MICROBIOLOGICAL TRANSFORMATIONS OF TERPENES

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

by
K. M. MADYASTHA, M.Sc.

Division of Biochemistry
National Chemical Laboratory
POONA 8

NOVEMBER 1968

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Chapter I

INTRODUCTION

INTRODUCTION

In connection with studies on the microbial degradation of terpenes initiated in this Laboratory, a soil pseudomonad was isolated which could grow not only on hydroaromatic terpenoid substrates such as α-pinene, β-pinene, limonene and Δ^3-p-menthene but also on the aromatic compound, p-cymene. Since the metabolism of these compounds may be considered to be related to that of hydrocarbons in general, it may be relevant to recapitulate briefly the data available in the literature on the fermentation of hydrocarbons, the aromatic ones in particular.

Metabolism of hydrocarbons

Although it has been known for more than sixty years that microorganisms can utilise hydrocarbons for growth, systematic studies have been carried out only in recent years on the action of microorganisms on a variety of types and classes of hydrocarbons in the aliphatic, olefinic and aromatic series. Nearly, a hundred species of bacteria, yeasts and molds representing some thirty genera have been isolated which attack one or more kinds of hydrocarbons (Zobell, 1946). The assimilation of hydrocarbons is of considerable significance in nature and of potential interest in industry. The microbial oxidation

of hydrocarbons which bring about rapid disappearance of petroleum that pollutes waterways (Bushnell and Haas, 1941; Tausson, 1929), the deterioration of certain rubber products and the corrosion of underground pipe-lines (Zobell and Grant, 1942; Dimond and Horsfall, 1943) are some of the few examples of the diverse foci of interest in this field. Besides, ingenious use has been made of these microorganisms for obtaining products of economic value such as fatty acids or proteins from hydrocarbons, useful organic acids from methane or higher hydrocarbons and for the elimination of industrial wastes around refineries (Strawinski and Stone, 1940; Taggart, 1948).

Although microorganisms have been utilized extensively for the production of various compounds of economic value, very little is known about the biodegradation of various classes of compounds by the microorganisms. Recent years have witnessed a widespread interest in the microbiological degradations of various compounds, notably that of aromatic hydrocarbons.

Metabolism of aromatic compounds

Aromatic compounds in general serve as carbon and energy sources for soil bacteria. The oxidative metabolism of these compounds by microorganisms is an essential biochemical step in the 'carbon' cycle in Nature. Bacteria are versatile in this respect, as compared to several yeasts and fungi which can degrade only a limited

range of benzenoid compounds. A number of excellent reviews have appeared covering various aspects of this subject (Fuhs, 1961; Evans, 1963; Zobell, 1946; Zobell, 1950; Happold, 1950; Rogoff, 1961; Stanier, 1955; Dagley, 1965; Hayaishi, 1957).

The fact that up to 1945 progress in the field of microbial hydrocarbon dissimilation lagged considerably behind as compared with the advancements made in regard to other classes of compounds is due mainly to (a) the non-physiological character of hydrocarbons, (b) the experimental difficulties involved in working with insoluble substrates, and (c) the difficulties encountered in the isolation and identification of products. But advances made since then in various directions have overcome the difficulties presented by these unfavourable aspects.

Stanier's method based on sequential induction (Stanier,1947) is used widely as a tool in the elucidation of adaptive metabolic patterns. The pioneering work of Mason (Mason 1956) and Hayaishi et al. (1955) have made it possible to study the oxygenases in bacteria which play a dominating role in the metabolism of aromatic and aliphatic compounds.

Studies on the bacterial degradation of aromatic compounds have also been conducted in many laberateries with a view to elucidating certain general metabolic pathways through which these aromatic degradations are carried out.

The methodology employed in these metabolic studies is mainly

one of isolation of various intermediates that are formed during the course of the fermentation and the establishment of induced enzyme systems. These studies have yielded some general ideas about the way in which aromatic compounds are metabolized by bacteria. Microbes produce, mostly as a result of induction, a whole sequence of enzymes which convert aromatic substrates into an ortho or para dihydroxy phenol derivatives prior to the ring fission. Next the benzene nucleus of the dihydroxy phenol is cleaved by an almost irreversible dioxygenase type reaction which incorporates both the atoms of a molecule of oxygen into its substrate. These ring fission products are then funnelled into the Krebs' cycle through a variety of pathways, depending on the organism and cultural conditions. Thus, phenol is hydroxylated to catechol. Similarly, polynuclear aromatic compounds are degraded by end-ring fission, the ultimate product of the ring cleavage being salicylic acid or an analogue. Salicylic acid, then undergoes a single oxidative decarboxylation step to catechol. So from most of the aromatic compounds, orthodihydroxy derivatives are formed which are then cleaved by different oxygenase systems.

Biodegradation of polynuclear aromatic hydrocarbons

Interest in the biochemical degradation of polynuclear aromatic hydrocarbons stems from the fact that some of these are carcinogens and their metabolism in mammals has been of considerable interest to biochemists and biologists. It is to be noted that there exists similarities

between the initial mode of attack by bacterial and by liver enzymes, as exemplified by the formation of arenediol in both the instances. However, in animals these polynuclear hydrocarbons are excreted in the form of various conjugated derivatives, while the soil pseudomonads can completely dissimilate them to carbon dioxide and water.

Rogoff and Wender (1957) initiated investigations towards understanding the biochemistry of the oxidative dissimilations of phenanthrene and anthracene. They observed that soil pseudomonads did not attack phenanthrene at the 9, 10 bond (i.e., the so-called K region) which is the chief loci of attack in mammalian system but the primary microbial activity was directed at an end ring. Colla et al. (1959) isolated 3,4-dihydro-3,4-dihydroxy phenanthrene from cultures of a Flavobacterium metabolizing phenanthrene, and Rogoff and Wender (1957) obtained 1-hydroxy-2-naphthoic acid from cultures of a soil pseudomonad. Both the groups showed by sequential induction technique that salicylate and catechol were the later intermediates in the oxidative pathway of phenanthrene.

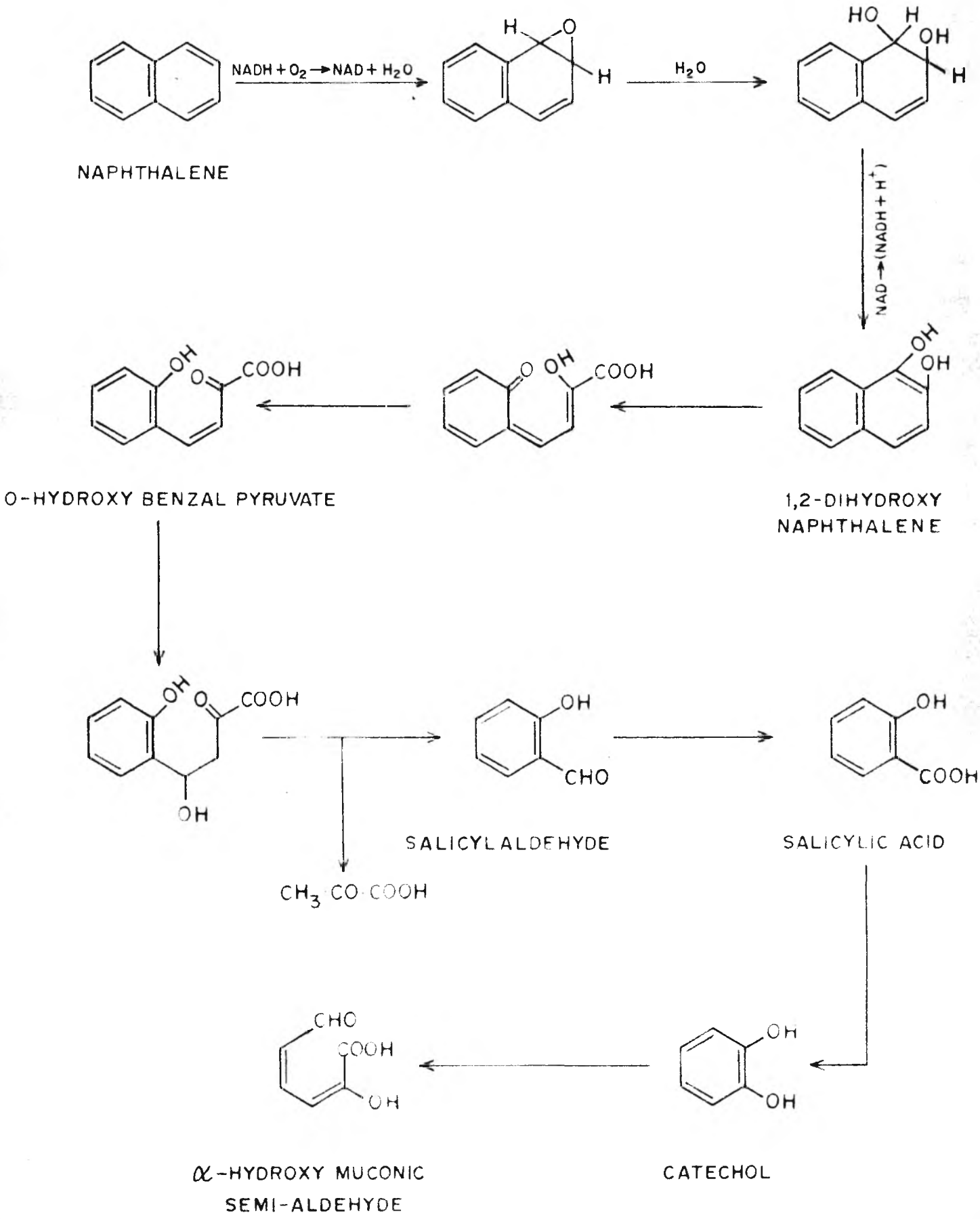
Fernly and Evans (1958) reported that naphthalene-grown cells sequentially induced the oxidation of trans 1,2-dihydro-1,2-dihydroxy naphthalene; 1,2-dihydroxy naphthalene and salicylic acid.

Evans et al. (1965) have shown that a Fe^{++} ion-dependent oxygenase converts 1,2-dihydroxy naphthalene to o-hydroxybenzalpyrvate,

which is subsequently converted to salicylaldehyde and pyruvate by a hydrolytic cleavage. A NAD-dependent dehydrogenase produces salicylate from the aldehyde and a salicylate hydroxylase gives catechol which is finally ruptured to yield α-hydroxymuconic semialdehyde by the catechol 2,3-oxygenase. It has been proposed that an epoxide is formed as a primary intermediate and subseqnent hydration is involved with the formation of trans 1,2-dihydro,1,2-dihydroxy naphthalene which is further oxidized to 1,2-dihydroxy naphthalene in presence of NAD. In general, most of the microorganisms utilizing naphthalene adopt the pathway represented schematically (Fig. I).

When a naphthalenic compound undergoing oxidation beare a substituent group (CH_3,Cl,Br) on one ring, oxidation is usually directed at the unsubstituted ring. The salicylic acid derivative produced will then bear the substituent group of the starting compound. Thus 3-chloro-salicylic acid was isolated as an intermediate from culture fluids of a gram-negative bacterium utilizing 1-chloronaphthalene as the source of carbon (Walker and Wiltshire, 1955). Similarly, Rogoff and Wender (1959) studied the oxidation of 1, and 2,methyl naphthalene by soil pseudomonads. They noticed that the cells grown either on naphthalene or on one of the methyl naphthalenes, were induced to oxidize salicylic acid, catechol and their methyl derivatives. This suggested that the same enzymes which are responsible for the oxidation of salicylic acid

FIG·1· BIODEGRADATION OF NAPHTHALENE BY A SOIL PSEUDOMONAS



and catechol, are involved in the oxidation of the methyl derivatives. So the presence of the methyl group on the benzene nucleus does not generally confer a specificity requirement for the enzymes involved.

Regeff (1962) has pointed out that when linearly arranged aromatic compounds such as naphthalene or anthracene are acted on by bacterial enzymes attachment to the enzyme and ring splitting may take place on the same ring i.e., at the C-C bonds of high electron density (1,2-position). Angular aromatic compounds such as phenanthrene afford attachment to the enzyme in the ring other than the one containing the ring splitting site.

Oxidative metabolism of benzenoid compounds

Before the benzene nucleus is cleaved it must first carry two hydroxyl groups, and if these groups are not already present, they must be introduced by the action of a dioxygenase or a mixed function oxygenase. Often, the hydroxyl groups are carried by adjacent (1,2) carbon atoms so that catechol or a substituted catechol is formed (Evans et al. 1949). Sometimes hydroxyl groups are introduced across the nucleus in the 1,4-position, as in the case of homogentisic acid (Jones et al.1952). But in all cases the presence of two hydroxyl groups is a prerequisite for ring fission.

1) Hydroxylation of benzene nucleus

Several workers (Mason, 1956; Hayaishi et al. 1956; Hayano et al. 1955) have reported that oxygen plays a more direct role in a diverse

groups of reactions including hydroxylations, cleavage of aromatic rings and cyclizations of steroids, the mechanism involves a direct oxygenation of the substrate. The enzymes which are responsible for the activation of molecular oxygen and its incorporation into organic substrates are called "oxygenases". They include two groups of enzymes which catalyze the incorporation of either one or two atoms of molecular oxygen per molecule of organic substrate and these two groups are referred to as:

(1) Monooxygenase ($Sub+O_2+H_2X \longrightarrow Sub-O+H_2O+X$), and

(2) Dioxygenase ($Sub + O_2 \longrightarrow Sub-O_2$) (Hayaishi, 1964).

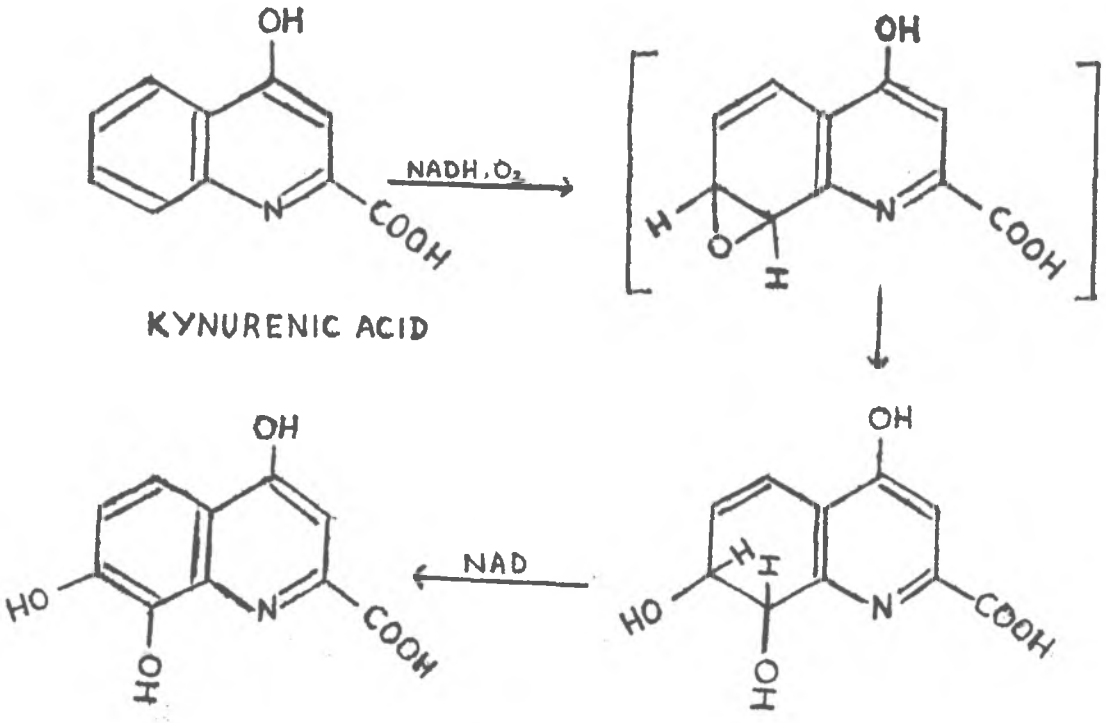
Here H_2X is an electron donor which includes reduced forms of pyridine nucleotides, flavin nucleotides, cytochromes, tetrahydropteridines and ascorbic acid. Mason (1956) has proposed the terms "mixed function oxidase" and "oxygen-transferase" for the above two classes of oxygenases. In general, most of the hydroxylases fall into the category of mixed function oxidase except for a few isolated cases as can be seen in the case of the formation of 6-hydroxynicotinic acid from nicotinic acid which was shown to involve hydration of a double bond followed by dehydrogenation (Hunt et al. 1957).

Monooxygenase (Mixed function oxidase)

Hydroxylation of kynurenic acid

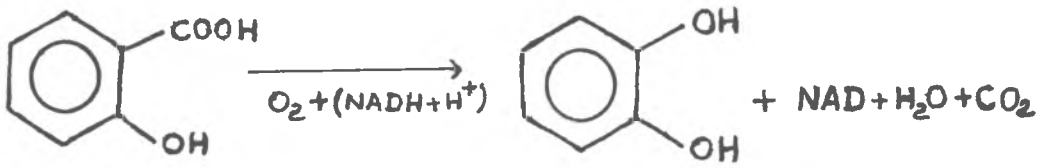
Taniuchi and Hayaishi (1963) have studied the hydroxylation of kynurenic acid by the action of kynurenic acid 7,8-hydroxylase. The

reaction catalyzed by this enzyme probably proceeds from kynurenic acid via kynurenic acid 7,8-epoxide, a hypothetical intermediate which can subsequently undergo hydration to yield 7,8-dihydro-7,8-dihydroxy kynurenic acid. The latter compound is further converted to 7,8-dihydroxy kynurenic acid by the action of a dehydrogenase. It was further shown that kynurenic acid is attacked by a NADH-dependent hydroxylase and the dihydrodiol was isolated as a reaction intermediate. They proposed that an epoxide is formed first and the dihydrodiol is subseqnently oxidized to 7,8-dihydroxy kynurenic acid with regeneration of NADH so that there is no overall loss of the reduced co-factor. The sequence of reaction is described below.



Hydroxylation of salicylic acid

Recently Hayaishi et al.(1963) have purified from /pseudomonad an enzyme which hydroxylates salicylic acid in presence of NADH to form catechol. One mole of FAD was associated with a mole of enzyme protein.



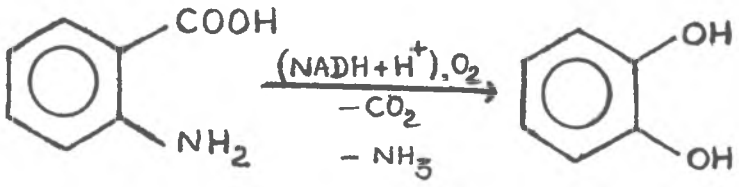
In this monooxygenase reaction, the substrate is oxygenated and is simultaneously reduced by the electron donor, thus cleaving the bond between the two oxygen atoms and yielding a monooxygenated product and water. However, Yano and Arima (1958) have reported that several strains of Pseudomonas show an alternate pathway for salicylic acid degradation via gentisic acid.

Many instances are known where the enzymatic hydroxylation of aromatic compounds that requires oxygen and reduced pyridine nucleotide. The hydroxylation of p-hydroxyphenylacetic acid also depends on the presence of both oxygen and reduced pyridine nucleotide (Adachi et al. 1964) and the hydroxylase has been classified as a mixed function oxidase type. In a similar manner, m-hydroxybenzoic acid is oxidised via protocatechuic acid by a strain of Pseudomonas (Yano and Arima, 1958).

Recently, Hosakava and Stanier (1966) have isolated a crystalline p-hydroxy benzoate hydroxylase from Pseudomonas putida which catalyzes the hydroxylation of p-hydroxy benzoate to protocatechuate. This enzyme also contains one mole of FAD but is NADPH dependent.

Dioxygenase (oxygen transferase)

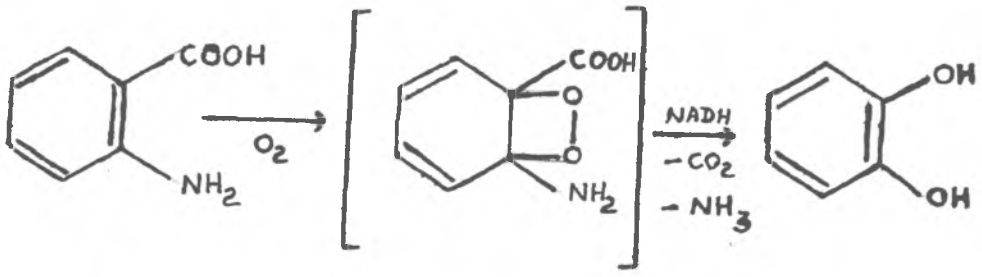
Ichihara et al. (1962) and Taniuchi et al. (1964) postulated the hypothetical mechanisms involving a possible intermediate for the enzymic formulation of catechol from anthranilic acid. According to



this hypothesis, an epoxide is formed as a primary intermediate and subsequent hydration is involved. This mechanism has been disproved by Hayaishi and his co-workers (Hayaishi et al. 1957; Kobayashi et al. 1964) who have given experimental evidence to prove that both the atoms of oxygen which are incorporated into catechol are derived exclusively from molecular oxygen, and none from the medium. The reaction involves the consumption of one molecule of oxygen and of NADH with the concomitant evolution of one mole each of carbon dioxide and ammonia per mole of the substrate utilized.

Available evidence indicates that two oxygen atoms add to

the double bond across carbon 1 and 2 to give a cyclic peroxide intermediate which is then reductively cleaved with the release of ammonia and carbon dioxide.



Such a cyclic peroxide intermediate has been postulated in the pyrocatechase reaction (Hayaishi, 1964).

2) Cleavage of the benzene nucleus

The benzene nucleus of the dihydric phenol is cleaved by a dioxygenase which incorporates both the atoms of a molecule of oxygen into the substrate as cleavage occurs. Two methods of cleavage of the aromatic nucleus by microbial enzymes have been clearly demonstrated:

1) by oxidative fission of the bond between carbon atoms bearing the hydroxyl groups of an ortho-dihydric phenol, for example in catechol and protocatechuic acid. In both the cases, the bacterial oxygenases catalyze the cleavage of the benzene ring of catechol or protocatechuic acid between the two hydroxyl groups to yield cis, cis-muconic acid and -carboxy-cis, cis-muconic acid, respectively. This enzyme has been named "pyrocatechase" (Hayaishi and Hashimoto, 1959). (Fig.II).

