Regarding the growth of pinene and limonene, it was found that the growth rate of the cells were comparable on these substrates for low substrate concentrations (Fig. 1). However, at 0.6% level of limonene a plateau on the growth curve was reached and remained stationery with increasing concentration. With α-pinene, however, a bell shaped growth curve was obtained with a maximum at 0.6% level. Beyond this level the substrate showed increasing toxicity. At higher levels of α-pinene considerable lyses of cells were observed. Later experiments with borneol indicated that probably this toxic effect is partly due to increasing accumulation of borneol in the medium with the progress of α-pinene fermentation. It may be

recalled (Dhavaliker 1964) that limonene did not produce borneol and, therefore, this toxicity was not observed.

The rates of growth of limonene with both α-pinene and limonene-adapted cells are identical (Fig. 2). This would indicate that the enzymes involved in the degradation of limonene are more or less produced in identical amounts irrespective of whether the cells were grown on limonene or pinene.

At the beginning, the α-pinene adapted cells grew on α-pinene faster than on limonene. However, a plateau was reached, abruptly at 12 hours while the logarithmic phase of growth on limonene continued upto 18 hours.

Limonene-adapted cells in this particular experiment failed to grow on α-pinene even after 24 hrs. However, this behaviour was not constant and in some subsequent experiments it was found that growth of limonene adapted cells on α-pinene started with a much shorter lag phase, or none at all. Hence the protonation system, (Shukla 1965) the extra enzymic set up needed for adaptation on α-pinene, was sometimes, but not always retained by the bacterium throughout its passage through limonene. In other words, the capacity

for metobolism of limonene is inherent in the cells grown on α-pinene but not necessarily vice-versa.

Utilization of carbohydrate for growth of α-pinene and limonene adapted cells

Since the organism showed a narrow range of substrate specificity towards the p-menthenoid compounds or compounds which can give rise to p-menthenoid structure by prototropic re-arrangement, it was considered worthwhile to determine whether such specificity also exists in its growth on carbohydrates and some related compounds.

The results (Fig. 4A) indicated that limonene-adapted cells could grow on glucose faster than on limonene but slower on fructose. The rates of growth on gluconic acid was the highest at the beginning but soon reaching a plateau after 8 hrs. The cells did not grow on sorbose, sucrose, sorbitol, mannitol or 5 keto-gluconic acid within the experimental period of 48 hrs. The growth behaviour of α-pinene adapted cells (Fig. 3) was surprisingly enough, somewhat different, from that of the limonene adapted cells. The growth rates on limonene, α-pinene, glucose and gluconic acid were similar for the first eight hours; with fructose the rate was slow in the beginning and

increased after 12 hours. With sorbitol the growth started after a lag phase of 18 hrs. and with sorbose after 36 hrs. Addition of a small amount of sorbitol to sorbose did not produce any change in the lag phase observed with either of these compounds indicating sorbose may not be in the pathway for sorbitol fermentation.

The behaviour of sucrose was rather unexpected. The growth curve showed a "diphasic" phenomenon or a "diauxie" which indicated that at least two sets of enzymes have to be induced for complete degradation of this substrate.(Oginsky & Umbriet, 1959) At first, growth was observed in 8 hrs. followed by stationary phase which continued for about 10 hrs. and then the growth started again. This peculiar behaviour of sucrose is completely reproducible. Perhaps this ready adaptation of pinene-grown cells on sucrose may indicate an allosteric effect where the same operon sites are activated through different regulatory mechanisms. (Hayes, W. 1964) The cross adaptation of the micro-organism on both pinene and sucrose will be discussed later on.

<u>Growth on different Krebs Cycle acids</u>

α-Pinene adapted cells grew on both the sodium salts and ammonium salts of succinic, malic, and citric acids as fast as they grew on limonene. Acetate, however, failed to support growth. In the case of sodium malate and sodium citrate and ammonium salts of succinic, malic and citric acids, a toxic effect was observed at 16 hrs. Examination of pH at this stage revealed that it was very alkaline and the organism changed its morphological characteristics to coccal forms, perhaps to survive under the unphysiological conditions. This morphological change was reversible and under more favourable condition the cocci reversed back to the rod form.