FIG. II. BACTERIAL DEGRADATION OF CATECHOL AND PROTOCATECHUIC ACID BY A PSEUDOMONAS SPECIES

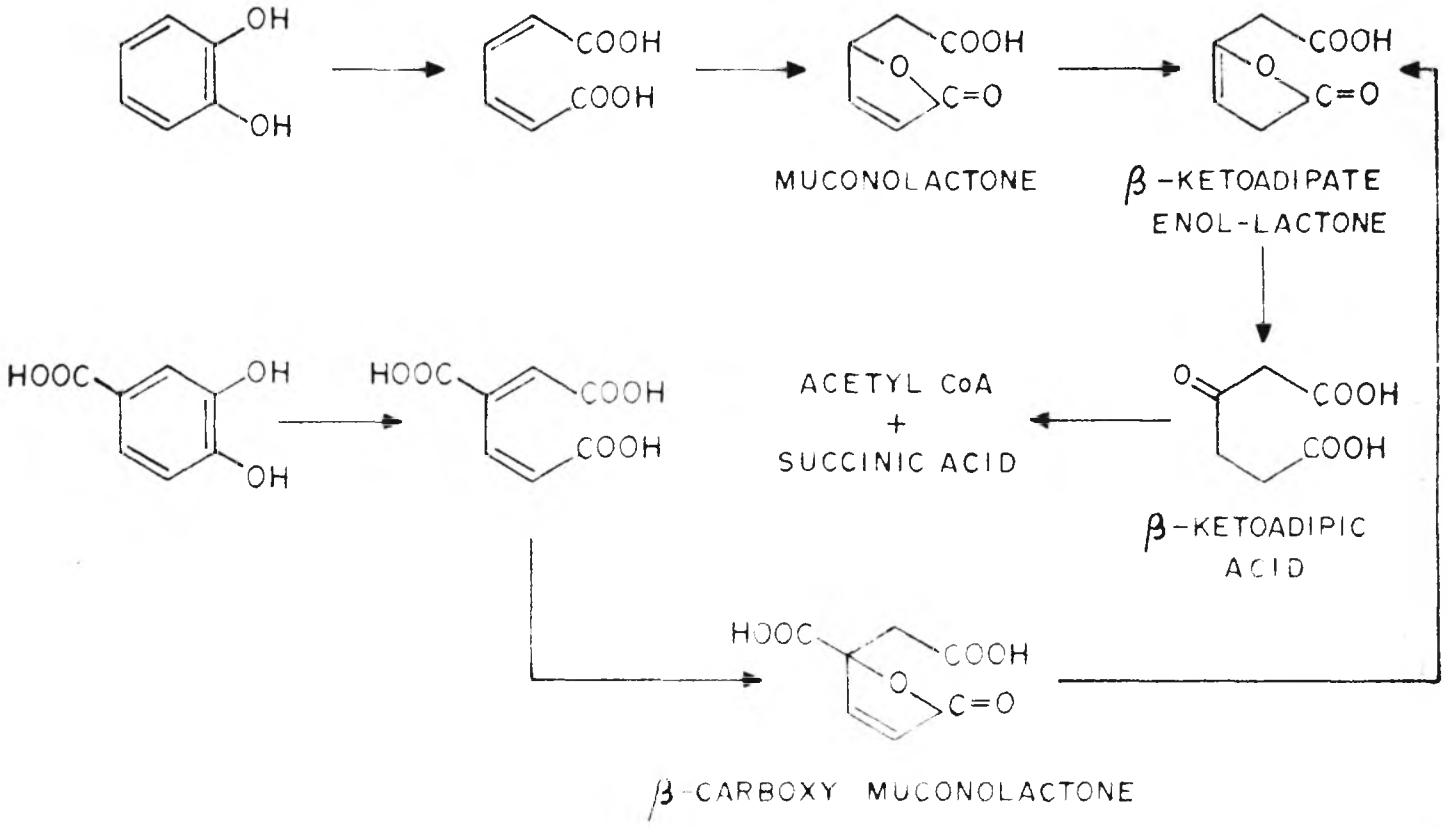
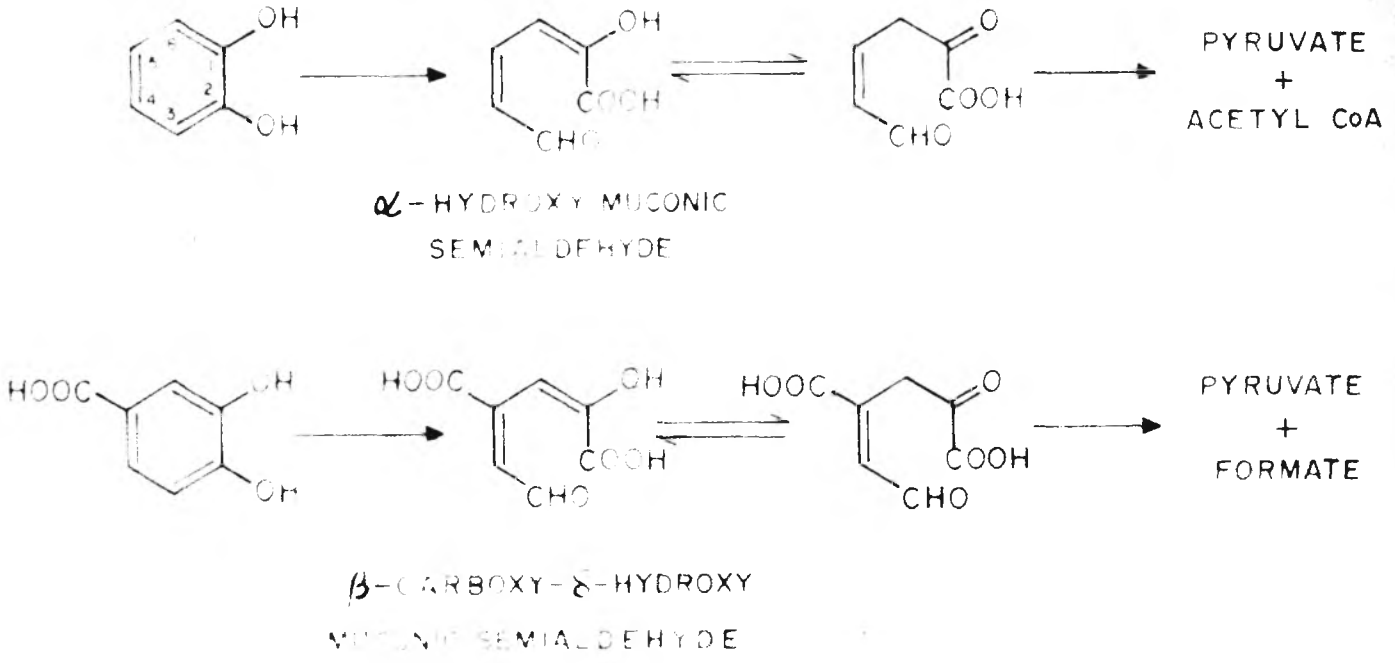


FIG. III. METAPYROCATECHASE-TYPE RING CLEAVAGE OF CATECHOL AND PROTOCATECHUIC ACID



(2) by oxidative fission of the bond between the carbon atom bearing the hydroxyl group and the carbon atom bearing a substituent (R) other than a hydroxyl group (R=H or CH_3 or CH_3-CH_2). This mode of ring fission was discovered by Dagley and Stopher (1959). In this case, as mentioned above, the benzene nucleus is not cleaved between the carbon atoms bearing hydroxy groups, but it opens in the 2,3 position, so that instead of muconic acid, α-hydroxymuconic semialdehyde, which is a half aldehyde, is the cleavage product. Protocatechuic acid is similarly attacked to give β-carboxy- δ-hydroxy muconic semialdehyde. However, this reaction is catalysed by a different oxygenase which does not attack catechol (Fig. III).

The enzyme which is responsible for the cleavage of catechol and protocatechuic acid at the 2,3-position was designated "catechol 2,3-oxygenase" by Dagley et al. (1960) and "Metapyrocatechase" by Hayaishi and co-workers (1961).

There are several reports on aromatic ring fission reactions of the metapyrocatechase type. Adachi et al. (1964) have shown that Pseudomonas ovalis when grown on p-hydroxyphenylacetic acid, cleaves the benzene nucleus of 3,4-dihydroxyphenyl acetic acid between C-2 and C-3 by means of an oxygenase and this enzyme has been crystallized by Kita et al. (1965). Similarly, Dagley and Wood (1965) have also isolated an enzyme of metapyrocatechase type from Pseudomonas which cleaves 3,4-dihydroxyphenylacetic acid between C-4 and C-5 (Fig. IV).

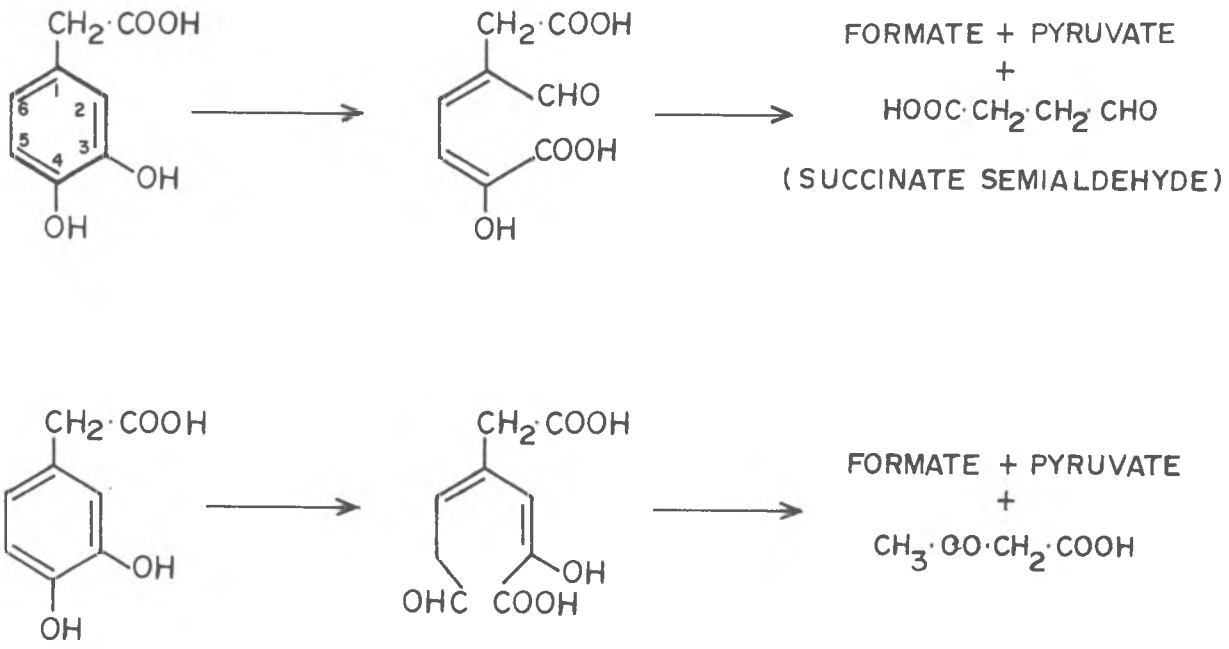
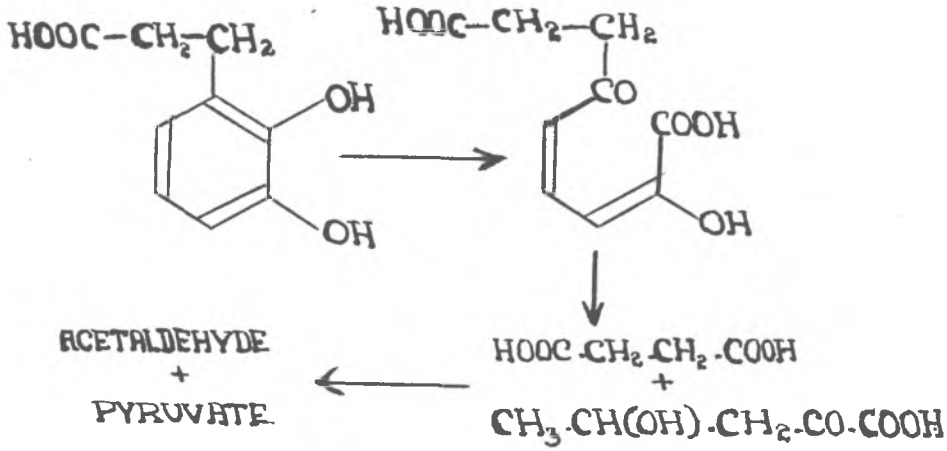


FIG. IV TWO MODES OF CLEAVAGE OF 3,4-DIHYDROXYPHENYL ACETIC ACID

It has been reported that the nucleus of 4-methyl catechol is ruptured between C-2 and C-3 by a ring fission oxygenase from Pseudomonas, whereas, 4-carboxy catechol is cleaved between the carbon atoms 4 and 5 (Dagley et al. 1964).

Dagley et al. (1965a) studying the metabolism of β-phenyl-propionic acid by an Achromobacter have shown that extracts of the cells contain enzymes that catalyze the oxidative fission of the benzene nucleus of 2,3-dihydroxy-β-phenylpropionic acid between the adjacent carbon atoms that bear the side-chain and one hydroxyl group. The cleaved product was then converted into succinate and 4-hydroxy-2-oxovalerate. The

latter compound was also formed when catechol was metabolized by a species of Pseudomonas.



In the bacterial oxidation of 2,3-dihydroxy benzoic acid (Ribbons, 1966) the presence of a new oxygenase was noticed which resembles 2,3-oxygenase or metapyrocatechase in so far as the mode of cleavage is concerned. This Pseudomonas utilises 2,3-dihydroxybenzoate as the sole source of carbon. It has been shown here that 2,3-dihydroxybenzoate is not decarboxylated to catechol by extracts of this strain and catechol itself is not oxidised. The extracts of the cells catalyzed the oxidation of 2,3-dihydroxybenzoate with the consumption of one mole of oxygen and the evolution of one mole of carbon dioxide per mole of the substrate. The formation of an oxo acid identified as its 2,4-dinitrophenylhydrazone which is identical with α-hydroxy muconic semialdehyde was also shown in the above experiment. So, it is assumed here that the oxygenase cleaves the benzene nucleus of 2,3-dihydroxybenzoic acid between the hydroxyl and carboxyl functions and after the cleavage decarboxylation

takes place to yield α-hydroxy muconic semialdehyde.

So the cleavage of the benzene nucleus of orthodihydric phenol is usually brought about by either pyrocatechase or metapyrocatechase type reactions. The nature and mode of attack of these two enzymes, namely, pyrocatechase and metapyrocatechase have been elegantly summarised by Hayaishi (1964).

(3) Further metabolism of the ring cleavage product into smaller carbon fragments

The conversion of either cis-cis muconic acid or -carboxy cis-cis muconic acid to β-ketoadipate is carried out by a lactonizing enzyme and a lactone-splitting enzyme. The recent work of Ornston and Stanier (1964; 1966) has now established that catechol and protocatechuic acid are both metabolised to give β-keto adipate enol-lactone which is then hydrolysed to β-ketoadipate. The final step before the entry of the compounds into the Krebs' cycle was shown by Kilby (1951) to be a C_4-C_2 split of β-ketoadipate to succinate and acetate.

It was noted by Gross et al.(1956) that although Neurospora crassa converts protocatechuic acid into β-oxoadipate, it does so by a different pathway from that used by Pseudomonas species (Fig. Va). They have shown that the enzyme from this mould catalysed the conversion of β-carboxy cis, cis-muconic acid into another lactone i.e., β-carboxy muconolactone which was not metabolized when exposed to cell extracts of Pseudomonas.

FIG. V a. CONVERSION OF β-CARBOXY CIS-CIS MUSCONATE INTO β-OXOADIPATE BY *NEUROSPORA CRASSA*

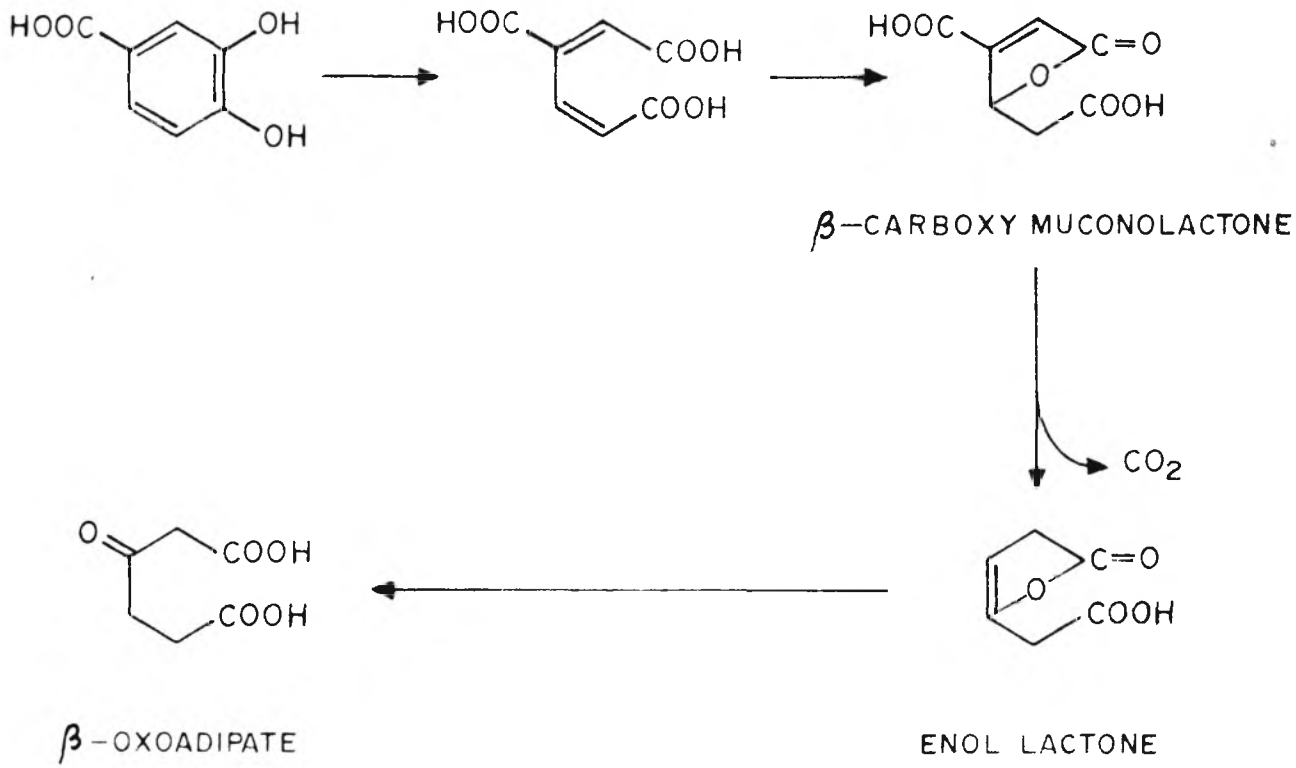
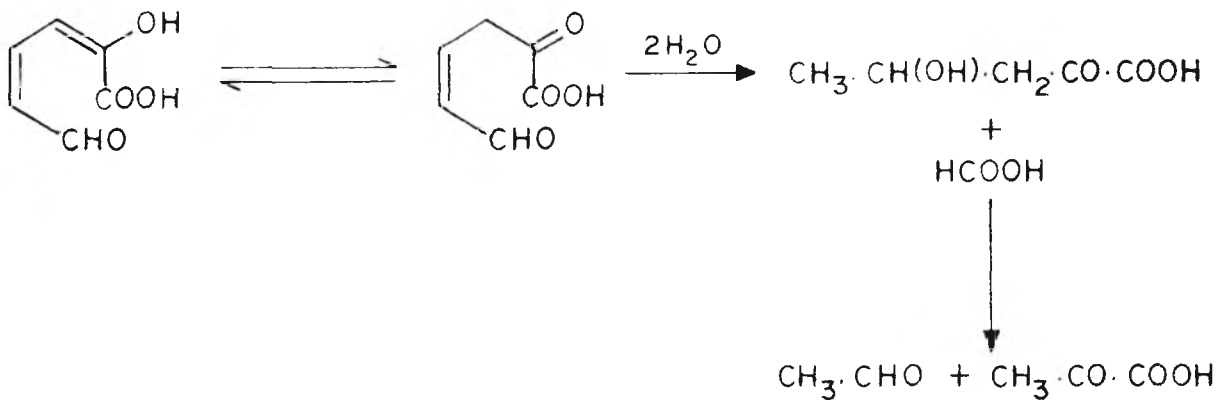


FIG. V b. METABOLISM OF α-HYDROXY MUCONIC SEMIALDEHYDE



Dagley and Gibson (1965) have shown that 2-hydroxy muconic semialdehyde is metabolized to formate, acetaldehyde and pyruvate by the following reaction sequence (Fig. Vb).

Other examples of the oxidative metabolism of benzenoid compounds

Catechol appears to be a common intermediate in the bacterial metabolism of phenol, benzoic acid, anthranilic acid and other aromatic compounds. Wieland et al.(1958) concluded that benzene was oxidized directly to catechol by a Nocardia strain. Similarly, Kleinzeller and Fenel (1953) have reported on the basis of the results of sequential induction experiments that phenol and catechol are the intermediates in benzene degradation.

Dagley and Patel (1957) showed that p-cresol was oxidized by a Pseudomonas species through p-hydroxy benzaldehyde, p-hydroxybenzoic acid and protocatechuic acid and that 2,4- and 3,4-xylenol were oxidized analogously by the same organism. Claus and Walker (1964) were able to isolate two strains of bacteria, a Pseudomonas and an Achromobacter, which utilized toluene, benzene and certain other related aromatic compounds as the sole source of carbon. 3-Methyl catechol, acetic acid and pyruvic acid were detected in toluene-oxidizing cultures. However, Kitagawa (1956) proposed a different pathway for the degradation of toluene by Pseudomonas aeruginosa. According to him oxidation occurs via the intermediates benzaldehyde, benzoic acid and catechol.

In general, it has been noticed that during the metabolism of aromatic hydrocarbons by bacteria catechol or substituted catechols are formed as the last intermediates possessing the aromatic character. This is further cleaved either by a metapyrocatechase or by a pyrocatechase type of oxygenases.

There are a few instances in the metabolism of aromatic compounds with side chain, wherein, the side chain is removed before the cleavage of the benzene nucleus by the stepwise degradation with the formation of either catechol or substituted catechol as one of the key intermediates. This is followed by the fission of the benzene nucleus to yield metabolites of the Krebs' cycle. The conversion of mandelic acid to catechol and p-hydroxy mandelic acid to protocatechuic acid are examples illustrative of side-chain degradation. The metabolism of mandelic acid has been described by Stanier (1948) and the pathway for the degradation of p-hydroxy mandelic acid was established by Gunter (1953). The enzymatic conversion of mandelic acid to benzoic acid was studied by Gunsalus and his co-workers (1953) and the following steps were clearly demonstrated (Fig. VI).

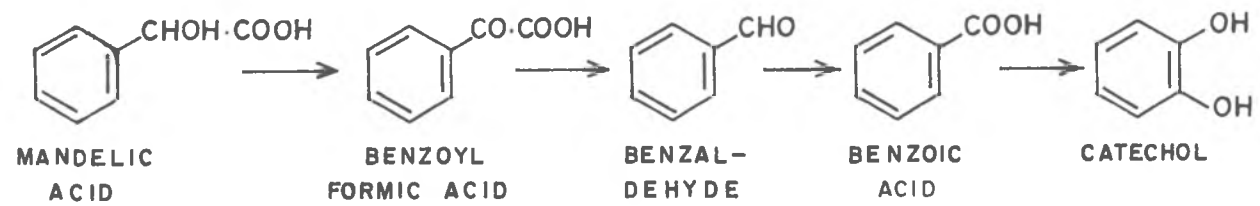
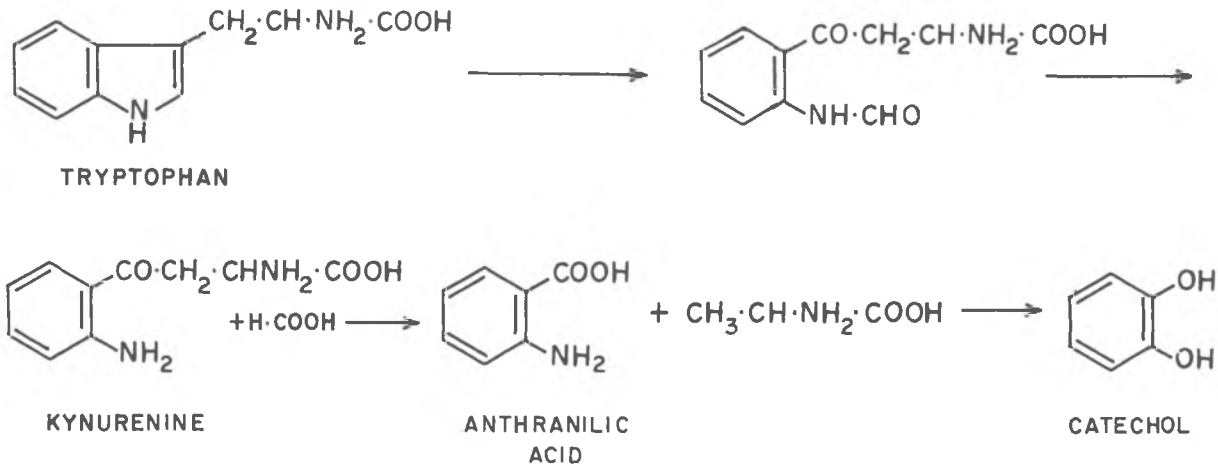


FIG. VI METABOLISM OF MANDELIC ACID

Certain species of *Pseudomonas* convert tryptophan into kynurenine (Hayaishi and Stanier, 1951) and the pathway for the degradation of tryptophan to catechol is as shown below:



CONVERSION OF TRYPTOPHAN INTO CATECHOL

In this particular case, an oxygenase initiates the degradative sequence by cleaving the pyrole ring.

TRANSFORMATIONS OF TERPENES BY MICROORGANISMS

In this connection it will be relevant to present a brief review of the microbial degradation of terpenes which can be visualised to be hydroaromatic compounds. The first report on the degradation of a terpene was by Mayer and Neuberg (1913) who obtained citronellol in 50 per cent yield by reduction of citronellal using bottom yeast. Molinari (1929) achieved with the help of *Azotobacter xylinum*.

The first systematic studies on the microbiological transformation of camphor were carried out by Gunsalus and co-workers (Bradshaw *et al.*1959).

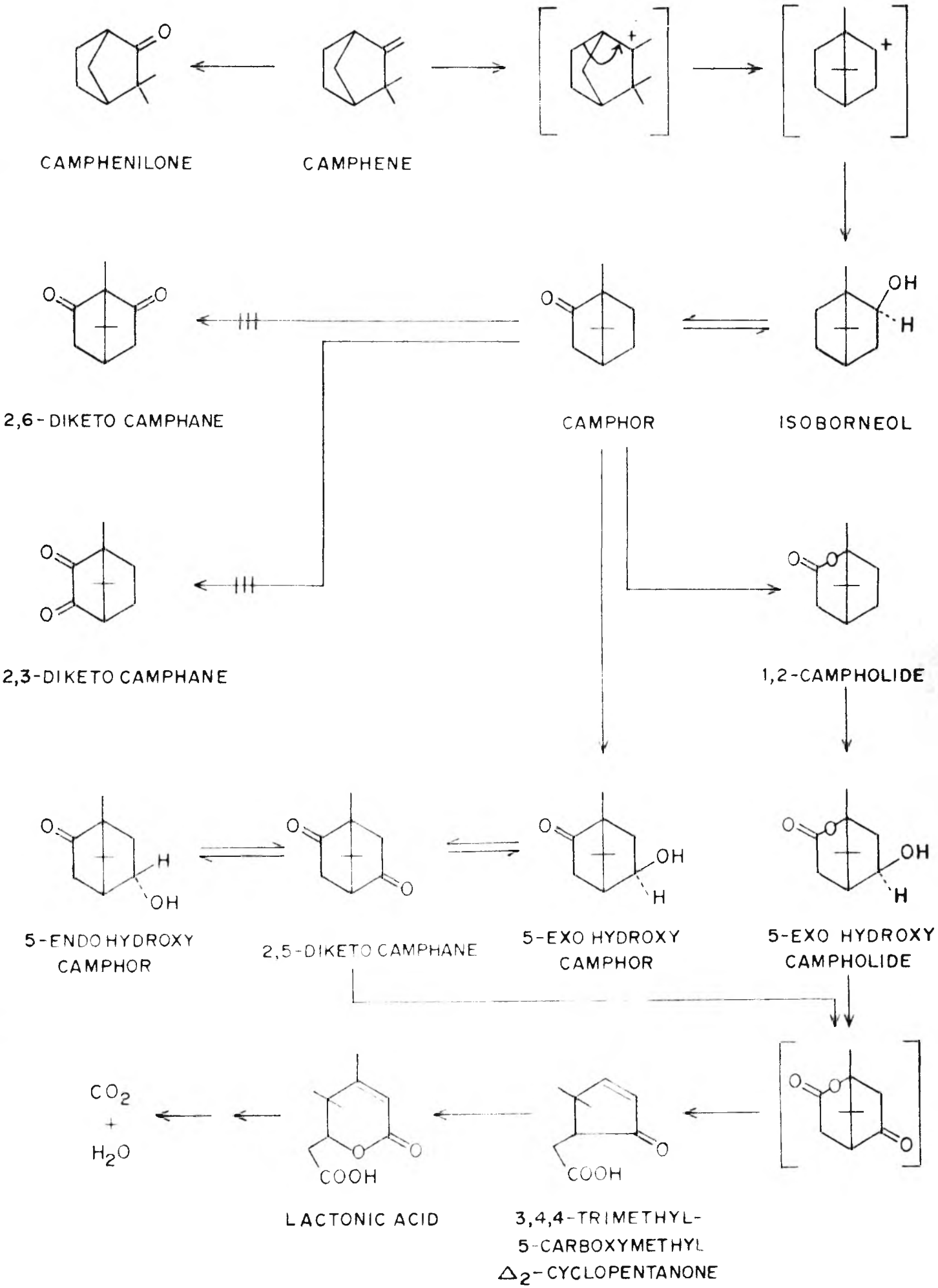
They were able to isolate two strains of Pseudomonas (C_1 and C_5) and a Diphtheroid strain (T_1) which were found to utilise camphor as the sole source of carbon. The complete pathway for the degradation of camphor and the detailed studies of the various enzymes which play dominating role in bringing about the biodegradation of camphor, were studied by several workers (Hedegaard et al. 1961; Conrad et al. 1961a; 1961b; 1962; 1964; 1965a; Baum and Gunsalus, 1962; Gunsalus et al. 1964). Khanchandani (1966) has studied the metabolism of camphene by a Pseudomonas isolated by enrichment technique on camphene as the sole source of carbon and has postulated a catabolic pathway involving isoborneol and camphor as the direct intermediates (Fig. VII) .

Systematic investigations on the microbial transformations of terpenoid hydrocarbons were started in this Laboratory a few years ago by Bhattacharyya and co-workers who studied extensively the degradation of compounds of the pinene series. They isolated a soil Pseudomonas by enrichment culture technique with α-pinene as the carbon source. This particular organism exhibited a certain degree of versatility by growing equally well on limonene, α-pinene, β-pinene, Δ^1-p-menthene and p-cymene.

Pathway for the degradation of limonene

The fermentation of limonene (1) by this strain yielded a number of neutral and acidic compounds which were separated into their components

FIG. VII PROBABLE PATHWAY FOR THE DEGRADATION OF CAMPHENE



and identified. The neutral fractions yielded carvone (2), dihydrocarvone (3), carveol (4) p-menth-8-ene-1-ol-2-one (5), p-menth-8-ene-1,2-diol (6) and p-menth-6-ene-2,9-diol (7). From the acidic fractions perillic acid (8), 2-hydroxy-p-menth-8-ene-7-oic acid (9), isopropenyl pimelic acid (10) and a dihydroxy acid (11) have been isolated and identified (Fig. VIII).

Growth and adaptive enzyme studies indicated that none of the neutral compounds lie on the direct pathway for the degradation of limonene. On the other hand, perillic acid, hydroxy acid and the dicarboxylic acid were found to be oxidized by limonene grown cells. Based on the above observation a pathway for the degradation of limonene has been suggested (Fig. IX). The enzymes responsible for these conversions have also been demonstrated (Bhattacharyya et al. 1964).

The organism which degrades limonene was also found to utilise α- and β-pinene as the carbon source. Shukla and Bhattacharyya (1964) have studied the degradation of these two terpenoid hydrocarbons and have characterised some of the metabolites obtained during the course of the fermentation. From the fermentation of -pinene (+) borneol (12) and myrtenol (13) were identified in the neutral fraction and from the acidic fraction a dicarboxylic acid (14) was isolated. Besides these the presence of myrtenic (15), phellandric (16) and perillic acids (17) were also demonstrated (Fig. X).

FIG. VIII. PRODUCTS OF BACTERIAL DEGRADATION OF LIMONENE

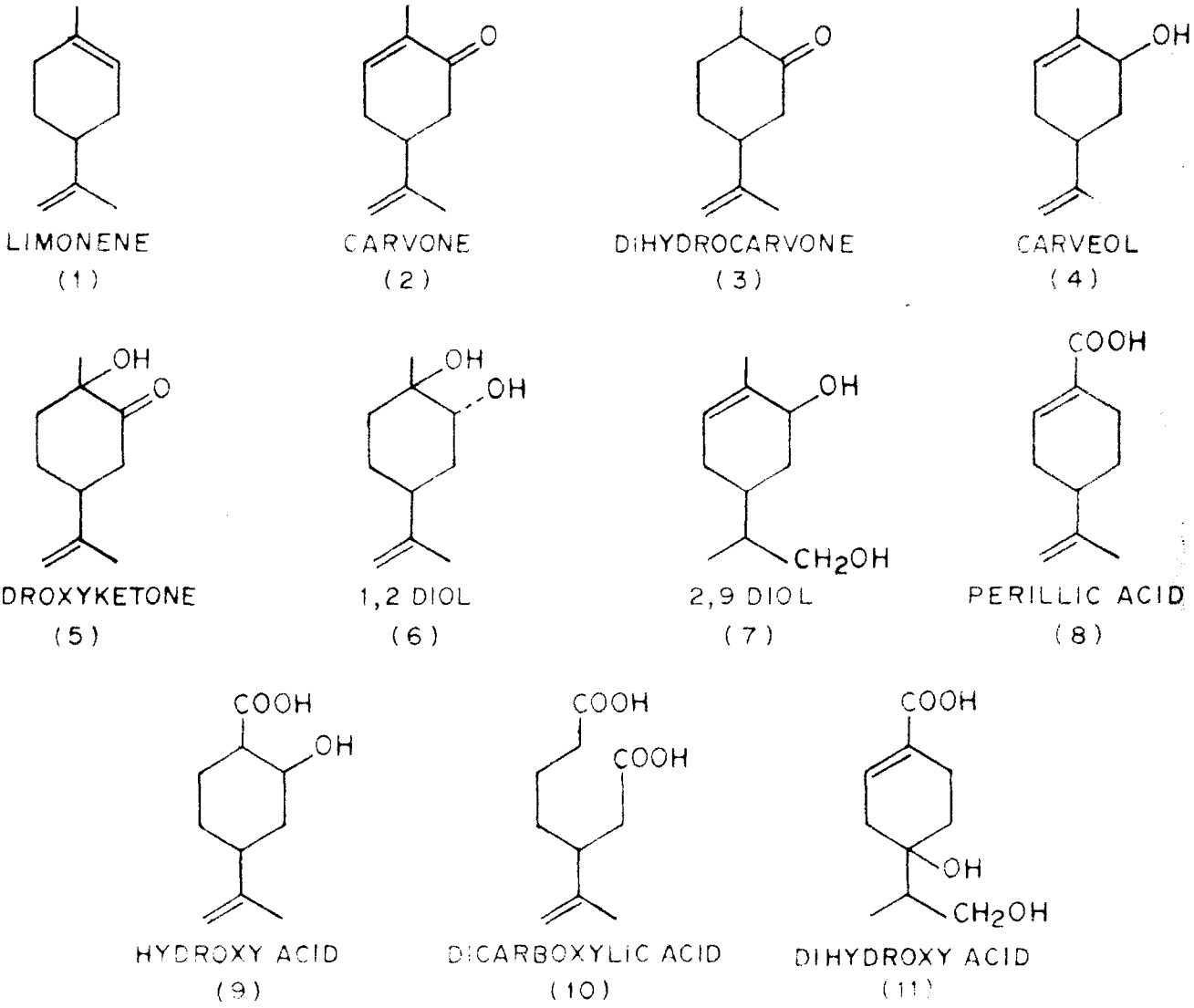
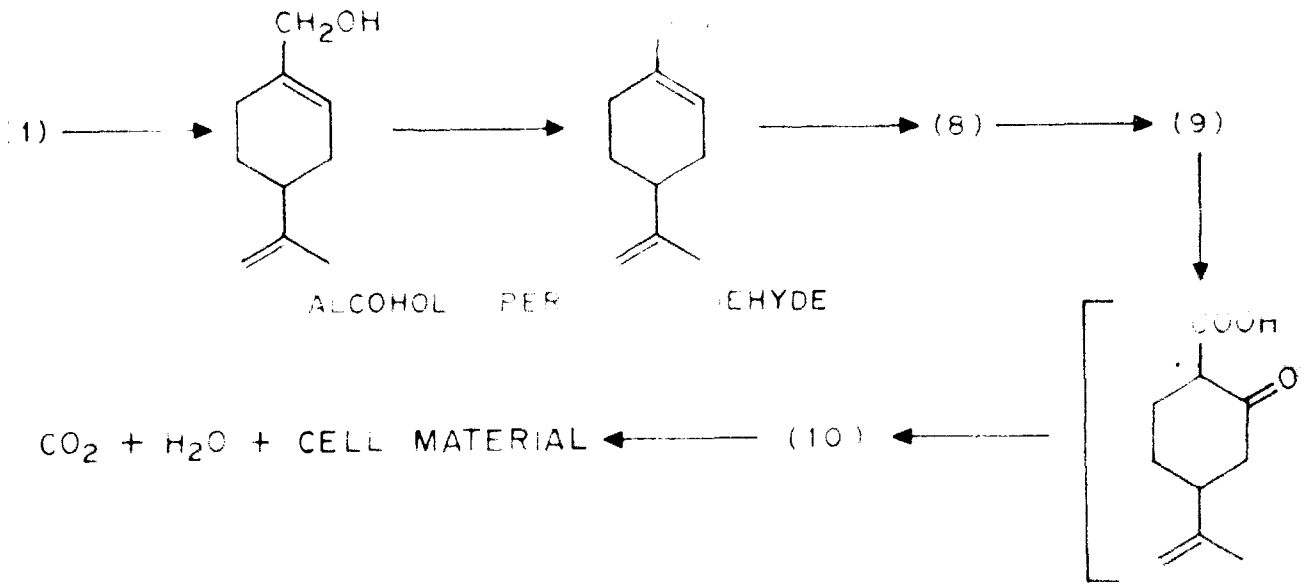


FIG IX. PATHWAY FOR LIMONENE DEGRADATION

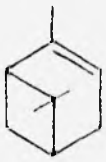


The fermentation of α-pinene gave (-) borneol, the saturated dicarboxylic acid (18) and appreciable amounts of oleuropeic acid (19) (Fig. X).

Adaptive enzyme studies revealed that neither borneol, myrtenol nor oleuropeic acid were oxidized by pinene-grown cells, whereas, the monocarboxylic acid fraction was oxidised very rapidly. The dicarboxylic acid was oxidized at a very slow rate. Growth and adaptive enzyme studies indicated that α - or β-pinene-grown cells are also capable of oxidising limonene and Δ^1-p-menthene with or without added chloramphenicol indicating the constitutive nature of limonene and Δ^1-p-menthene degrading enzymes in these cells. It was also observed that pinene-grown cells rapidly oxidized the probable intermediates in the oxidation of limonene and Δ^1-p-menthene. Based on the above observations a hypothetical scheme for the degradation of pinenes has been postulated (Shukla, 1965).

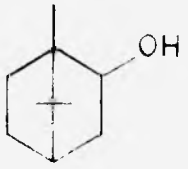
Although complete enzymatic evidence in support of the above pathway is not available, Shukla (1965) has shown the presence of an NAD-alcohol dehydrogenase (for dehydrogenation of perillic alcohol, phellendrol and oleuropeic alcohol) and aldehyde dehydrogenase (for perillic aldehyde, phellandral,[&]/oleuropeic aldehyde) in the cell-free extracts. Ballal *et al.* (1966) have purified perillic alcohol dehydrogenase 10-20 folds and have worked out the cofactor and substrate requirements of this dehydrogenase.

FIG·X· METABOLITES ISOLATED FROM α- AND β-PINENE FERMENTATION

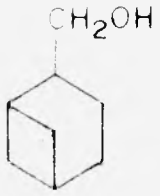


α-PINENE

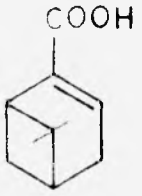
β-PINENE



(12)



(13)



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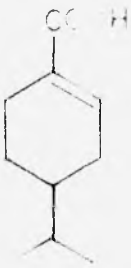

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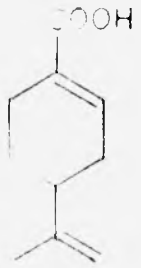

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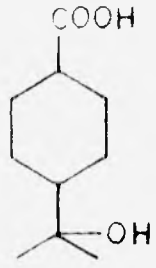
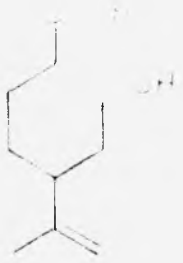


(19)

PERILLIC ALCOHOL



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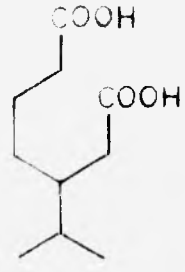
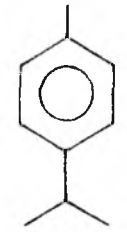


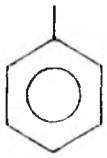
(18)

The organism which ntilizes α and β-pinene, limonene and Δ^1-p-menthene as the sole source of carbon, is very versatile in nature. Also it has been noticed that the organism is capable of adapting more rapidly to an aromatic hydrocarbon, p-cymene. This offered an unique opportunity of testing a large number of easily accessible model compounds as substrates and deriving more information regarding the structural features essential in a compound before it can serve as a growth substrate for this bacterium. Shukla (1965) has reported that this organism grows freely on 4-sec-butyltoluene (20) and 4-n-propyl toluene (21). But it took sufficiently longer time to adapt itself to 1-methyl-4-ethyl-benzene (22) and 4-isopropenyl toluene (23), whereas,4-isobutyl toluene (24) and 4-tert butyl toluene (25) were very poor growth supporters. It seems probable that the compound 1-methyl-4-ethyl benzene (22) may possess the basic minimum requirement before it can be acceptable as a growth substrate for the organism. None of the compounds (Fig. XI) tested was capable of supporting growth of the organism. It was concluded from these studies that the introduction of an extra methyl group in positions 2 or 3 in the p-cymene molecule renders the compound unacceptable as a growth substrate to the organism. The presence of an oxygen function at position 8 resulted in a total loss of growth-promoting activity (Shukla, 1965, Shukla et al. 1965).

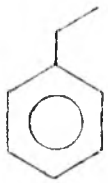
FIG. X] AROMATIC MODEL COMPOUNDS



p-CYMENE



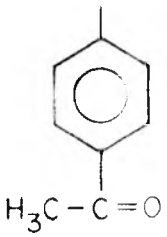
TOLUENE



ETHYL BENZENE

CUMENE

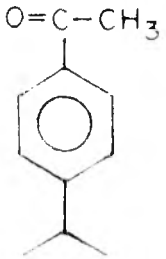
p-XYLENE



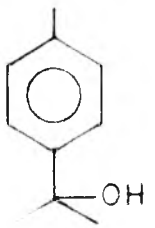
4-METHYL ACETO-PHENONE

1,4-DIETHYL BENZENE

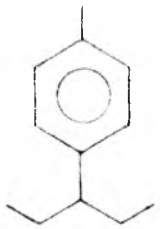
ETHYL-p-ISOPR. BENZENE



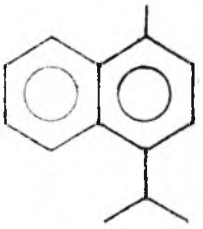
4-ISOPR ACET PHENONE



METHYL,p-TOLYL CARBINOL

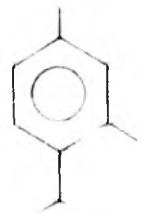


1-METHYL-4-(1 ETHYL-PROPYL) BENZENE

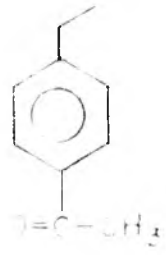


APOCADALENE

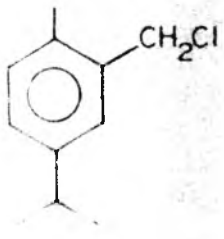
2-METHYL p-CYMENE



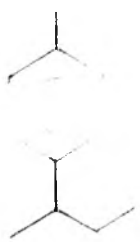
3-METHYL,p-CYMENE



p-ETHYL,ACETO PHENONE



2-CHLORO METHYL, p-CYMENE



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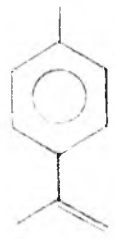
(21)

(22)



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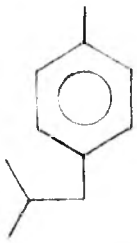

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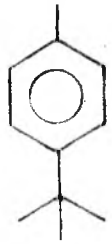


(25)

PRESENT STUDIES

The ability of the Pseudomonad, 'PL-strain', isolated in this Laboratory to grow on p-cymene poses an interesting problem in ring degradation. It has been established that the same organism finally degrades the alicyclic hydroaromatic rings in compounds such as limonene and Δ^1-p-menthene through hydration of a double bond. Obviously the same hydration process is expected to require much higher energies in aromatic compounds as compared to the alicyclic hydrocarbons. Regarding p-cymene degradation no data is available in literature on the microbial metabolism of this hydrocarbon when provided as the sole source of carbon. However, Davis and Raymond (1945) have reported on the oxidation of p-cymene by a Nocardia species when this hydrocarbon was added after the organism was initially grown on n-alkanes and under these conditions, p-cymene was oxidized to give cumic acid. Handler and Perlzweig (1945) have also reported the oxidation of p-cymene to cumic acid when fed to sheep. It had also been reported earlier by Mathews (1924) that certain soil bacteria can obtain their energy and carbon requirement from p-cymene. He claimed that the introduction of a methyl group into the benzene ring rendered the resulting compound more susceptible to bacterial oxidation probably because of the greater potential energy of the methylated compound. Furthermore, as against the enormous data accumulated in

the field of bacterial degradation of several polynuclear aromatic and substituted benzenoid compounds such as, salicylic acid, catechol, naphthalene, anthranilic acid and protocatechuic acid, relatively no information is available as to the exact mechanism by which p-cymene is degraded into smaller fragments. The present studies were, therefore, undertaken to investigate the degradation of p-cymene by the 'PL-strain' and to ascertain the exact position of the cleavage of the benzene nucleus of the dihydroxyphenol by the oxygenase. The results obtained during the course of these investigations are presented in this thesis.

Chapter II

MATERIALS AND METHODS

MATERIALS AND METHODS

All chemicals used for the preparation of media were of chemical grade and those used for enzymatic purpose were of analytical reagent grade. p-Cymene was obtained from M/s. Riedel de Haan Ltd., Germany, and was further purified by fractional distillation at atmospheric pressure. The fraction distilling between 165-68°/710 mm was collected and further purified by refluxing over metallic sodium and then distilled. Based on VPC analysis p-cymene used in these studies was 99.5% pure. Neutral alumina Grade-I for column chromatography and silica gel for thin layer chromatography were obtained from the Fine Chemicals Project, National Chemical Laboratory.

Maintenance and propagation of culture

The organism was isolated from soil and was found to be Gram-negative motile rods having characteristics of *Pseudomonas* species. This organism growing on p-cymene as the sole source of carbon was propagated on a nutrient agar slant of the following composition (Mackie & McCartney, 1949).

Peptone (Armour)	1.0 g
Sodium chloride (B.D.H.)	0.5 g
Yeast extract (Oxoid)	0.5 g
Beef extract (Difco)	0.5 g
Agar	2.0 g
Total volume	100 ml

The first four constituents were dissolved in water and the pH

was adjusted to 7.0 with 4N sodium hydroxide. The final volume was then made to 100 ml. Two per cent agar (Algaden or Difco) was added and the mixture was steamed for one hour. For preparation of slants 6-7 ml aliquots were distributed into 19 X 150 mm Pyrex test tubes, autoclaved at 15 p.s.i. (120°) for 20 minutes and were slanted. The slants were resteamed for one hour to destroy any germinated spores and were reslanted again. These slants were inoculated either from broth or stock culture and incubated at 28° for 24 to 48 hr.

For purification of culture by streaking method, nutrient agar of the above composition was poured aseptically while hot in previously autoclaved (20 p.s.i. for 1 hr) petri dishes. The culture can be propagated even on glucose-mineral salt-agar slants. The composition of the mineral salt is described below and to this 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°). For routine work the culture was maintained by regular transfers in liquid culture.