That the toxicity was not due to the anion was demonstrated by growing the organism on graded levels of the organic acids. Fig 4 B would indicate that growth remains more or less proportional to the substrate concentration upto a level of 0.3% then the growth curves flatten out somewhat with increasing concentration.

As far as the Krebs cycle acids are concerned the limonene-grown cells behaved in an almost identical manner as the pinene-grown cells, both towards the sodium salts and the ammonium salts.

MANOMETRIC STUDIES

The growth studies on various substrates were followed up by manometric studies with cells adapted to each of these substrates.

α-Pinene grown cells seemed to adapt more readily to oxidise fructose and glucose than limonene-grown cells (Fig. 7 A & 7 B). A smaller lag phase was observed in the oxidation of glucose and fructose with α-pinene grown cells. However, the oxidation of these carbohydrates was a substrate-induced phenomenon in these cells since the oxygen uptake was completely abolished by added chloramphenicol. These cells did not get adapted to oxidise any of the other carbohydrates during the experimental period.

In both these cases the adaptation to fructose appeared to be faster than that to glucose.

Fructose-grown cells on the other hand freely oxidised α-pinene, limonene, fructose and glucose with or without chloramphenicol (Fig. 8 A & B). It should be noted, even with these cells the observed rates of oxidation of limonene and α-pinene were much higher than that on fructose itself. With chloramphenicol a small lag phase was observed with α-pinene. Additions of chloramphenicol did not materially affect the rate of limonene-oxidation (not shown).

Sorbose was oxidised very slowly but this oxidation was not sensitive to chloramphenicol.

It can be noticed that adaptation to fructose is a phenomenon which is associated with a pronounced lowering of the QO_2 values of the cells on all the substrates. The reason for this behaviour is rather obscure.

Glucose adapted cells, however, oxidised glucose and limonene at comparable rates and the rate of limonene oxidation was unaffected by added chloramphenicol (Fig. 9 A & B). The lowering of QO_2 values observed with fructose-grown cells was much less pronounced with glucose-grown cells which oxidised fructose at a faster rate than fructose-grown cells. Even sorbose was oxidised at a faster rate. These rates were unaffected and in some cases increased with added chloramphenicol. This anamolous behaviour with chloramphenicol has been observed in other cases also, and is by no means an uncommon phenomenon (Ladd, 1962).

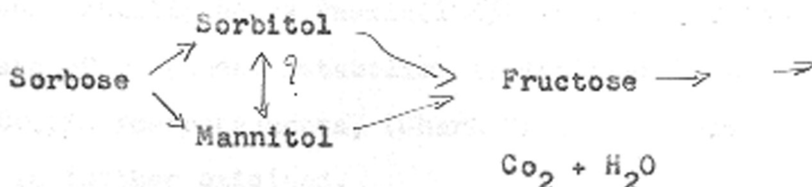
Qualitatively, however, there was very little difference between fructose and glucose-grown cells, both oxidising the same substrates although at different rates.

Growth on sorbose seemed to induce some new enzymes in the pseudomonad since the sorbose-grown cells oxidised sorbitol and mannitol besides those substrates which are oxidised by cells grown on limonene, α-pinene, fructose and glucose (Fig. 10 A & B).

It is interesting to note that in these cells the rates of oxidation of limonene and α-pinene were very slow as compared to the rates of oxidation of these substrates encountered so far (excepting in the case of fructose-grown cells). It is also apparent that the oxidations of fructose, glucose as well as sorbitol and mannitol are constitutive phenomena inherent in sorbose-grown cells.

Sorbitol and mannitol grown cells behave, at least qualitatively in a manner similar to the sorbose-grown cells (Fig. 11 A & B and 12). A curious phenomenon with these cells is that these cells show high rates with fructose and very slow rates with sorbose. A teleological explanation for this behaviour would be that sorbose does not lie in the pathway for oxidation of sorbitol. And a comparison of the rate pattern observed with cells grown on fructose, sorbose, sorbitol and mannitol on these substrates would indicate the following alternative pathways for the metabolism

of sorbose in this bacterium.



The data, however, do not conclusively establish a direct isomerisation of sorbitol to mannitol.

It is also noteworthy that the cells grown on sorbitol and mannitol show cross-adaptation on sucrose, this property appearing for the first time. Very probably this may be an allo-steric effect (Monod, J., 1963). A slow rate of sucrose oxidation is observed with sorbose-grown cells also. (Fig. 10 A).

The pattern observed with sucrose grown cells (Fig. 13) is strikingly different from that observed with cells-adapted to other substrates, in as much as the limonene oxidising property, a constitutive phenomenon with cells grown on other substrates, seems to have been reduced to an almost insignificant level. It may be recalled in this connection that Dhavlikar (1964) found that sucrose-grown cells mainly oxidised limonene to perillic acid (12) and the rate of oxidation of perillic acid is very low in these cells.

In this context the ready oxidation of α-pinene by sucrose-grown cells is rather surprising as it has been established by Shukla(1965) that one of the pathways of α-pinene metabolism is mediated by a prototropic rearrangements, (Chart 2) to limonene which is further oxidised.

It will, therefore, be interesting to study the products of fermentation of α-pinene by sucrose-grown cells.

It has been mentioned earlier that α-pinene grown cells grow readily on sucrose whereas limonene-grown cells take a very long time to establish themselves on sucrose. It is highly probable that this is also a phenomenon of cross adaptation on structurally dissimilar substrates.

Another striking phenomenon is that the highest rates of oxidation is obtained with sorbitol; mannitol on the other hand is not oxidised at all. These cells oxidise fructose but at much lower rates.

The significance of these findings is not clear at this stage and more work remains to be done with the isolated enzymes from sucrose-grown cells.

Oxidation of Krebs Cycle Acids

Limonene- and α-pinene grown cells behave similarly towards succinate, malate and citrate (Fig.15 & 16). The oxidation of citrate is inhibited by chloramphenicol in these cells. There are two possible reasons for this.

A) The entry of citrate into the cell is an active transport phenomena requiring perhaps ATP and a specific permease. Such behaviour has been observed with yeast and other bacteria which are known to have the complete Krebs cycle (Krebs et.al. 1952).

B) The entire Krebs Cycle is not present in this bacterium when grown on terpenes. It can be argued that the acetate units produced by degradation of terpenes are channelled for the synthesis of carbophydrates through the glyoxylate by pass (Kornberg, 1957).

Manometric experiments were also performed with cells grown on citrate and glucose (Fig. 18, 16) with these Krebs Cycle substrates, to determine whether the behaviour of the cells would show any difference. It is noteworthy that glucose-grown cells which are likely to have the complete Krebs Cycle did not oxidise citrate in presence of chloramphenicol.

It is possible, therefore, that chloramphenicol completely blocks the synthesis of enzymes responsible for the entry of citrate inside the cells.

Citrate grown cells were unique in their failure to metabolise either α-pinene or limonene (Fig. 18). Growth on citrate, therefore, changes the enzymic pattern of these cells rather drastically.

Acetate grown cells on the other hand metabolised limonene readily and α-pinene slowly after a prolonged lag phase. A lag was also observed with citrate but not with succinate and malate. (Fig. 17)

These studies were undertaken with a view to understand the basic pathway in this organism particularly with respect to the terminal oxidative mechanisms for terpene and carbohydrate degradation.

It has been established that at least part of the citric acid-cycle is operative in the oxidation of fragments from these substrates. No definite evidence has been obtained as yet to prove conclusively the existence of either Krebs Cycle or the Glyoxylate bypass in this organism.

CHAPTER VI

SUMMARY

SUMMARY AND CONCLUSION

A soil pseudomonand was found to grow on α-pinene, β-pinene, limonene, Δ^1-p-menthene, p-cymene but not on camphene, borneol, camphor and longifolene. Cells adapted to α-pinene utilized β-pinene and limonene very easily but not necessarily vice-versa. High concentrations of α-pinene were toxic to the bacterium while limonene showed no toxic effect. This toxic effect could be associated in part with the accumulation of borneol, one of the neutral product of fermentation in the medium.

The organism could grow readily on 1% glucose, fructose and sodium gluconate but a lag phase was observed on sorbose, sucrose, sorbitol and mannitol.