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 at a favourable hydrogen-ion concentration. So the organism was grown in a chemically-defined medium as described by Seubert (1960) having the following composition:

1) Dipotassium hydrogen phosphate (Danpha grade, India)	.....	63.0 g
2) Potassium dihydrogen phosphate	.....	18.2 g
3) Ammonium nitrate (E.Merck)	.....	10.0 g
4) Magnesium sulphate (B.D.H.) anhydrous	.....	1.0 g
5) Calcium chloride ($CaCl_2.2H_2O$ - E.Merck)	...	1.0 g
6) Ferrous sulphate ($FeSO_4.7H_2O$ - B.D.H.)	.....	1.0 g
7) Manganese sulphate (Riedel de Haan)	.....	0.006 g
8) Sodium molybdate (B.D.H.)	.....	0.006 g

Made up to 10 litres, pH 7.0.

All the constituents were dissolved separately excepting the phosphates which were dissolved together and diluted to 6 litres. Solutions of other mineral salts were mixed together, added to phosphate solution and the total volume was made upto 10 litres. Aliquots of 100 ml medium were taken in 500 ml Erlenmeyer flasks and were autoclaved at 15 p.s.i. for 20 minutes. The flasks were then inoculated under aseptic condition with 1-2% inoculum and 1 ml of the respective carbon sources were added to each flask. The flasks were incubated at 28°±1 on a rotary shaker at 220 r.p.m. for 24-72 hr.

Extraction and separation of compounds:

At the end of the incubation period (24-72 hr), the flasks were pooled together and the broth was then acidified with 6N hydrochloric

acid and extracted thrice with ether. The ether layers were pooled together and shaken with hyflo supercel to remove the suspended cells. The clear yellow broth and the supercel cake containing the cells were extracted separately with n-butanol.

The ether and n-butanol extracts were separated into neutral (N_1 and N_2) and acidic (A_1 and A_2) fractions by washing with aqueous 5% sodium carbonate. The acids were regenerated from sodium carbonate layer by acidification and were extracted with ether. The neutral fractions were further subjected to a four-transfer modified Craig distribution between n-hexane and 90% aqueous methanol (Prema and Bhattacharyya, 1962). The polar compounds from the methanolic layers were further purified either by column chromatography or by preparative TLC methods. The purity of the fractions were established both by TLC and VPC techniques.

The acids were converted to their corresponding methyl esters by treatment with diazomethane in ether. The methyl esters were then purified by chromatography over alumina (neutral Grade I) and then by preparative TLC in hexane:ethylacetate, 90:10 (System I). The separation of different acids in the mixture was also tried using silica gel partition column as well as by fractional crystallization. The separation and identification of these compounds were monitored by various physico-chemical methods such as TLC, VPC and spectroscopic methods.

The extraction procedure and the methods of separation are summarised in Fig. XII.

Chromatography

Thin layer chromatography was carried out with 85% silica gel (supplied by the Fine Chemicals Project of this Laboratory) using 15% plaster of Paris as the binder.

Resolution of the neutral fraction and the methyl esters of acids was achieved by using hexane:ethylacetate, 95:5 (System I), 90:10 (System II) or 85:15 (System IV) as the solvent systems. For acidic compounds a mixture of benzene;methanol:acetic acid 45:8:4 (System III) was generally used. The compounds were detected either by spraying the plate with concentrated sulphuric acid and developing the spots by heating for 10 to 15 min at 110-160° or by keeping the plate in an iodine chamber.

Identification of 2,4-dinitrophenylhydrazones of oxo-acids and neutral carbonyl compounds by TLC and paper chromatography

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

Solvent systems for oxo-acids

1) Benzene:tetrahydrofuran:acetic acid (60:36:4) (System V).

EXTRACTION PROCEDURE

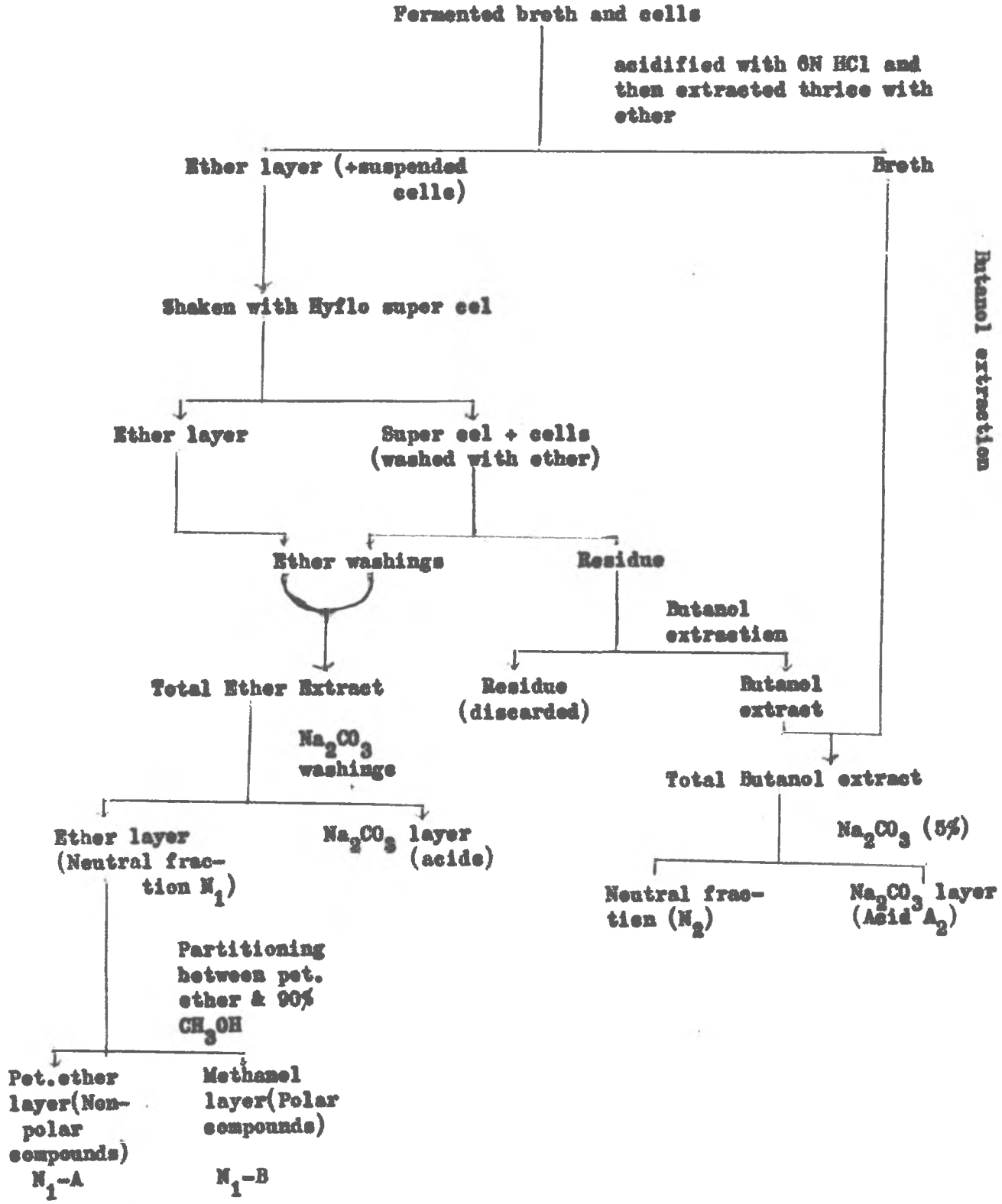


FIG. XII

Solvent system for neutral carbonyl compounds

Benzene:tetrahydrofuran 60:40 (System VI).

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-dinitrophenylhydrazine solution

2,4-Dinitrophenylhydrazine (100 mg) was dissolved in 100 ml of 2N hydrochloric acid (approx.). The solution was filtered and kept in refrigerator when not in use.

Growth Studies

In the experiments where growth pattern of the organism was studied on different compounds, the cells adapted to p-cymene were used. p-Cymene grown cells were freed from adhering substrates by centrifugation, washed with 0.05 M phosphate buffer and resuspended in sterile medium. Centrifugations and washings were done under aseptic conditions. Suitable aliquots of 2 ml cell suspension with a turbidity reading of 250-260 (Klett readings) at 660 mμ (Red filter) were inoculated in flasks containing the respective substrates (100 to 200 mg) and incubated in the usual manner.

Turbidity measurements for growth studies were recorded either visually or colorimetrically at 660 mμ with a Klett-Summerson colorimeter using a flask reported by Shukla (Shukla, 1965).

Manometric Studies

Measurements of oxygen uptake by cell preparations were carried out manometrically (Umbreit et al. 1959) using Warburg flasks (25 ml capacity) with a single side arm. Each flask in a total volume of 3 ml contained 1.5 ml cell suspension corresponding to 1-2 mg (dry wt.) cells, 1 ml of 0.05 M phosphate buffer, pH 7.0, 0.2 ml of 20% potassium hydroxide in the central well and water to make the total volume 3 ml. The substrates were taken in the side arm and added by gradual tipping. Temperature of the bath was maintained at 28°±0.2 and the flasks were shaken at 110 strokes per min. Cells for manometric studies were harvested by centrifuging at 15,000 X g for 30 min at 0-4°, washed twice with 0.05 M phosphate buffer, pH 7.0 and resuspended in the same buffer for the respiration experiments.

Preparation of cell-free extracts from p-cymene grown cells

Freshly grown cells (24 hr) were harvested and washed twice with 0.05 M phosphate buffer. The harvested cells were resuspended in phosphate buffer, 0.05 M, pH 7.0 and 10 ml of buffer was used for each 1 g of cell paste. It was then subjected to sonication (Raytheon Magneto striction sonic oscillator, Model DF 101, KC-10, 250 W) for 10-12 min and the sonicate was centrifuged at 15,000 X g for 45 min. The supernatant was used for alcohol dehydrogenase and aldehyde dehydrogenase assays. The same supernatant was employed to show the presence of hydroxylating and cleavage enzyme systems. These extracts usually contain 10-15 mg of

protein per ml.

Protein concentrations were estimated according to Warburg and Christian equation (1941) for the estimation of protein, based on the extinction coefficients at 280 and 260 mμ.

$\frac{4}{7}$ 2.3 X (optical density at 280 mμ)-(optical density at 260 mμ) = mg protein per ml of solution

Substrates used in the present studies

Benzoic acid, benzaldehyde, p-cresol, salicylaldehyde, salicylic acid, m-toluic acid, p-toluic acid, orcinol and catechol were products of B.D.H. and E. Merck. All these compounds were further purified either by distillation or by crystallization. 3,4-, 2,6-, 2,4-, 2,5-, 3,5-, Dihydroxybenzoic acids were kindly supplied by Dr. K. Ganapathy, Portland, Oregon, U.S.A. Besides these several other compounds were used and their syntheses are given below.

2,3-Dihydroxy benzoic acid and 2,3-dihydroxy terephthalic acid

These two acids were prepared by carbonation of a mixture of anhydrous potassium carbonate and catechol (Baine et al. 1954). Anhydrous potassium carbonate and dry catechol were mixed in the ratio 3:1 and the mixture was heated to 175° for 4 hr under a carbon dioxide pressure of 1200 to 2000 p.s.i. The reaction mixture after acidification gave mostly 2,3-dihydroxy benzoic acid (70% yield) and little amount of 2,3-dihydroxy terephthalic acid. The mono- and di-carboxylic acids were separated by fractional crystallization from water. The mono-carboxylic acid being

much more soluble than the dicarboxylic acid. 2,3-Dihydroxy benzoic acid was recrystallized from water to a m.p. 202-203°(literature 203-205°, Cason and Dyke, 1950). The above reaction when carried out at 225° for 8 hr mostly 2,3-dihydroxy terephthalic acid (80% yield) was obtained. The product was crystallized from aqueous alcohol. It melted with decomposition at about 270° (literature 270°, Cason and Dyke, 1950).

2-Hydroxy cumic acid

All attempts to prepare 2-hydroxy cumic acid by the selenium dioxide oxidation of carvacrol were not successful. Finally it was prepared by the alkali fusion of carvacrol. However, the yield was very poor by this method. One part of carvacrol was mixed with 3 parts of potassium hydroxide and the mixture was heated to 400° for 3 hr. The fused product was dissolved in water and on acidification yielded 2-hydroxy cumic acid (30% yield). It was crystallized from hot water, m.p. 95-96° (literature, m.p. 96-97°, Heymann and Königs, 1886).

Isopropyl pyruvic acid

Six grams of leucine were suspended in 75 ml of water containing 15 ml of concentrated sulphuric acid. The mixture was cooled to 0° and then it was diazotized with a solution of sodium nitrite (3.12 g) in 10 ml of water. The diazotised solution was then refluxed for an hour after adding 5 ml of concentrated sulphuric acid. The reaction mixture was then saturated with ammonium sulphate and extracted

with ether. The residue (4g) obtained from the ether extract was taken in dry acetone (50 ml) and subjected to Jones' oxidation according to the procedure of Djerassi et al (1956). After 4 hr, the reaction mixture was diluted with water, filtered, excess of acetone present was removed under suction and then extracted with ether. The ether layer on evaporation yielded 2 g of isopropyl pyruvic acid, b.p. 84-5°/15 mm.

2-Hydroxy p-cymene (Carvacrol)

The first step was the preparation of barium cymene-2-sulphonate prepared according to Feurs (1934).

The second step involved the alkali fusion of barium cymene-2-sulphonate. Three parts of potassium hydroxide was mixed with one part of barium cymene-2-sulphonate and the mixture was heated to 320° in a bath consisting of an eutectic mixture of sodium and potassium nitrates with a fusion period of one hour. After the fusion the alkaline mixture was dissolved in water, acidified and extracted with ether. The ether layer yielded carvacrol, b.p. 234-236° (literature b.p. 237-238°, Wagner, 1928).

2,3-Dihydroxy cumic alcohol

2,3-Dihydroxy cumic acid methyl ester (200 mg) was reduced with lithium aluminium hydride (600 mg) as described by Nystrom and Brown (1947). At the end of the reaction period (about 2 hr refluxing) the mixture was processed as usual. This compound was freed of minor impurities by passing

over an alumina column (Grade I, 20 times). The column was washed with ether. The compound (40 mg) was eluted with methanol.

Cumic alcohol (27)

This was prepared by reducing cumic acid (300 mg) with lithium aluminium hydride (600 mg) as described earlier. The compound (27) distilled at 270° (bath temp.). The NMR spectrum (Fig.XLIII) indicated the presence of isopropyl group (doublet at 1.23δ), proton of tertiary carbon (2.85δ), protons of hydroxymethylene group (doublet at 4.4δ) and four aromatic protons (singlet at 7.12δ).

Cumic aldehyde (38)

Cumic aldehyde was prepared by oxidation of cumic alcohol in presence of chromium trioxide-pyridine complex as described by Poos et al. (1953).

The preparation of α-p-tolyl propanol (9-hydroxy-p-cymene) and 3-isopropyl catechol have been dissussed in Chapter III.

All the melting and boiling points reported are uncorrected. The infrared spectra were recorded on a Perkin-Elmer Infracord model 137B and E er on a Perkin-Elmer model 221 in liquid film or in nujol. All NMR spectra were taken on a Varian Associates A-60 spectrometer using carbon tetrachloride as solvent and tetramethyl silane as the internal reference standard.

Chapter III

FERMENTATION OF p-CYMENE

FERMENTATION OF p-CYMENE

DISCUSSION

The pseudomonad, "PL-strain" isolated by enrichment culture technique on α-pinene as the carbon source was used in the course of the present studies. This has been found to grow freely on glucose, sucrose, citrate, succinate and other members of the tricarboxylic acid cycle with or without lag phase. Besides these compounds it also utilises a number of terpenoid hydrocarbons, such as α-pinene, β-pinene, limonene and Δ^1-p-menthene (Shukla, 1965). It is interesting to note that the same strain grows remarkably well on the aromatic hydrocarbon, p-cymene, in a mineral salt medium at rates comparable to those on glucose. In order to elucidate the catabolic pathway of this hydrocarbon, large scale fermentations were carried out to identify the various metabolites formed during the course of the fermentation. Optimum conditions were established from different sets of fermentations using the pseudomonad "PL-strain". The rate of growth of the bacterium was practically steady between 27°-37°. However, a temperature of 28° was chosen arbitrarily with a view to minimizing the losses due to evaporation. The organism exhibited a pH optimum of 6.8 to 7.2 and for the fermentations a neutral pH (7.0) was chosen. It was found that p-cymene appeared to be non-toxic up to a level of 1.5% in the medium (Table 1). As there was practically no difference in the growth rate of the organism at

concentrations of 1.0 and 1.5%, an initial level of 1.0% p-cymene was chosen for the fermentations. It is appropriate to mention in this connection that when the initial concentration of the substrate was between 0.8-1.5% the culture medium developed a pale pink colour, possibly an indication of the accumulation of phenolic compounds. However, on reducing the concentration below this level the pink colour was not observed.

The quantity of neutral products formed at any time was quite insignificant as compared to the acidic products which increased considerably with the incubation period, although the amount of neutral products formed was comparatively more in the initial stages (Table ii). However, the accumulation of different acids in the culture medium depended mainly on the period of incubation. In a 24-hr fermentation, cumic acid was the major product of accumulation while in a 72-hr fermentation, 2,3-dihydroxy cumic acid was the major component. With prolonged incubation i.e., beyond 72 hr, considerable lyses of the cells were observed. Different sets of fermentations were carried out at different time intervals of 24 and 72 hr in order to accumulate the two major acids in fairly large quantities. The amount of acidic and neutral products formed during the course of fermentations with different time intervals is listed in Table ii.

Isolation and identification of metabolites

The extraction procedure and the separation of metabolites into polar and non-polar neutral and acidic products have been schematically outlined earlier in the chapter on "Materials and Methods"(Fig.XII). Briefly, the fermented broth along with the cells was acidified and extracted with ether and n-butanol. The aqueous layer was not subjected to continuous extraction with ether (Shukla, 1965) since the residue obtained by this procedure was too small to carry out any chemical work. The ether and the n-butanol fractions were then separated into their acidic and neutral constituents to obtain the following major fractions:

a) Ether extract: less polar neutral (N_1) and acidic fractions (A_1);

b) n-Butanol extract: more polar neutral (N_2) and acidic fractions (A_2).

Examination of each of these fractions by TLC revealed the presence of at least four to six components. These four fractions were subjected to solvent-solvent partition, chromatographic methods and fractional crystallization (in the case of acids) for the isolation of individual metabolites.

N_1 (less polar neutral) fraction

Chromatography of the fraction N_1 over neutral alumina (Grade I) gave four fractions, namely, N_1-a, N_1-b, N_1-c and N_1-d which were eluted by solvents of increasing polarity.

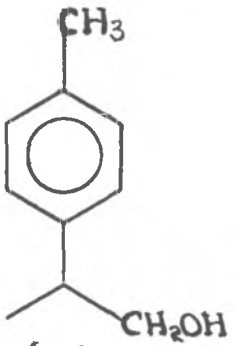
Fraction (N_1-a) eluted with petroleum ether (40-60°) was found

to contain unreacted p-cymene and fatty materials. Examination of this fraction in System I indicated the presence of two compounds of varying polarity; the relatively non polar compound corresponded to p-cymene in mobility pattern. The polar compound was separated from the mixture by careful rechromatography over neutral alumina (Grade I). The IR spectrum of this compound showed the presence of a strong absorption due to aliphatic CH stretching (γ_{max} 2950 cm^{-1}), an ester band (γ_{max} 1760 cm^{-1}), an ether linkage (γ_{max} 1160 cm^{-1}), a characteristic absorption for the presence of a long aliphatic chain with more than four methylene groups (γ_{max} 730 cm^{-1}) and the absence of any hydroxyl group. This suggested that the compound may probably be an ester of a fatty acid.

Fraction (N_1-b) eluted with benzene was also found to be a mixture of two components of very close mobilities in TLC (System I) and could not be resolved further by chromatographic methods. One of the components, however, corresponded in mobility pattern with that of the fatty acid ester obtained in the petroleum ether fraction (N_1-a).

The (N_1-c) fraction eluted with ether was found to comprise most of the major neutral metabolites. Preliminary examination by TLC (System II) showed the presence of three components. The metabolite with R_F 0.32 was isolated in a pure form by preparative TLC. This compound distilled at 155-60°/30 mm (bath temp.) and analysed for $C_{10}H_{14}O$. The IR spectrum (Fig.XIIE) exhibited a hydroxyl absorption (γ_{max} 3450, 1045 cm^{-1}).

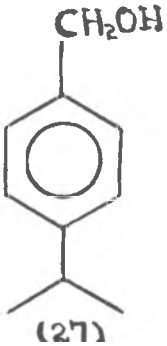
The absorption at 820 cm^{-1} probably indicates 1:4 substitution pattern on an aromatic nucleus. The NMR spectrum (Fig.XIV) indicated the presence of four aromatic protons (singlet of 4 proton intensity at 7.1 δ), the protons of hydroxy methylene group (a doublet of 2 proton intensity at 3.48 δ), proton of tertiary carbon (multiplet of one proton intensity around 2.8 δ), one hydroxyl proton (at 2.57 δ) which disappeared on D_2O exchange, an aromatic methyl (singlet of 3 proton intensity at 2.3 δ) and a secondary methyl (a doublet of 3 proton intensity at 1.17 δ). The above data indicated that p-cymene has undergone hydroxylation and the analysis of NMR spectra corroborated the structure, α-p-tolyl-propyl alcohol (26).



(26)

The IR spectrum of the compound was superimposable with that of the authentic sample of α-p-tolyl-propyl alcohol (Fig.XV).

From this fraction (N_1-e) a second neutral compound was identified by VPC as cumic alcohol (27). The observed peak intensity was enhanced



(27)

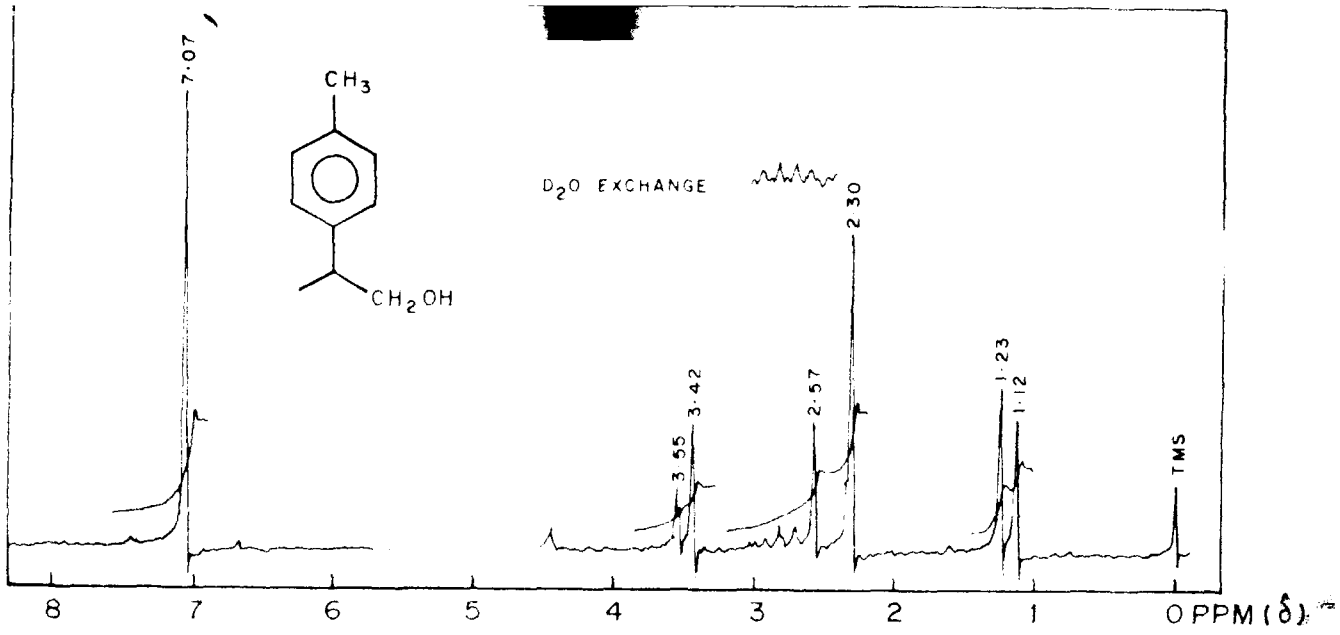


FIG XIV

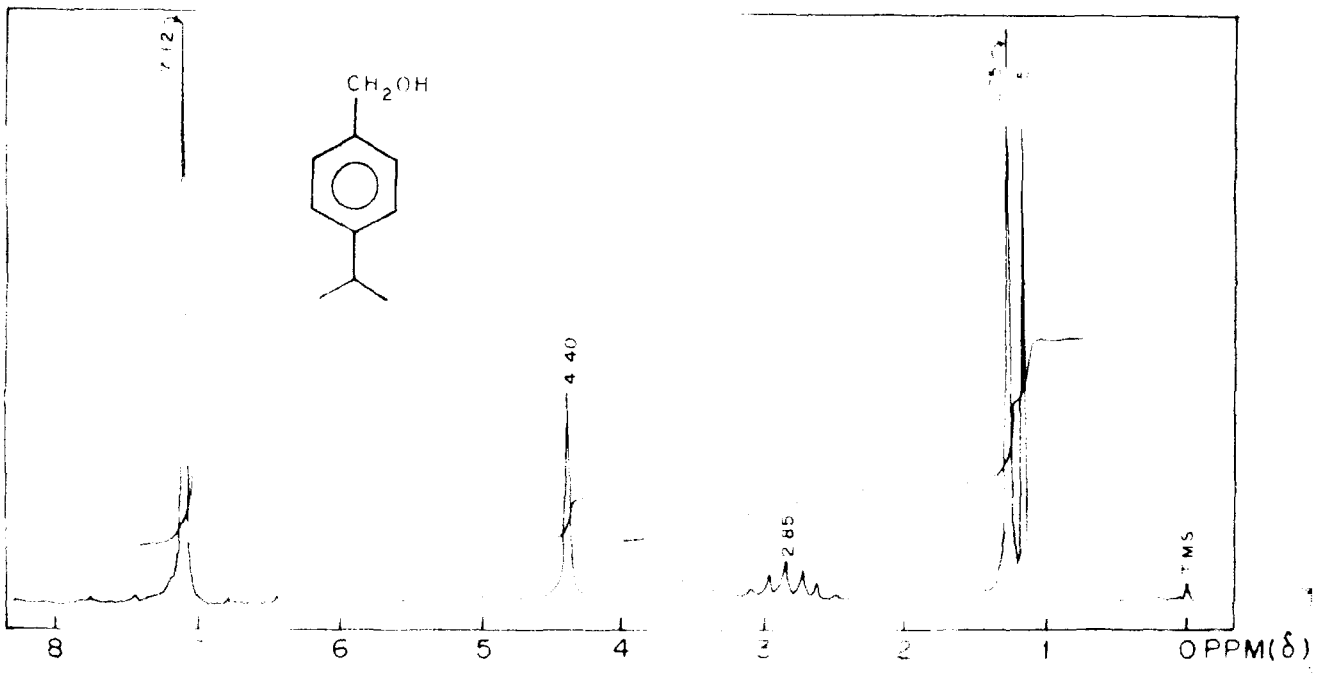


FIG. XLIII

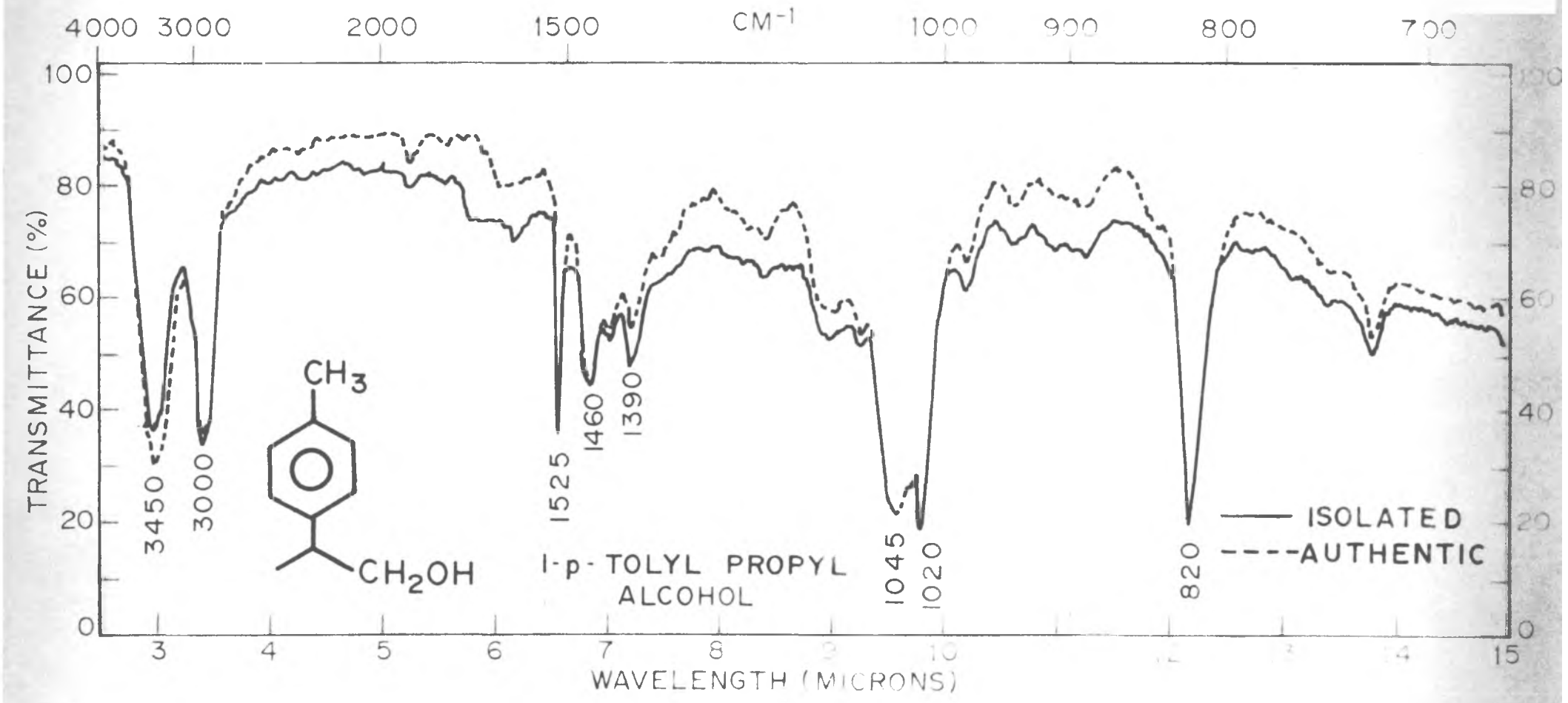

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
3450
3000
1525
1460
1390
1045
1020
820
CH3
CH2OH
1-p-TOLYL PROPYL ALCOHOL
ISOLATED
AUTHENTIC
3 4 5 6 7 8 9 10 12 13 14 15
WAVELENGTH (MICRONS)

FIG XV

when injected together with the authentic sample of cumic alcohol (Fig.XVI). Owing to the paucity of this alcohol further chemical studies were not possible.

The last fraction from the column (N_1-d) eluted with methanol yielded a dark coloured gum which was found to be a mixture of many components on TLC. Since this fraction was isolated in very small amounts further fractionation of this residue was not attempted.

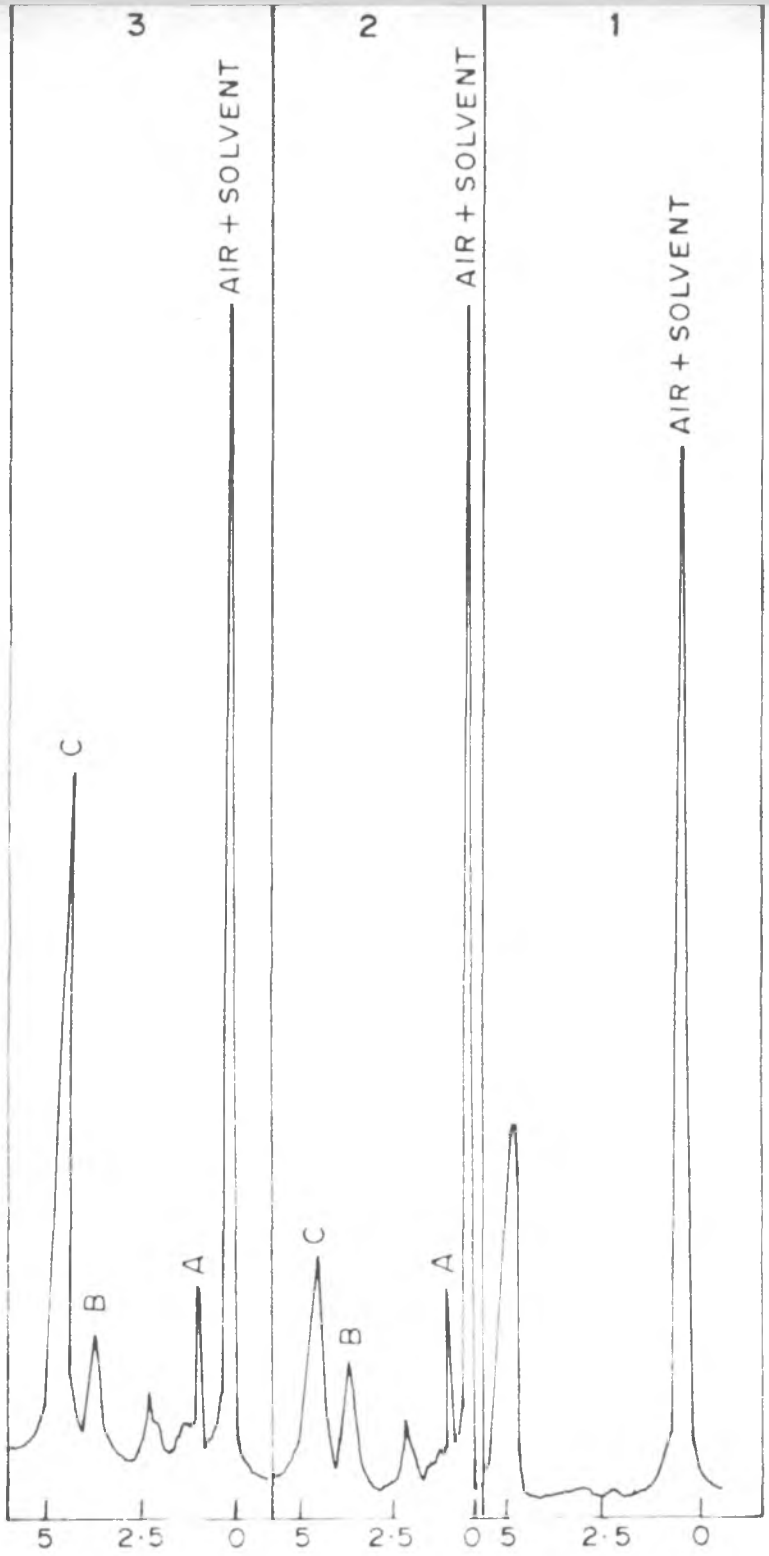
N_2 (more polar neutral) fraction

The neutral n-butanol extract (N_2) was found to separate into 4 to 5 compounds on TLC. The spot having a R_F 0.32 corresponded to that of α-p-tolyl propyl alcohol (System II). As the total yield of the metabolites in this fraction was very poor it was not possible to separate and identify the individual components. Nevertheless, the Warburg studies indicated that this fraction could not be oxidised and so, the failure to identify the various components probably did not hamper the main objective of the studies, namely, the elucidation of the pathway for the biodegradation of p-cymene.

ACIDIC FRACTIONS

Since at two different periods of fermentation, two acids accumulate in major proportions, their isolation was carried out by crystallisation and therefore, they are discussed in the beginning.

The acidic fraction obtained from the 24-hr fermentation was found to contain an acid as the major product which was separated from the other

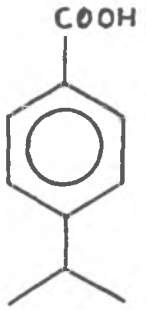


IDENTIFICATION OF CUMIC ALCOHOL (27) IN 100 % ETHER FRACTION BY VPC. (TEMP 130°, CARRIER GAS HYDROGEN; FLOW RATE 30cc/min; COLUMN F, CHART SPEED 12"/hr)

(1) CUMIC ALCOHOL (2) 100 % ETHER FRACTION (3) CUMIC ALCOHOL PLUS 100 % ETHER FRACTION.

FIG. XVI

acidic components present in minor quantities by crystallization from water as a white crystalline material with a m.p. 113-114°. The elementary analysis of this compound suggested an empirical formula of $C_{10}H_{12}O_2$. The IR spectrum (Fig. XVII) showed characteristic absorption due to an aromatic carboxyl group at γ_{max} 2550 and 1680 cm^{-1} and a strong doublet of equal intensity due to isopropyl group at γ_{max} 1330-1300 cm^{-1}. The NMR spectrum (Fig.XVIII) of the compound revealed an isopropyl group (split doublet of six proton intensity at 1.28δ), a complex signal due to proton on tertiary carbon (around 3.00δ), four aromatic protons (two doublets each of two proton intensity at 8.01 and 7.25δ) and the hydroxyl proton of the carboxyl group (at 12.66δ). The physical and spectral data fully support the following structure of cumic acid (28).



(28)

The acidic fractions obtained in the 72 hr fermentation, on TLC examination (System III) showed a single acid in major quantity. This acid fraction was suspended in minimum quantity of carbon tetrachloride to remove the relatively non-polar acids from the more polar acid. The carbon tetrachloride solution on evaporation and chromatography over thin layer showed the presence of small amounts of cumic acid (28). The

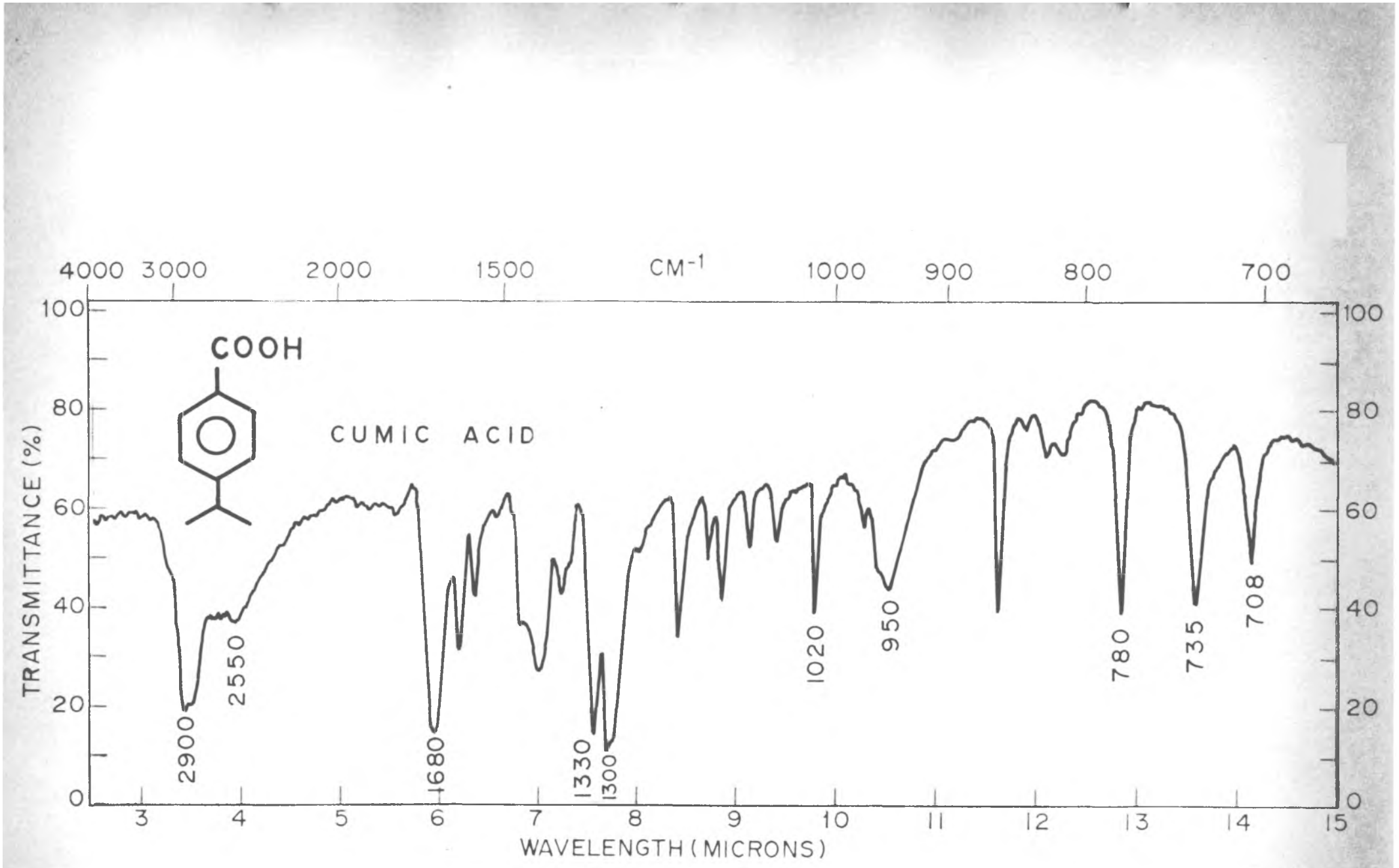
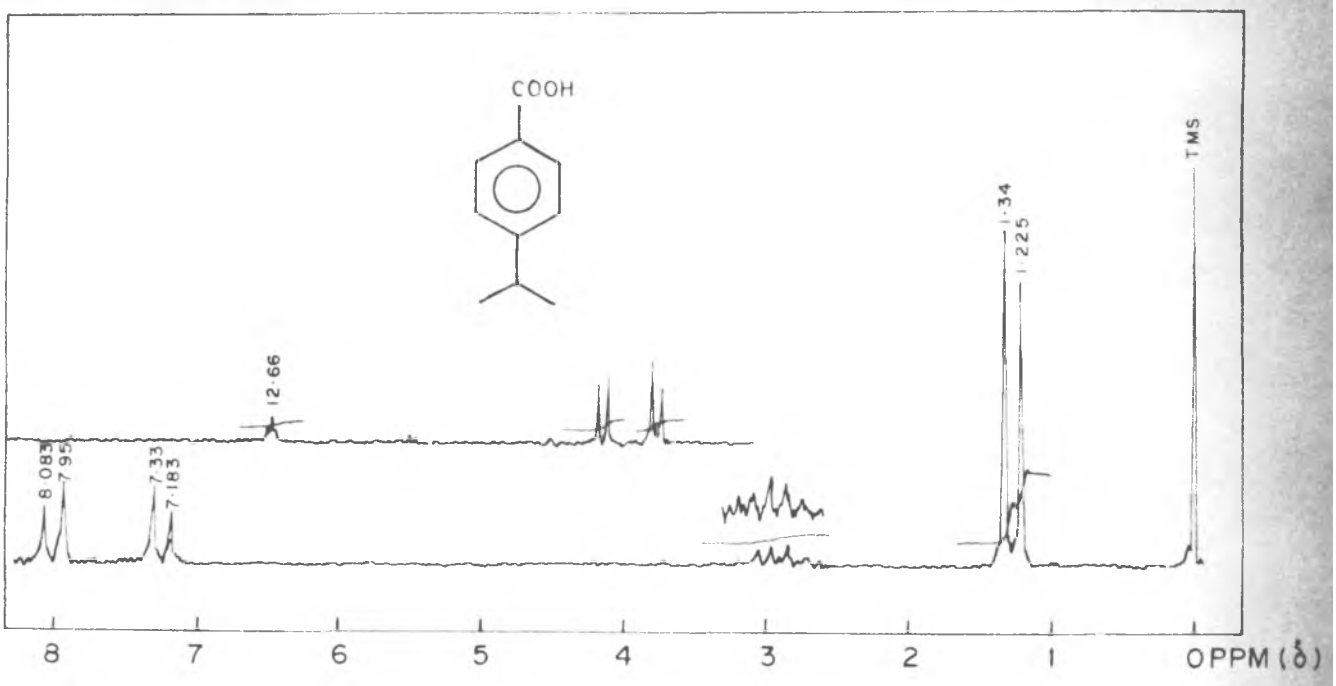

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
COOH
CUMIC ACID
2900
2550
1680
1330
1300
1020
950
780
735
708
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG XVII



FIG·XVIII

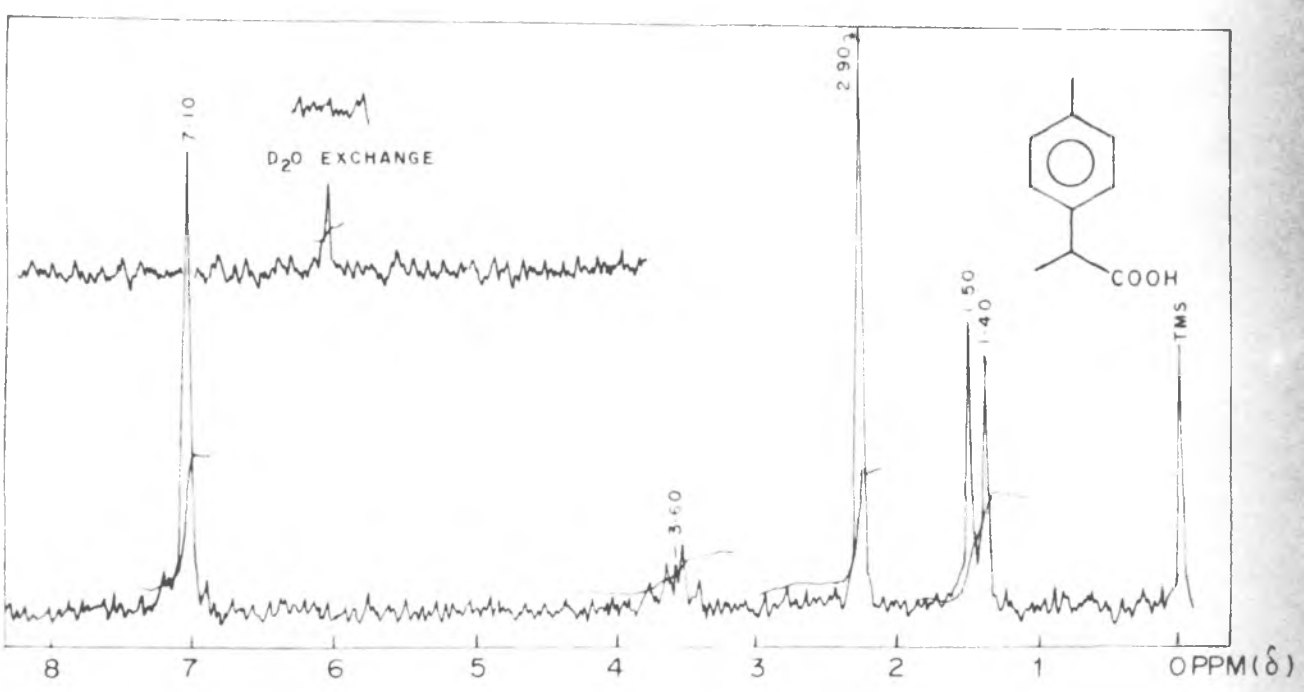
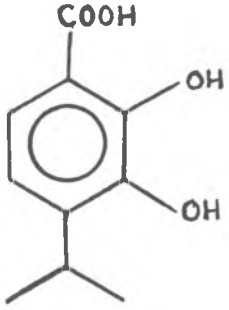


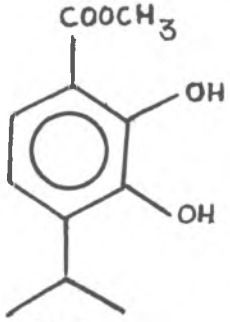
FIG. XXVI

more polar acid was crystallized repeatedly from aqueous ethanol to a constant m.p. 153-54° and showed a single spot in TLC (System III, R_f 0.51). This compound (29) gave a positive ferric chloride test, and analysed for $C_{10}H_{12}O_4$.