The organism could utilize sodium and ammonium salts of succinic malic, citric acid whereas a prolonged lag was observed on sodium and ammonium acetate. The organism, however, changed its morphological characteristic to coccal forms when grown on these salts due to the changes in alkalinity of the medium. This phenomenon was completely reversible.

Manometric studies indicated that the cells adapted to α-pinene oxidised α-pinene and limonene whereas limonene-adapted cells oxidised α-pinene more

slowly with or without a lag phase. These rates were chloramphenicol insensitive. The oxidation of α-pinene and limonene by cells adapted to sugars and sugar alcohols was also chloramphenicol insensitive.

Acetate-adapted cells oxidized limonene and α-pinene with a lag phase whereas citrate-grown cells failed to oxidise α-pinene and limonene during the experimental period.

Cells adapted to α-pinene oxidised glucose and fructose but with a lag phase. This phase was prolonged with limonene grown cells. The rates of oxidation were chloramphenicol sensitive. These cells did not show any oxygen uptake with sorbose, sorbitol, mannitol or sucrose upto 4 hours.

Cells grown on fructose appeared to metabolise limonene and α-pinene at a faster rate than fructose and glucose. These cells oxidised sorbose after a lag period of 90 minutes and showed no oxygen uptake with sucrose, sorbitol and mannitol.

Cells grown on glucose oxidised limonene and glucose at the same rates, but showed a lag phase of 30 minutes to 2 hours with pinene, sorbose and fructose, whereas sucrose, sorbitol and mannitol were not oxidised.

Cells adapted to sorbose oxidised sorbitol, mannitol, fructose, sorbose and glucose at fast rates but

the rates of oxidation of limonene was much slower. These cells oxidised pinene and sucrose only after a considerable lag period.

Sorbitol and mannitol-grown cells oxidised sorbitol, mannitol, and fructose. The rate of glucose oxidation was slower. However, sorbose was oxidised very slowly by mannitol grown cells. These cells also oxidised α-pinene and limonene.

Sucrose grown cells oxidised, sorbitol, sucrose, glucose, sorbose and fructose with decreasing rates of oxidation. Mannitol was not oxidised. These cells oxidised α-pinene faster than limonene. All these oxidations were chloramphenicol insensitive.

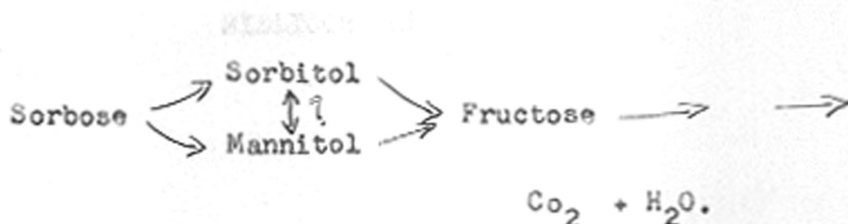
The organism adapted to α-pinene, limonene or glucose, oxidised sodium succinate, malate and citrate. Addition of chloramphenicol inhibited the oxidation of citrate.

Cells adapted to acetate oxidised succinate, malate without any lag phase whereas citrate was oxidised with a lag phase. These cells also oxidised glucose with a lag phase.

Citrate-grown cells also oxidised succinate and malate very fast but acetate was oxidised very slowly. These cells showed no oxygen uptake with glucose, α-pinene and limonene.

From the earlier and the present studies it is established that the capacity of oxidation of limonene is an inherent, characteristic of this bacterium when it is grown on a large number of substractes whereas α-pinene oxidation is an induced phenomenon. The bacterium is also stereospecific in its action towards the terpene substrates. Some cross adaptation was observed between sucrose and α-pinene. The oxidation of carbohydrates as well as citrate by this organism is an adaptive phenomenon.

The organism appeared to oxidise sorbose by the following pathway:



Finally, it is construed that the dicarboxylic acids obtained by the degradation of α-pinene and limonene by this organism is broken up into two carbon fragments such as acetyl Co A which condenses with malonyl Co-A or enter the oxidative cycle. It was not possible to determine whether the entire Kerbs cycle or the glyoxylate bypass was present in this bacterium.

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