The IR spectrum (Fig.XIX) of this acid (29) indicated the presence of a free hydroxyl group (ν_{max} 3450 cm^{-1}), a hydrogen bonded hydroxyl group (ν_{max} 3100 cm^{-1}), a free carboxyl group (ν_{max} 2500 and 1670 cm^{-1}) and an isopropyl side chain (ν_{max} 1330-1305 cm^{-1}). The NMR of the methyl ester of the acid (30) (Fig.XX) showed the presence of six protons corresponding to two methyl groups of isopropyl side chain (doublet at 1.22δ) and the methyl group of the carbomethoxy group showing three proton intensity (a singlet at 3.9δ). The presence of two aromatic protons were shown by two doublets (at 6.6, 7.2δ). Two sharp peaks each of one proton intensity were seen (at 5.6 and 10.6δ) due to the two hydroxyl protons the presence of which were confirmed by equilibration with D_2O. On the basis of the above observations it was concluded that the phenolic acid isolated was 2,3-dihydroxy cumic acid (29) and the corresponding methyl ester as (30).



(29)



(30)

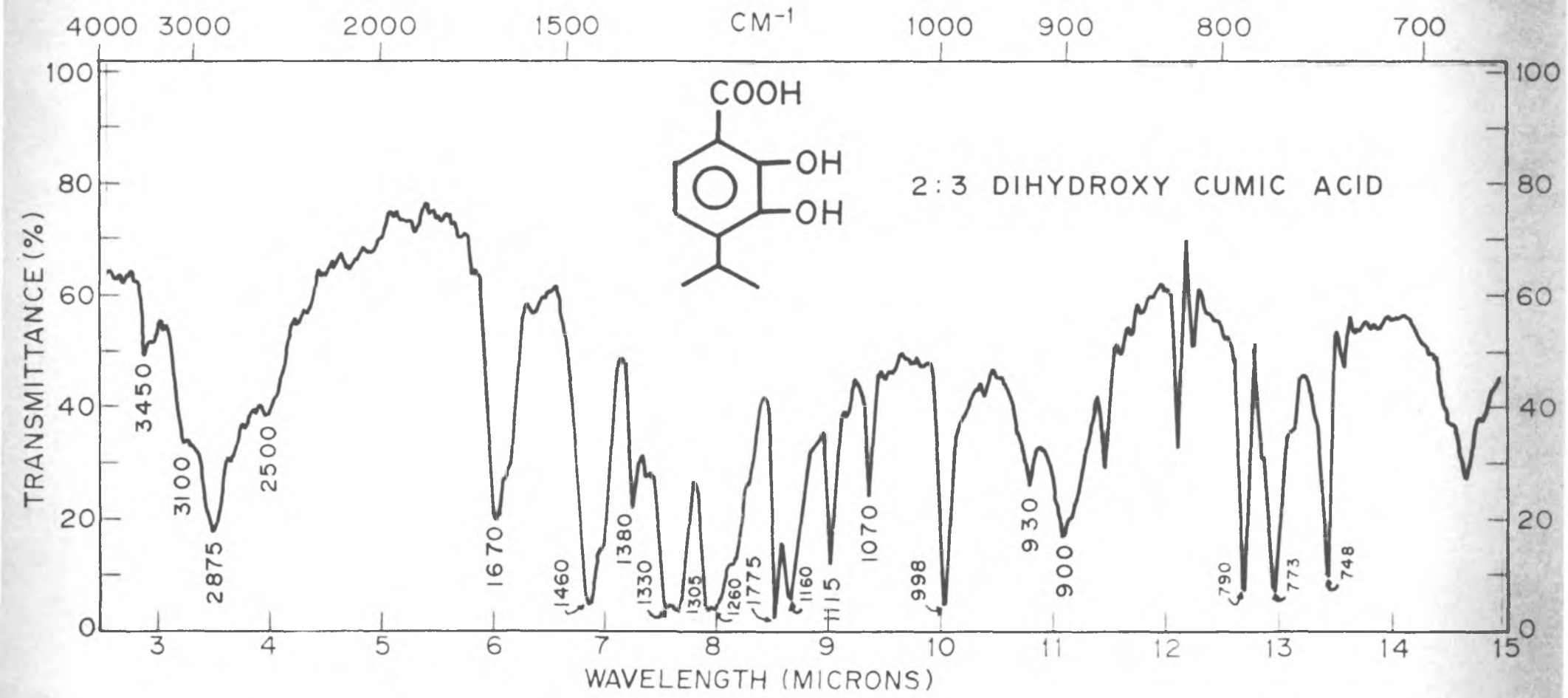

4000 3000 2000 1500 CM-1 1000 900 800 700
COOH
OH
OH
2:3 DIHYDROXY CUMIC ACID
TRANSMITTANCE (%)
100
80
60
40
20
0
3450
3100
2875
2500
1670
1460
1380
1330
1305
1260
1775
1160
1115
1070
998
930
900
790
773
748
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XJX

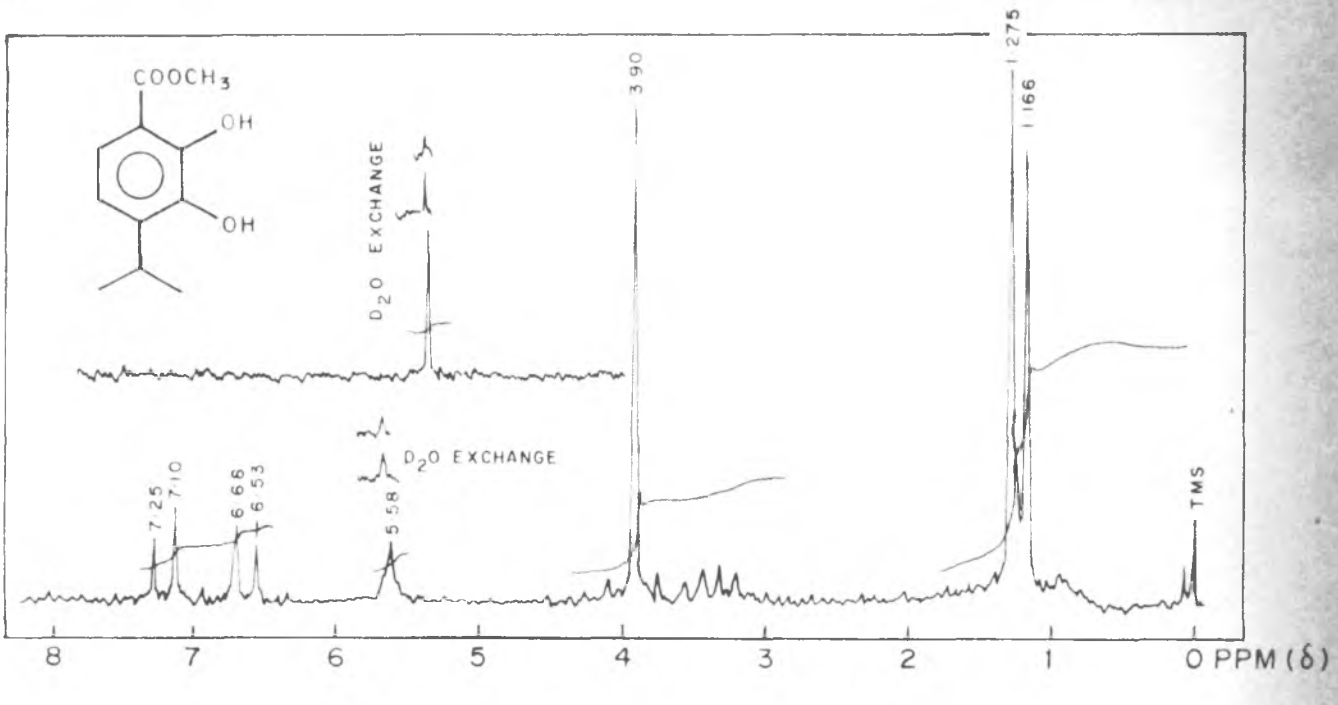


FIG. XX

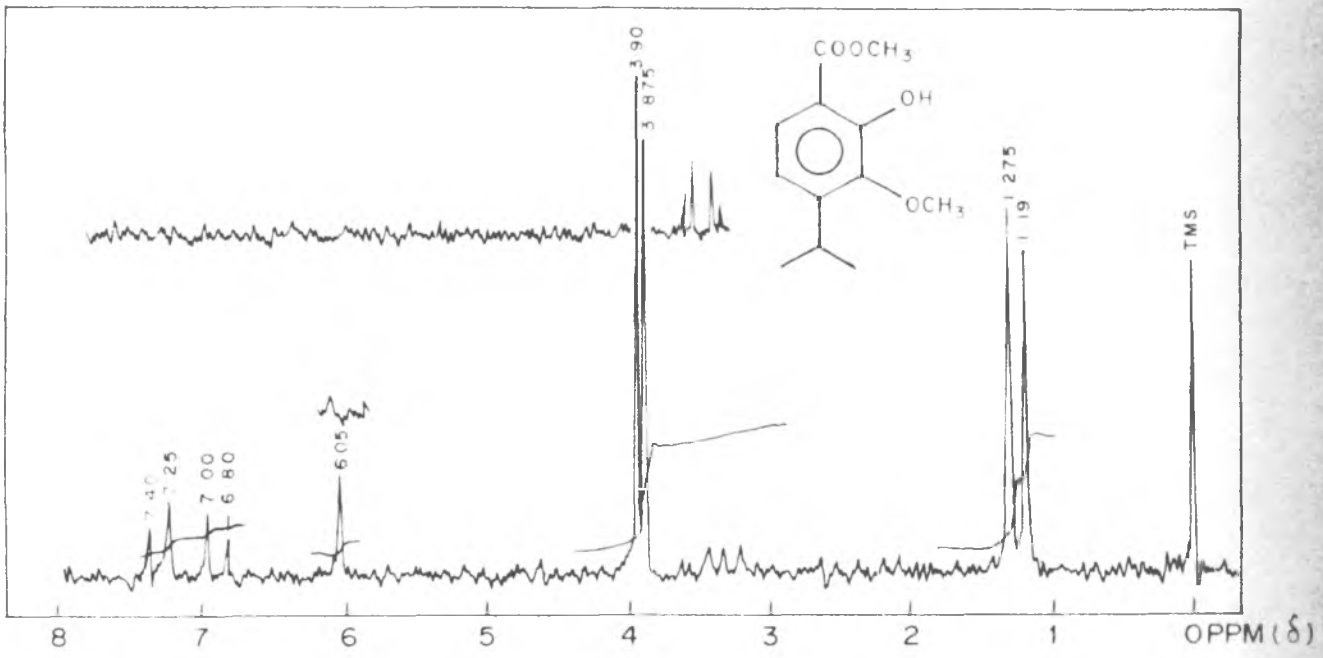
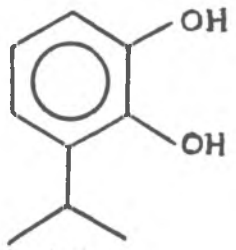


FIG. XXVIII

The physical properties and the spectral data of this phenolic acid compared well with the values that have been reported for this acid by Adams and Hunt (1939).

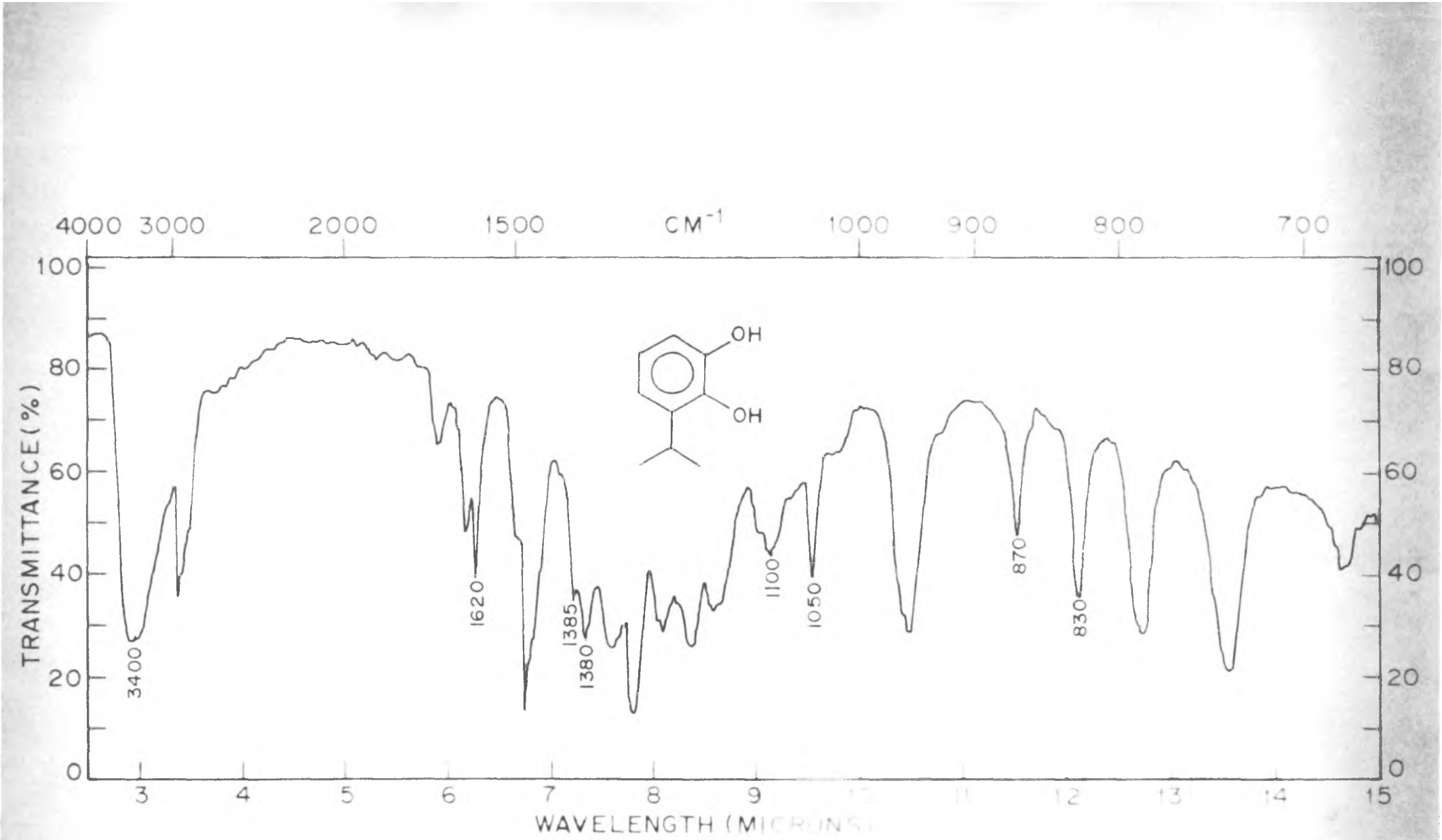
The above conclusion was further substnatiated by decarboxylating the phenolic acid to 3-isopropyl catechol (31). This compound analysed for $C_9H_{12}O_2$. The IR spectrum of this compound (Fig.XXI) indicated the presence of hydroxyl group (γ_{max} 3400 cm^{-1}), the isopropyl group (γ_{max} 1385-1390 cm^{-1}) and the absence of any carbonyl function. The NMR spectrum (Fig.XXII) revealed the presence of an isopropyl group (split doublet of six proton intensity at 1.2δ), a multiplet due to the proton on tertiary carbon (at 3.2δ), two hydroxyl protons (at 4.9δ) which were reduced in intensity by equilibration with D_2O, and three aromatic protons (appeared as a multiplet at 6.65δ). All data are



(31)

consistent with 3-isopropyl catechol structure. Based on this evidence the structure assigned to the phenolic acid was fully confirmed.

Besides these two compounds a few other metabolites were present in acidic fraction in minor proportions. Chromatography on silica gel


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE(%)
OH
OH
3400
1620
1385
1380
1100
1050
870
830
3 4 5 6 7 8 9 12 13 14 15
WAVELENGTH (MICRONS)

FIG·XXI

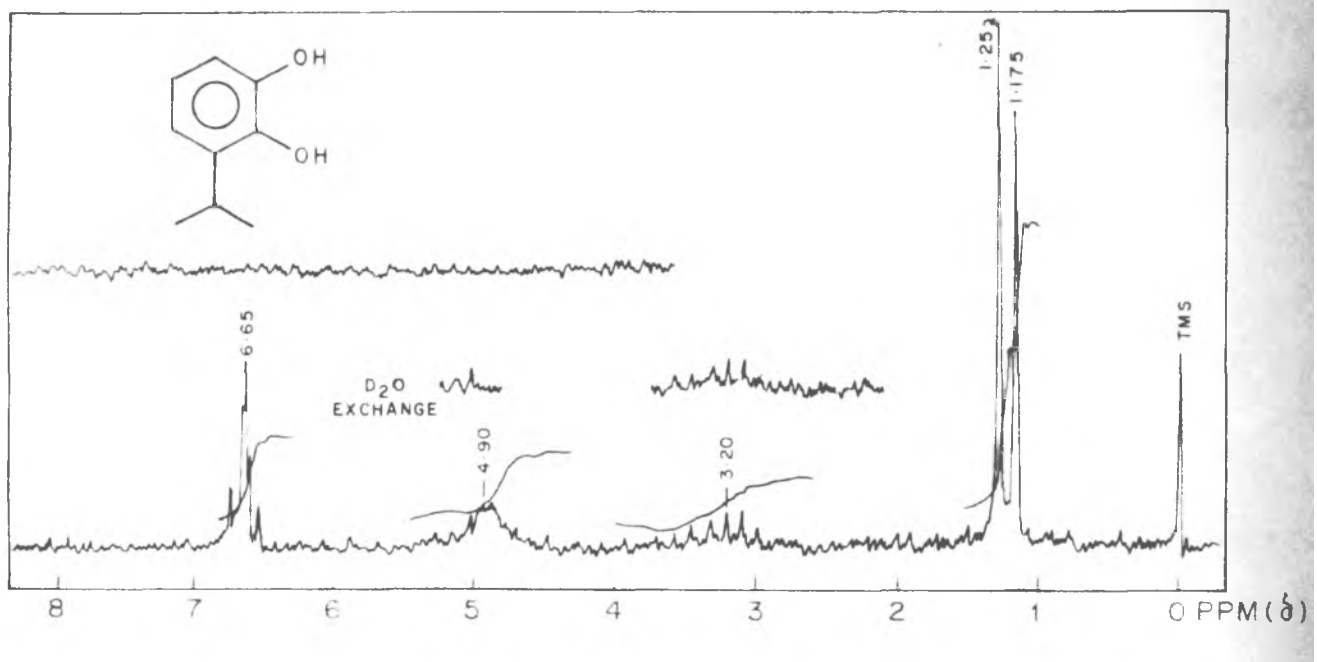


FIG. XXII

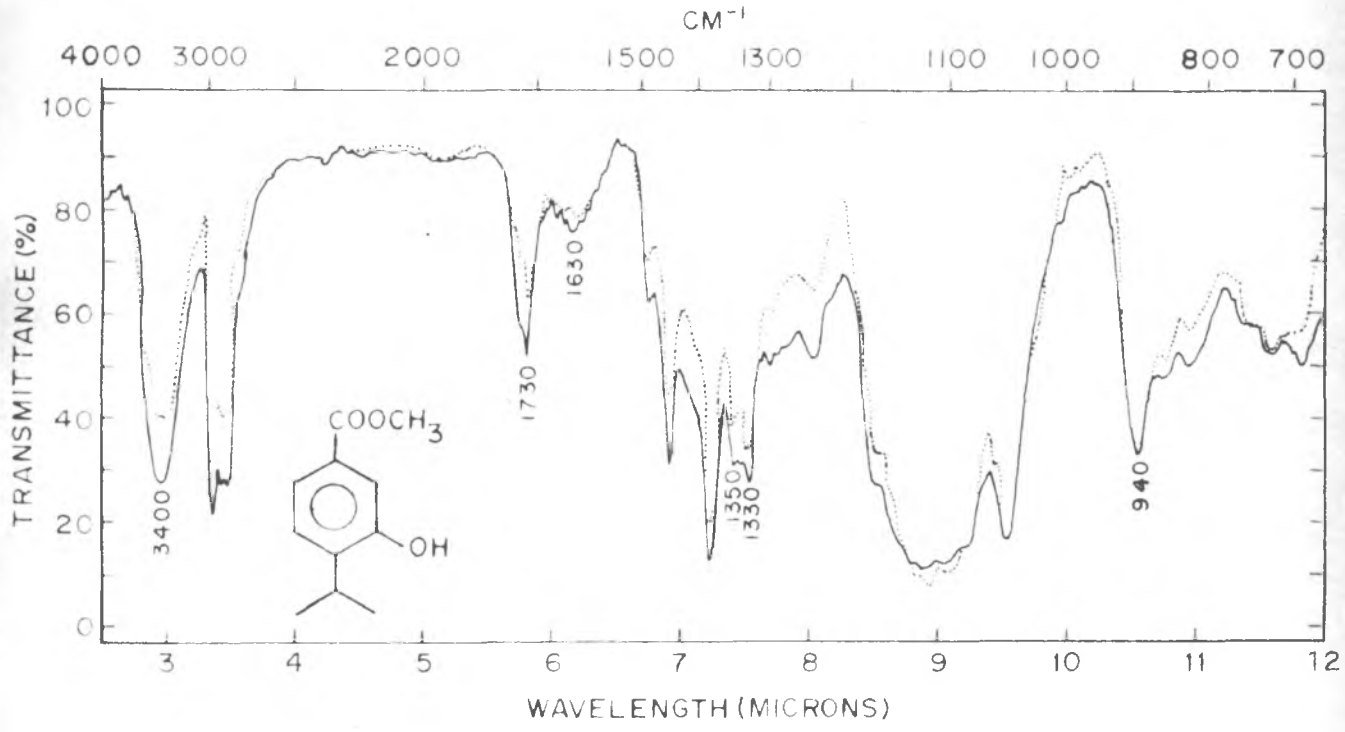


FIG. XXIX

failed to resolve them any further.

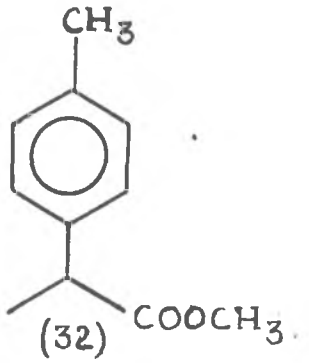
After studying these two acids, namely, cumic and 2,3-dihydroxy cumic acids, the acidic fractions A_1 and A_2 obtained from ether and butanol extracts, respectively (as mentioned before) were studied systematically for the identification of all the components, present. Acidic fraction (A_1) was directly converted into methyl esters with diazomethane and the resulting methyl esters were subjected to a four-transfer modified Craig distribution between 90% aq. methanol and petroleum ether (40-60°) to separate the less polar (fraction A_1-a) from the more polar esters (fraction A_1-b). These fractions were then processed separately.

Fraction (A_1-a): Preliminary examination of this fraction on TLC indicated that it was a mixture of three components. This entire fraction was distilled under vacuum as one fraction and was subjected to chromatography over neutral alumina (Grade I). The first petroleum ether (40-60°) eluates (A_1-a_1) yielded a residue which was found to contain only one compound as revealed by TLC (System I). The combined residue from the later petroleum ether fractions (A_1-a_2) on TLC (System I) showed the presence of two metabolites.

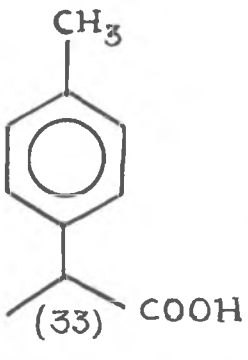
The (A_1-a_1) fraction on saponification gave a low melting solid which analysed for $C_{14}H_{28}O_2$. The IR spectrum (not shown) of the acid exhibited a free carboxyl group (γ_{max} 2600 and 1720 cm^{-1}) and a long methylene chain (γ_{max} 730 cm^{-1}). The spectrum appeared to be that of a fatty acid and this conclusion was supported by NMR studies. For this

reason no further attempts were made to determine the exact nature of the compound.

Fraction (A_1-a_2) was distilled under reduced pressure and the two acids from this fraction were separated on a preparative TLC (System I). The slightly polar acid was found to correspond to cumic acid ester on the basis of TLC and other spectral data. The other acidic compound, was isolated by preparative TLC analysed for $C_{11}H_{14}O_2$. The IR spectrum (Fig.XXIII) showed an aliphatic ester band (γ_{max} 1750 cm^{-1}) and a 1,4 aromatic substitution pattern (γ_{max} 830 cm^{-1}). The NMR spectrum of this compound (Fig.XXIV) showed the presence of four aromatic protons (singlet at 7.1δ), a methyl signal of a carbomethoxy group showing three proton intensity (3.6δ), an aromatic methyl signal (singlet at 2.33δ) and a secondary methyl group (a doublet of three proton intensity at 1.41δ). On the basis of this data the following structure could be assigned to the methyl ester (32).



(32)



(33)

The free acid (33) was obtained as a white crystalline solid, with a m.p. 60-61° on saponification of the methyl ester and it analysed for $C_{10}H_{12}O_2$. The IR spectrum of this acid (Fig.XXV) showed the presence

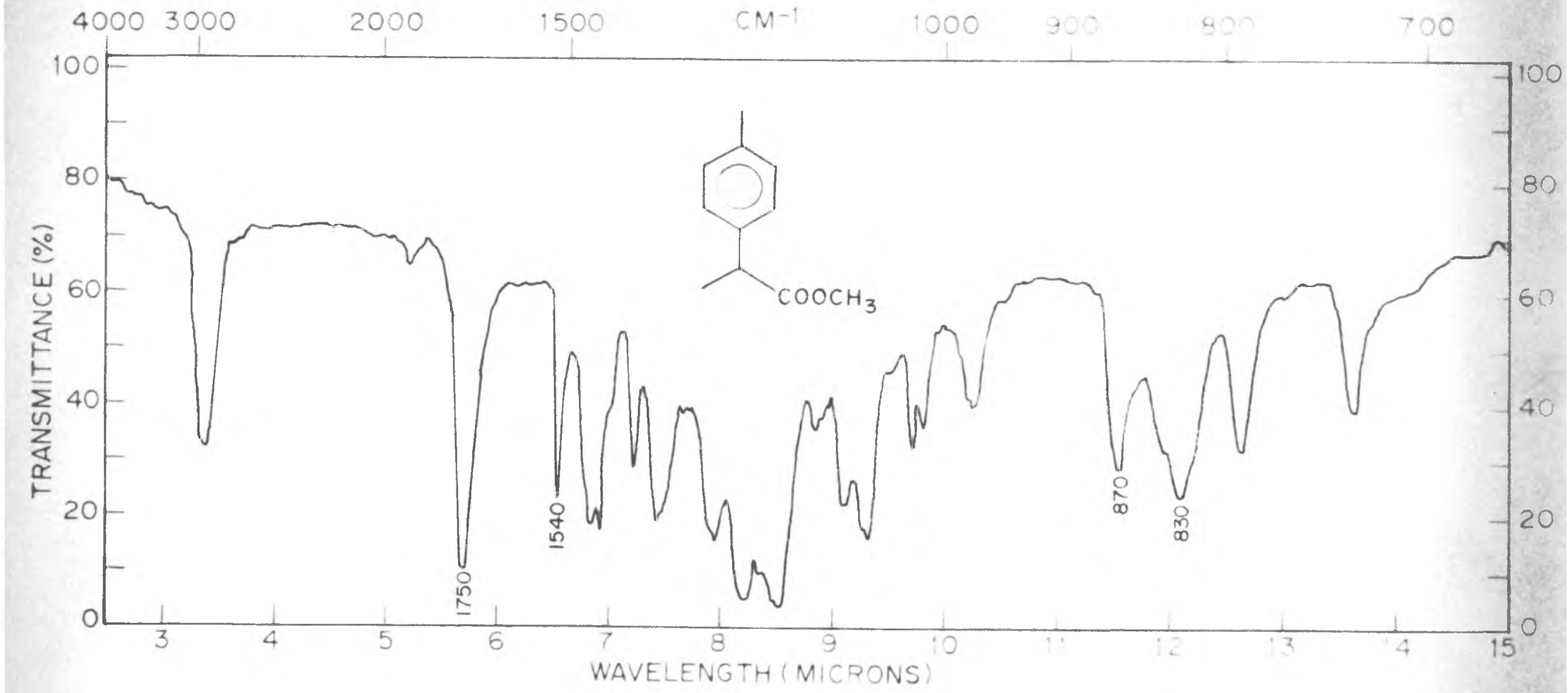

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
COOCH3
1750
1540
870
830
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XXIII

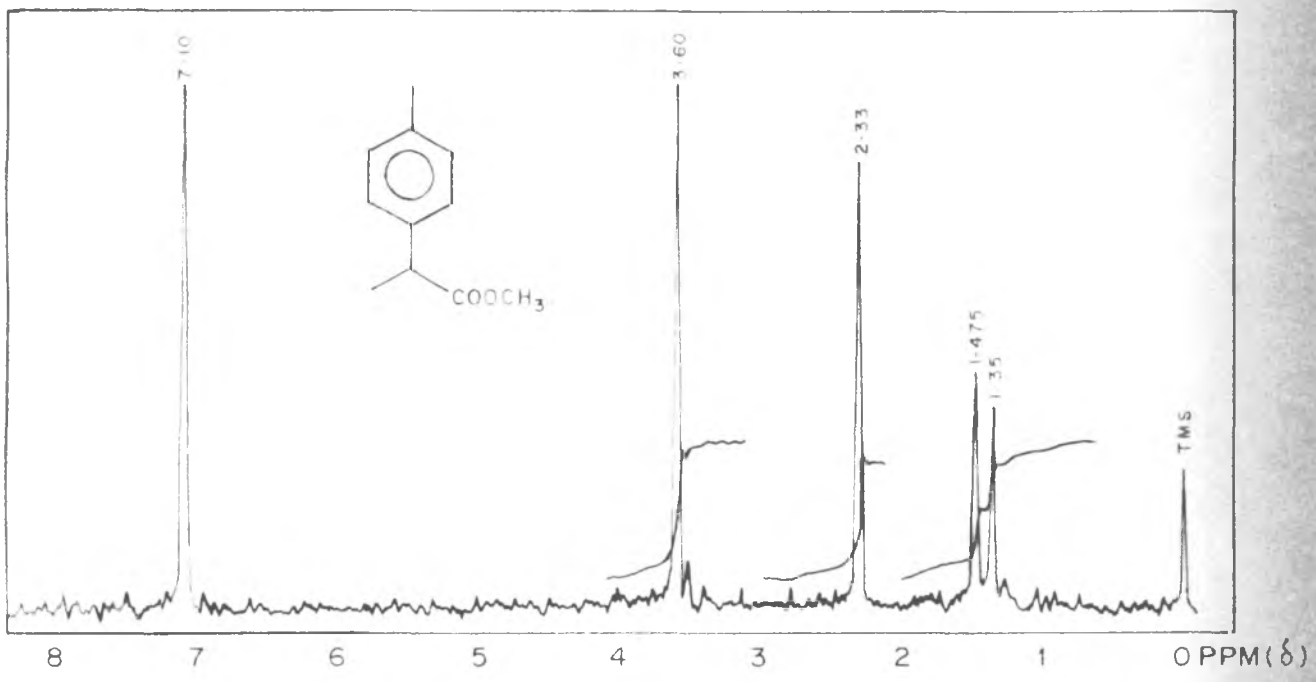


FIG.XXIV

of a free carboxyl group (ν_{max} 2700 cm^{-1}, 1700 cm^{-1}), and 1,4 aromatic substitution pattern (ν_{max} 829 cm^{-1}). The NMR spectrum (Fig.XXVI) also showed the same pattern as that of the methyl ester except for the absence of the methyl of the carbomethoxy signal and instead, the presence of an acidic proton (at 11.6δ) was seen.

Fraction (A_1-b)

TLC of this fraction (A_1-b) obtained from the Craig fractionation in System IV was found to contain at least four components. It was subjected to chromatography over neutral alumina (Grade II). Examination of petroleum ether:ether eluate (A_1-b_1) on TLC revealed the presence of two components having very close R_f values, and the ether eluate (A_1-b_2) on TLC was found to contain three components.

The compounds of the (A_1-b_1) fraction were separated by preparative TLC. The metabolite with the R_f value 0.59 in System IV corresponded to the methyl ester of 2,3-dihydroxy cumic acid. IR and NMR spectra were identical with those of 2,3-dihydroxycumic acid. The second component was a solid, $C_{12}H_{16}O_4$, and was crystallized from aqueous ethanol, m.p. 57-58°. The IR spectrum (Fig.XXVII) of this solid ester exhibited the presence of a hydroxyl group (ν_{max}3400, 1220 cm^{-1}), an ester band (ν_{max}1750 cm^{-1}) and an isopropyl group (ν_{max}1385-1370 cm^{-1}). The NMR spectrum (Fig.XXVIII) of this compound to some extent agreed with that of 2,3-dihydroxy cumic acid ester. Nevertheless, the disappearance

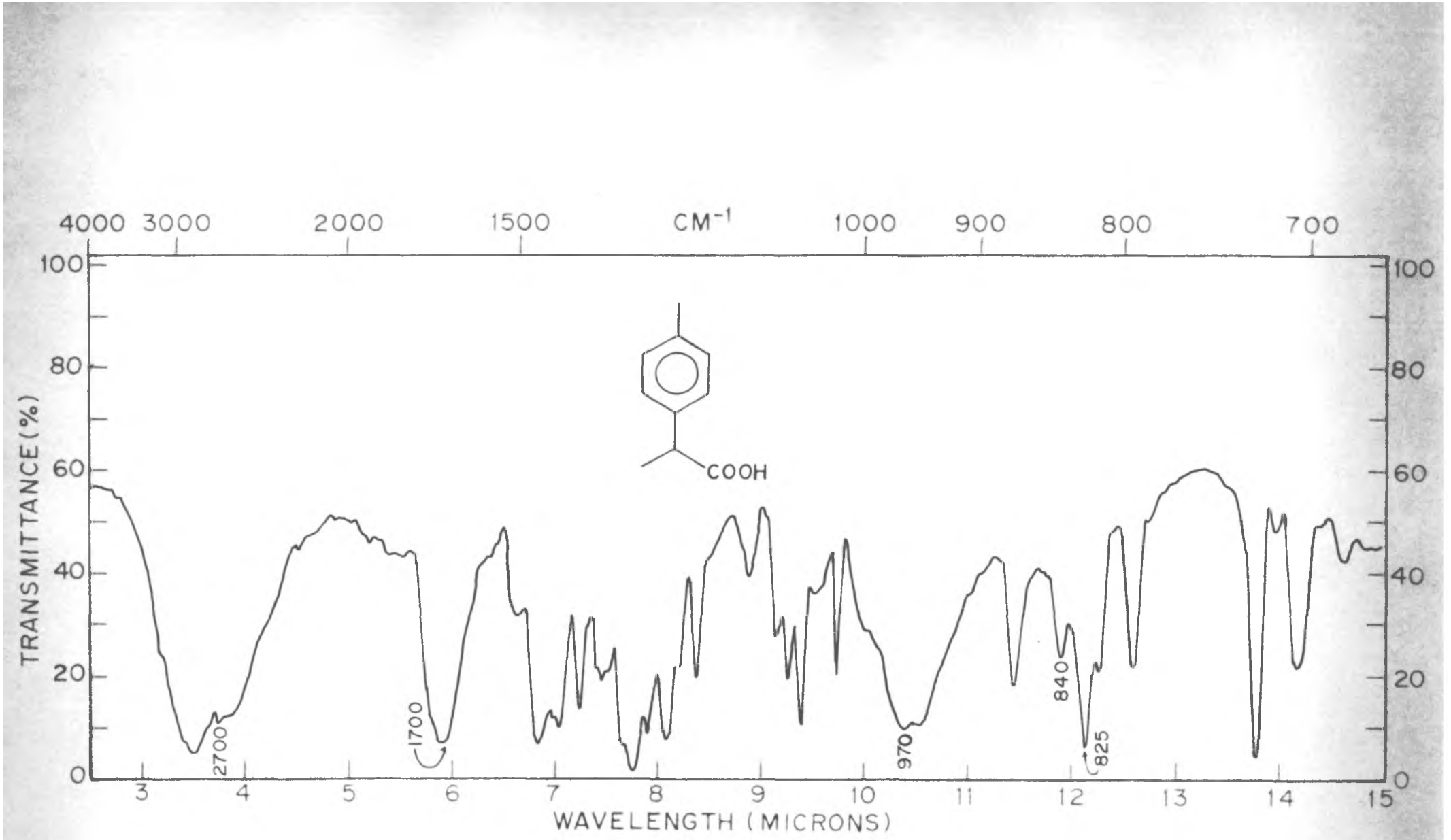
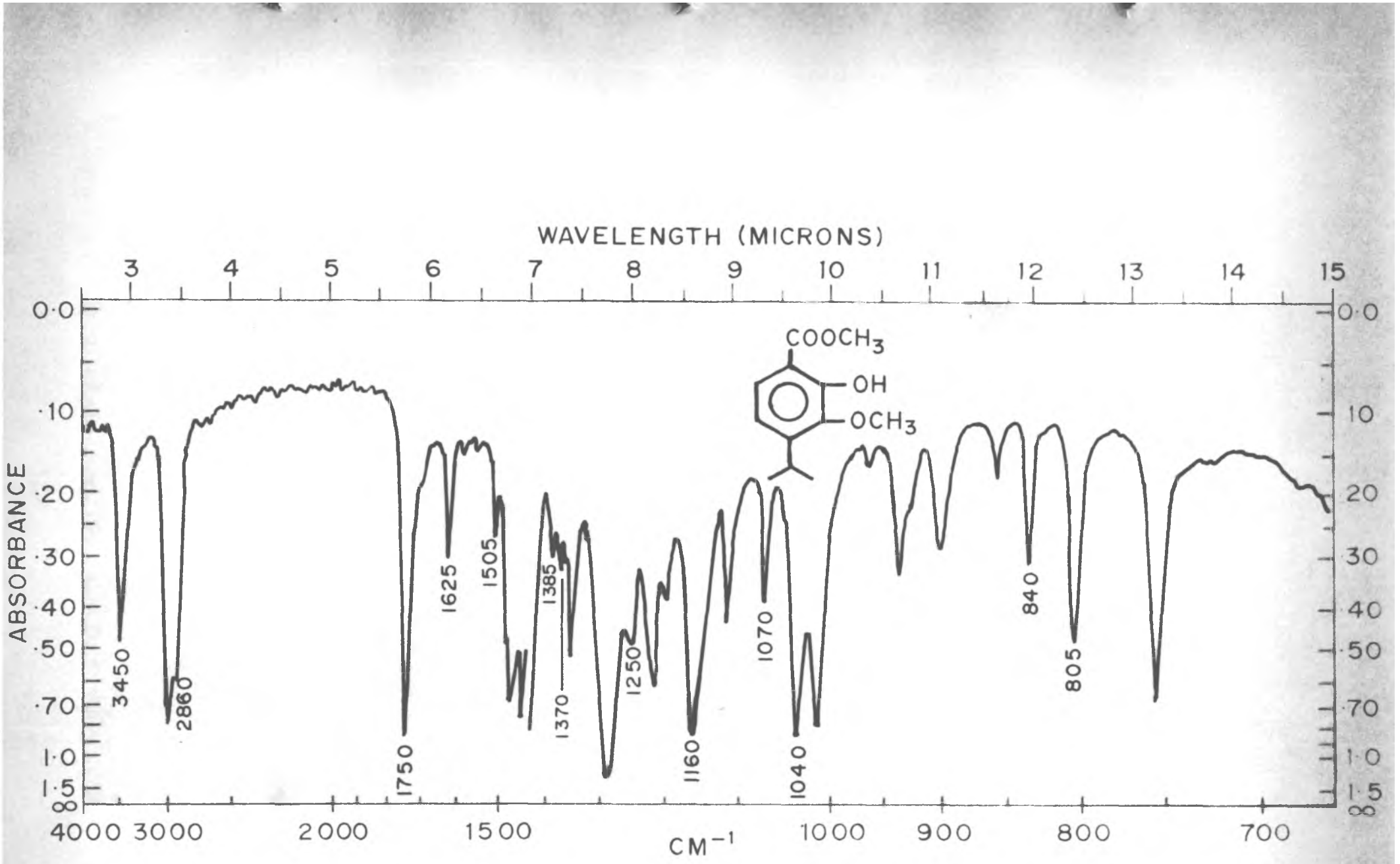
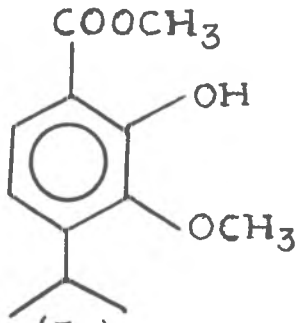

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
COOH
2700
1700
970
840
825
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XXV


WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
11
12
13
14
15
ABSORBANCE
0·0
·10
·20
·30
·40
·50
·70
1·0
1·5
COOCH3
OH
OCH3
3450
2860
1750
1625
1505
1385
1370
1250
1160
1070
1040
840
805
4000
3000
2000
1500
1000
900
800
700
CM-1

FIG· XXVII

of the strong acidic phenolic group and the appearance of methoxy protons were observed. This spectrum indicated the presence of an isopropyl side chain (doublet of 8 proton intensity at 1.23δ), carbomethoxy protons (singlet of three proton intensity at 3.9δ) methoxy protons (singlet at 3.87δ), a hydroxyl proton (singlet at 6.05δ, on equilibration with D_2O the peak disappeared) and two aromatic protons (two doublets at 6.90 and 7.82δ). On the basis of these data a probable structure of the type (34) was assigned to the solid methyl ester. However, this compound (34) may not be a true transformation

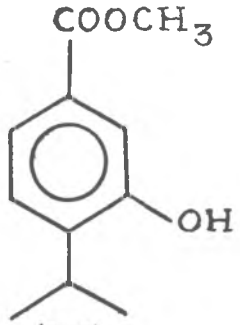


(34)

product but more likely it could have been formed while esterifying 2,3-dihydroxy cumic acid present in the acidic fraction with diazomethane. It was also observed with 2,3-dihydroxy cumic acid that when it was allowed to react with an excess of diazomethane for a longer time, the more acidic hydroxyl group at position 3 was methylated.

The (A_1-b_2) fraction on TLC examination (System IV) showed the presence of two compounds (30) and (34). Besides these the mixture also contained another ester which was separated on preparative TLC. This ester was found to be a very low boiling liquid, distilling at 98°/50 cm (bath temp.). The mobility pattern of this compound on TLC corresponded

with that of the authentic 3-hydroxy cumic acid methyl ester. The identity of this compound with authentic 3-hydroxy cumic acid ester (35) was established by spectral comparison (Fig.XXIX).

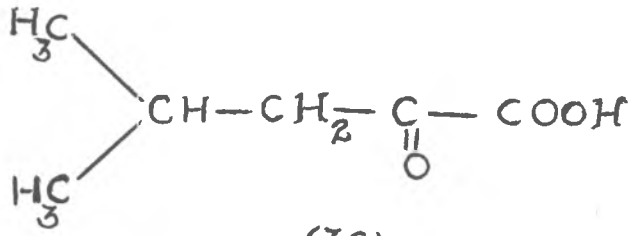


(35)

Butanol fraction (A_2)

Examination of the butanol fraction on TLC (System III) indicated the presence of five to six components. Two compounds had the same R_F as that of cumic acid and 2,3-dihydroxy cumic acid, respectively. The amount of this fraction was very small and hence the other metabolites could not be isolated for structure determination. Since the cleavage of the benzene nucleus of 2,3-dihydroxy cumic acid should result in the formation of either ketonic or aldehydic intermediates, the entire butanol acidic fraction was treated with 2,4-dinitrophenyl hydrazone to convert any such carbonyl compounds, into their respective hydrazones. The hydrazones formed were taken into ethyl acetate which when examined on TLC (System V) was found to contain a mixture of three hydrazones. The hydrazone with the R_F value 0.48 corresponded to that of the hydrazone of isopropyl pyruvic acid (36). Due to paucity of the material it

could not be isolated in a pure form for further studies. The other two hydrazones present in the mixture could not be identified. Furthermore,



(36)

enzymatic studies as reported in Chapter IV have shown that isopropyl pyruvic acid is formed when a cell free extract was incubated with 2,3-dihydroxy cumic acid for a period of two hours. This accords further proof that isopropyl pyruvic acid is formed during the biodegradation of p-cymene.

Fermentation of p-cymene with glucose grown cells

When the fermentation of p-cymene was carried out with glucose grown cells substantial amounts of acidic products accumulated in the medium. It is interesting to note that when fully adapted glucose grown cells are used for the fermentation of p-cymene the amount of the acidic metabolites formed was decreased considerably. On the other hand when partially adapted cells were used 2,3-dihydroxy cumic acid was found to accumulate in large quantities.

EXPERIMENTAL

FERMENTATION OF p-CYMENE

Effect of substrate concentration

The effect of concentration of the substrate on the growth of the organism was tested by inoculating 100 ml of sterile mineral salt medium with 1.0% inoculum (24 hr old culture grown on p-cymene) and adding p-cymene in various concentrations ranging from 0.2 to 2.0%. The results presented in Table (i) indicated that the growth rate reached a maximum when the initial concentration of the substrate reached a level of 1.0% and the remained steady with increasing concentration up to a level of 2.0%. Beyond this level the substrate was found to be toxic to the organism. The growth rate also increased as the period of incubation was increased and reached a maximum at a period of 96 hr. Beyond this period considerable lyses of the cells take place. As a result of these observations an initial concentration of 1.0% of p-cymene was employed throughout the fermentation.

Optimum conditions

Inoculum, pH and temperature: A pH of 7.0 was found to be optimum for purposes of growth and fermentation. All fermentations were carried out at a constant temperature of 28°±2 on a rotary shaker (220 r.p.m.).

TABLE (1)

EFFECT OF CONCENTRATION OF THE SUBSTRATE ON THE GROWTH OF THE ORGANISM

Concentration (%)	Growth (Klett reading)			
	24 hr	48 hr	72 hr	96 hr
1) nil	37	40	42	44
2) 0.2	275	310	305	265
3) 0.4	285	315	305	285
4) 0.6	290	375	395	260
5) 0.8	295	400	455	350
6) 1.0	305	405	590	630
7) 1.5	305	395	575	610
8) 2.0	300	400	490	510

The salt medium of the composition as mentioned in Chapter II was found to be suitable for the fermentations.

Accumulation of products as a function of time

A batch of 70 flasks each containing 100 ml of sterile medium and 1.0 ml of the substrate were inoculated with 1 ml of inoculum of a 24 hr old culture. Five flasks were removed at zero hour and the remaining 65 flasks were incubated at 28° on a shaker. 10 flasks were removed every time at an interval of 4, 8, 12, 24, 48, 72 and 96 hr. The broth was acidified, saturated with sodium chloride and extracted with ether. The ether layer was separated into acidic and neutral fractions. The neutral fraction was chromatographed over alumina (Grade I) and eluted with petroleum ether (40-60°) and ether. The ether eluates were concentrated to yield comparatively more polar compounds and taken as polar neutral fraction which was free from unreacted p-cymene.

The acids were obtained from the sodium bicarbonate extract by acidification followed by ether extraction. The results of this experiment are tabulated in Table (ii). It is evident from this table that the amount of the acidic fraction reached a maximum when the incubation period was 72 hr. In the initial period comparatively more neutral products were formed and it was, therefore, decided to carry out fermentations of p-cymene up to 72 hr.

TABLE (ii)

EFFECT OF TIME ON THE ACCUMULATION OF PRODUCTS

Time (hr)	Total neutral fraction* (g)	Acidic fraction (g)	Polar neutral fraction (g)
4	3.10	0.028	0.075
8	2.63	0.060	0.355
12	2.53	0.071	0.235
24	1.50	0.089	0.140
48	0.60	1.200	0.225
72	0.20	1.450	0.135
96	0.21	0.580	0.131

*before chromatography

Large scale fermentation

50 flasks each with 100 ml of mineral salt medium were inoculated with 1.0% inoculum from a 24-hr old culture. One ml of p-cymene was added to each flask and then incubated on a rotary shaker for 72 hr at 28°±2. At the end of the desired incubation period (72 hr) the contents of the flasks were pooled, acidified and extracted with ether. The broth was reextracted with n-butanol. The ether and n-butanol extracts were concentrated and separated into acidic and neutral fractions by washing with 5.0% aqueous sodium carbonate.

In order to get large quantities of metabolites for isolation and identification, the contents from five batches each of 50 flasks were pooled together and processed. The total ether extract on concentration afforded 35.05 g of products. This on separation into neutral and acidic products gave a neutral (N_1) fraction (3.8 g) and an acidic (A_1) fraction (31.25 g).

The n-butanol extract was concentrated on a water bath under vacuum. The residue was taken up in chloroform and separated into neutral (N_2, 1.56 g) and acidic (A_2, 0.31 g) fractions.

Chromatography of the neutral fraction

The neutral fractions were as such chromatographed over alumina. In a typical experiment the neutral fraction obtained from 100 flasks (7 g) was chromatographed on an alumina column (Grade I, neutral, 210 g) and the following fractions each of 1000 ml volume were collected.

N_1-a: petroleum ether fraction

N_1-b: benzene fraction

N_1-c: ether fraction

N_1-d: methanol fraction

The petroleum ether fraction (N_1-a; 5.6 g) on TLC examination (System I) showed only two spots, one of them corresponded to p-cymene. The (N_1-a) fraction was then rechromatographed over neutral alumina (Grade I) and eluted with dry petroleum ether (40-60°). The initial petroleum ether eluate on evaporation yielded unreacted p-cymene (4.9 g).

From the tail fractions a small amount (30 mg) of a semi solid material was isolated. IR (γ_{max} 1760, 1485, 1390, 1160, 825 and 730 cm^{-1}).

The next fraction (N_1-b; 0.380 g) was found to be a mixture of two components with very close mobilities (R_f 0.91; 0.88) in TLC (System I) and so it was not possible to separate them further.

The major neutral metabolite was obtained from (N_1-c) fraction (0.610 g). This fraction was distilled under reduced pressure (b.p. 155-160°/30 mm, bath temp.) to give a material (0.47 g) which showed three components on TLC (System II). The distilled material (0.47 g) was chromatographed on an alumina column (Grade I) 25 g, eluted with dry ether and 25 ml fractions were collected. The first seven fractions showed the presence of two components with R_f 0.32, 0.35 on TLC (System II). Out of which compound with R_f 0.32 was in major quantity. These fractions were pooled together (0.250 g) and was subjected to a preparative TLC. Compound with a R_f 0.32 (0.150 g) was separated and was distilled at 155-60°/30 mm (bath temp.). Found C, 80.03, H, 9.38%; $C_{10}H_{14}O$ requires, C, 80.0, H, 9.33%. IR (Fig.XIII) γ_{max} 3450 cm^{-1} (OH). The NMR spectrum (Fig.XIV) indicated that the compound isolated is α-p-tolyl-propyl alcohol (26). The identity of (26) was further established by spectral comparisons (Fig.XV) with an authentic sample.

Preparation of ∝-p-tolyl-propyl alcohol

This compound was prepared from ∝ -p-tolyl propaldehyde which was prepared essentially according to method of Henderson and Cameron (1909). When dilute solution of p-cymene (15.8 ml, 0.1 M) in dry carbon disulphide was treated with a similar solution of chromyl chloride (16 ml, 0.2 M), a brown oily liquid was formed which was then subjected to steam distillation. From the distillate ∝-p-tolyl propaldehyde was separated as its bisulphite compound from which the free aldehyde (5 ml) was obtained [illegible]. The aldehyde was then reduced to ∝-p-tolyl propyl alcohol (3.5 ml) by lithium aluminium hydride. The alcohol distilled at 128-30°/35 mm.

The second fraction from the preparative TLC (50 mg) on further analysis in VPC showed three peaks, namely, a, b, c. Peak c represented cumic alcohol (27) as demonstrated by the enhancement of the peak intensity when mixed with an authentic sample of cumic alcohol (Fig. XVI) (Temp.130°, column P, carrier gas H_2, flow rate 30 ml/min.). This compound could not be obtained in pure form from the mixture.

The last fraction (N_1-d; 0.230 g) was a dark coloured gum which could not be further separated.

Acidic fraction (A_1)

Since the two major acids, namely, cumic acid and 2,3-dihydroxy cumic acid were formed at the end of 24 and 72 hr of fermentations,

respectively each was identified by carrying out fermentations at intervals of 24 and 72 hr.

In a typical fermentation 40 flasks each with 100 ml of mineral salt medium were inoculated with 1.0% inoculum from a 24-hr old culture. One ml of p-cymene was added to each flask and incubated on a rotary shaker. Twenty flasks were removed after 24 hr and the remaining 20 flasks were removed after the completion of 72 hr. The metabolites obtained in these two batches were processed separately.

1) Wt. of the acidic fraction from a 24 hr fermentation (20 flasks) = 1.5 g

2) Wt. of the acidic fraction from a 72 hr fermentation (20 flasks) = 2.85 g

The acidic fraction obtained from the 24 hr fermentation (1.5 g) was found to contain mostly a single component. This after repeated crystallisation from hot water resulted in a white crystalline material (1.15 g) which melted at 113-114°. R_f value of the acid 0.61 (System III). Found C, 73.09, H, 7.38%; $C_{10}H_{12}O_2$ requires C, 73.17, H, 7.32%. IR γ_{max} 2550 1680, 1330-1300 cm^{-1} (Fig. XVII). NMR (Fig. XVIII). The compound was identified as cumic acid (28) and it agreed with the properties as mentioned in the literature (Crounse, 1949).

The acidic fraction obtained from the 72 hr fermentation (2.85 g) was suspended in a minimum amount of carbon tetrachloride and then filtered. The residue (1.8 g) was repeatedly crystallised from aqueous ethanol to a

constant m.p. 153-54°. It gave a characteristic phenolic test with ferric chloride and had a R_F value 0.51 in System III. Found C, 61.23, H, 6.21%. $C_{10}H_{12}O_4$ requires C, 61.19, H, 6.17%. IR γ_{max} 3450, 3100, 2500, 1670, 1330-1305, 1115 and 1070 cm^{-1} (Fig. XIX).

Methyl ester: The acid (29) was esterified with diazomethane in ether and the resulting methyl ester (30) distilled at 130-133°/1 mm. TLC showed a single spot with R_F 0.59 in System IV. Analysis; Found C, 63.05, H, 6.7, $C_{11}H_{14}O_4$ requires C, 63.0, H, 6.66%. The NMR spectrum (Fig.XX) indicated that the compound is the methyl ester of 2,3-dihydroxy cumic acid (30).

Decarboxylation of 2,3-dihydroxy cumic acid to 3-isopropyl catechol:

200 mg of the substance was taken in a small distillation unit and heated at 200° for 30 min. When the bath temperature was raised to 300° a pale yellow liquid, 3-isopropyl catechol (31) distilled (75 mg). Found, C, 71.1, H, 7.96%; $C_9H_{12}O_2$ requires C, 71.05, H, 7.99%. IR γ_{max} 3400, 1620, 1385-1380 and 830 cm^{-1} (Fig.XXI), NMR (Fig.XXII).

Separation and identification of the components of the acidic fraction (A_1):

The acidic fraction (A_1, 5 g) was converted into its methyl ester (4.8 g) by treatment with diazomethane in ether solution. The methyl esters were further separated into polar and nonpolar esters by partitioning between petroleum ether and 90% aqueous methanol. The petroleum ether layer after drying and evaporation gave nonpolar methyl esters (A_1-n, 1.8 g).

The methanolic layer yielded the polar methyl esters (A_1-b, 2.5 g).

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

This fraction was distilled under reduced pressure and the entire amount (1.5 g) was then subjected to chromatography over alumina (Grade I, 45 g). The column was eluted with petroleum ether (40-60°) and fractions of 25 ml each were collected. The first six fractions (A_1-a_1) were pooled together as it showed only one spot on TLC (System I). TLC of the later five fractions (A_1-a_2) showed the presence of two components in System I.

The (A_1-a_1) fraction (0.20 g) was hydrolysed and the free acid was further purified by washing with cold acetonitrile (yield 0.110 g). The IR spectrum showed absorption bands at ν_{max} 2600, 1720 and 730 cm^{-1}. The NMR spectrum also suggested that the compound is a long chain fatty acid. So no further attempts were made to identify this compound.

The fraction (A_1-a_2; 1.21 g) distilled at 130-35°/30 mm (bath temp.). This was subjected to preparative TLC in System I, and the two components were separated. One of the components was found to be cumic acid ester. The other fraction (0.40 g) was distilled at 140-45°/ 1 mm (bath temp.). Found C, 74.20, H, 7.88%. $C_{11}H_{14}O_2$ requires C, 74.16, H, 7.86%. The IR spectrum (Fig.XXIII) showed bands at ν_{max} 1750, 1540 and 830 cm^{-1}. The NMR spectrum (Fig.XXIV) of this compound agreed fully with the structure of α-p-tolyl propionic acid methyl ester (32).

Regeneration of the free acid (33)

The methyl ester (0.20 g) was refluxed with an excess of 10.0% methanolic potassium hydroxide on a water bath for three hours. Methanol was removed under vacuum and the residue was diluted with water and extracted with ether to remove the unreacted methyl ester. The aqueous layer was acidified with hydrochloric acid and extracted with ether. The ether layer on evaporation yielded the free acid (33; 0.110 g). It was crystallised from aqueous ethanol as a colourless crystalline solid, m.p. 60-61°. Analysis, C, 73.13, H, 7.5%. $C_{10}H_{12}O_2$ requires C, 73.17, H, 7.32%. The IR spectrum (Fig. XXV) showed the presence of a free carboxyl group (ν_{max} 2700 and 1700 cm^{-1}). The NMR spectrum (Fig.XXVI) proved it to be α-p-tolyl propionic acid. In the earlier literature (Rupe and Wiederkehr, 1924) a mention has been made that this compound melts at 40-41°. However, the compound that has been isolated in the present studies has a different melting point (60-61°). But other physical data fully agree with the structure (33).

Fraction (A_1-b)

The total fraction (2.50 g) was chromatographed over neutral alumina column (Grade II, 75 g) and the following fractions were collected.

i) Petroleum ether: ether (1:1) ..... (A_1-b_1)

ii) Ether fraction ..... (A_1-b_2)

The (A_1-b_1) fraction (1.8 g) was found to contain two components. Compound with the R_F value 0.59 in System IV corresponded with the methyl

ester of 2,3-dihydroxy cumic acid. The other component (R_f 0.52) was isolated (50 mg) in a pure form by subjecting the mixture to preparative TLC (System II). Melting point of the pure compound was 57-58°. Analysis, C, 64.36, H, 7.26%. $C_{12}H_{16}O_4$ requires C, 64.3, H, 7.15%. The IR spectrum (Fig. XXVII) γ_{max} at 3400, 1730, 1610, 1150 and 1115 cm^{-1}. The NMR spectrum (Fig.XXVIII) of this compound confirmed its structure as (34).

The (A_1-b_2) fraction (0.20 g) when subjected to preparative TLC yielded a compound (35) which gave a superimposable IR spectrum with an authentic sample of 3-hydroxy cumic acid methyl ester (Fig.XXIX). Further properties could not be studied due to paucity of the material.

Preparation of 3-hydroxy cumic acid (35):

Cumic acid was nitrated in 80.0% yield using a mixture of concentrated sulphuric acid and nitric acid. The obtained 3-nitro cumic acid (m.p. 154-55°) was converted into its corresponding ester using diazomethane in ether (yield 85%).

Reduction of the nitro ester

3-Nitro cumic acid ester (1.1 g, 0.005 M) was dissolved in 5-6 ml of ethanol and then poured into 80.0 ml of 10.0% ammonium chloride solution. To this mixture 2.20 g (0.04 gram atom) of finely powdered iron was added and stirred at room temperature for 4 hr. It was then made acidic to congo red and extracted with ether. The ether layer was dried over sodium sulphate and distilled. Amount of amino ester obtained was 0.905 g.

Conversion of amino compound to hydroxy compound:

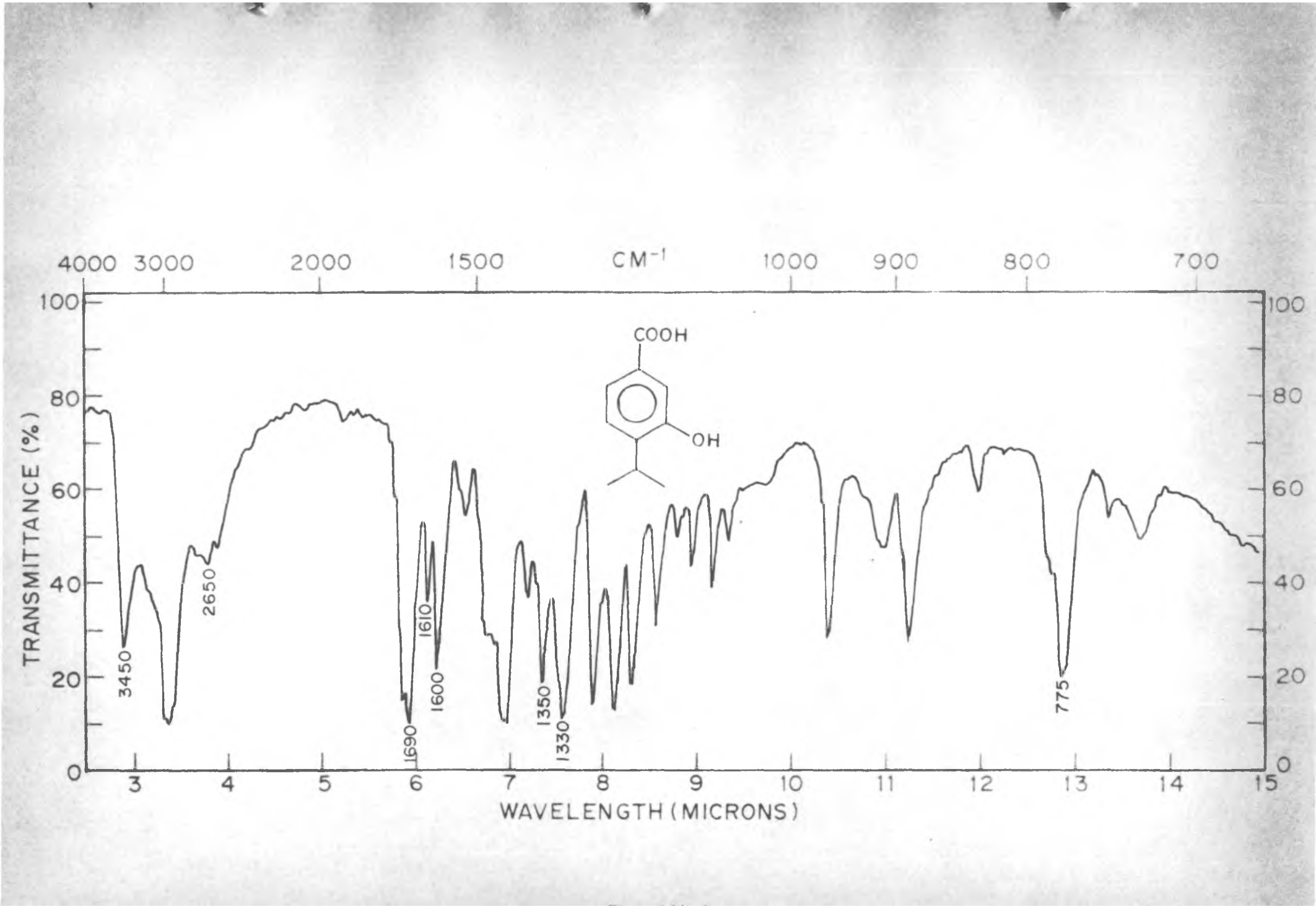
3-Amino cumic acid ester (1 mol) was dissolved in 50.0% sulphuric acid (3 moles), cooled to 5-10° and then diazotised with a cold solution of sodium nitrite (1 mol). The resulting diazonium salt was refluxed with 50% sulphuric acid for an hour and then extracted with ether. The ether layer yielded 3-hydroxy cumic acid (60%). The crude acid was crystallized from aqueous ethanol, m.p. 133-34°. IR spectrum (Fig. XLIV) ν_{max} at 3450, 2650, 1690, 1610, 1600, 1350, 1330 and 775 cm^{-1}.

n-Butanol fraction (A_2)

The total acids from butanol fraction (0.31 g) was treated with 2,4-dinitro phenyl hydrazine in ethanol (20 ml). The hydrazones formed were filtered (60mg) and taken in ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulphate and was concentrated under vacuum. TLC of this fraction (System V) found to contain three components out of which the component with R_f 0.48 had a similar mobility pattern as that of the hydrazone of isopropyl pyruvic acid (R_f 0.50). Due to the non-availability of the material, the other two hydrazones could not be separated and identified.

Fermentation of p-cymene with glucose grown cells

Twenty flasks each containing 90 ml of basal medium were sterilized and to each of the flasks 10 ml of 10% sterile glucose solution was added. The flasks were inoculated with p-cymene grown cells (1 ml inoculum to each flask) and incubated for 24 hr on a rotary shaker. After 24 hr


4000 3000 2000 1500 CM⁻¹ 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
COOH
OH
3450
2650
1690
1610
1600
1350
1330
775
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

1 ml of p-cymene was added to each flask and the fermentation was continued for another period of 24 hr. At the end of this period (total 48 hr) the contents were pooled, acidified and extracted with ether (250 ml X 3). The ether layer was separated into acidic and neutral fractions in the manner as described in Chapter II.

Similarly, another set of above experiment was carried out with cells fully adapted to glucose. These cells were obtained after two or three transfers on a medium containing 1% glucose. Other conditions were the same as those used for the above experiment.

TABLE (iii)

ACCUMULATION OF ACIDIC PRODUCTS WHEN FULLY AND PARTIALLY ADAPTED CELLS TO GLUCOSE WERE USED

Cells adapted to p-cymene	Cells partially adapted to glucose	Cells fully adapted to glucose
*2.01 g	*9.4 g	*1.4 g

*Obtained from a fermentation of 20 flasks

Chapter IV

ENZYMATIC STUDIES

ENZYMATIC STUDIES

DISCUSSION

(A) GROWTH AND MANOMETRIC STUDIES

It is evident from the nature of the compounds formed during the fermentation of p-cymene by the 'PL-strain' that the initial stages of the biodegradation of this hydrocarbon which is the oxidation of C_7-methyl group follows a pattern similar to that seen in organic oxidation reactions. The C_7-methyl group of p-cymene undergoes successive oxidation to cumic acid (C_7-COOH) via the intermediates cumic alcohol and cumic aldehyde. Identification of cumic alcohol and cumic acid from the culture medium has substantiated the above mode of oxidation. Also from the fermentation medium 3-hydroxy and 2,3-dihydroxy cumic acids were identified which suggests that after the formation of cumic acid it is progressively hydroxylated at C_3 and C_2. Failure to identify any aromatic compounds without an isopropyl side chain, from the fermented broth indicated that the hydroxylation is followed by the ring-cleavage with the isopropyl side-chain intact. In order to get further insight into the catabolic pathway of p-cymene, it was necessary to undertake growth and oxidation studies. The respirometric work was undertaken to determine whether the organism adapted to utilize p-cymene could degrade structurally related compounds or the suspected intermediates obtained during the degradation of p-cymene, with

or without any 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 the growth and the oxidation patterns of the intermediates that lie in the catabolic pathway of an aromatic compound by the organism grown on the parent hydrocarbon could indicate, but not prove, the probable intermediates and pathway of degradation.

Growth Studies

Washed suspension of 'PL-strain' grown on p-cymene, was found to grow without any lag period on cumic alcohol, cumic aldehyde, cumic acid, 3-hydroxy cumic acid, 2,3-dihydroxy cumic acid, isopropyl pyruvic acid and as well on 2,3-dihydroxy benzoic acid. On the other hand, the organism was incapable of utilizing 2-hydroxy, 3-hydroxy and 9-hydroxy p-cymene, 2-hydroxy cumic acid, 3-isopropyl catechol, catechol and α-p-tolyl propionic acid as the growth substrates (Table iv). These observations rule out the possibility of ring hydroxylation prior to the oxidation of the C_7-methyl group and also clearly indicate the possible sequential hydroxylation pattern.

Cross-adaptation studies indicated that cumic and 2,3-dihydroxy cumic acid grown cells grew on p-cymene after a lag period of 18 and 24 hr, respectively. From the Table(v) it is evident that cumic acid and 2,3-dihydroxy cumic acid adapted cells grew less rapidly on p-cymene than the cells adapted to p-cymene itself.

Growth pattern of this strain adapted to p-cymene indicated that it has a significant ability to utilize many aromatic compounds structurally similar to p-cymene as the sole source of carbon (Table vi) although they may not lie on the pathway of p-cymene degradation. Of the compounds tested p-hydroxy benzaldehyde, p-hydroxy, 2,3-dihydroxy, 2,5-dihydroxy and 3,4-dihydroxy benzoic acids were found to be good carbon sources for the organism. In contrast, salicylic acid, 3,5 dihydroxy and 2,4-dihydroxy benzoic acids were found to be utilized by the organism only after a lag period of 48 hr. However, m-toluic, p-toluic, 2,6-dihydroxy benzoic, 2,3-dihydroxy terephthalic acids and salicylaldehyde were quite resistant to dissimilation. Similarly, p-cresol at different concentrations failed to serve as the carbon source for the organism. Perhaps the presence of the methyl group para to the hydroxy may hinder the normal oxidation of the compound. The organism was capable of utilizing benzaldehyde and benzoic acid equally well, provided they are used at very low concentrations (0.1%).

The above studies indicated that the hydroxy benzoic acids were relatively easily oxidized by the organism than the methyl-substituted benzoic acids and phenolic compounds. Furthermore , the above data lend additional support to the fact that compounds with a hydroxyl group para to the carboxyl could serve as better growth substrates than those with a hydroxy group ortho to the carboxylic function.

Manometric Studies

The above results have been further confirmed by oxidation studies using manometric techniques. p-Cymene grown cells were incubated with a number of compounds and the rates of oxygen consumption were measured. The manometric studies with the various compounds substantiated the observations that were made in the case of growth pattern.

It is apparent from the QO_2 values listed in Table vii that p-cymene grown cells oxidize readily cumic alcohol, cumic acid, 3-hydroxy cumic acid, 2,3-dihydroxy cumic acid, 2,3-dihydroxy benzoic acid and isopropyl pyruvic acid. On the other hand, compounds such as 2-hydroxy p-cymene, 3-hydroxy p-cymene, 2-hydroxy cumic acid, 3-isopropyl catechol, catechol, α-p-tolyl propyl alcohol and α-p-tolyl propionic acid gave an oxygen uptake slightly above the endogenous value, but this uptake was not convincingly positive (Fig.XXX). Since there was no measurable biological oxygen consumption with these compounds, it was reasonable to conclude that they probably do not appear as intermediates in the catabolism of p-cymene. These results strongly suggest that the ring hydroxylation takes place after the progressive oxidation of the 7-methyl group of p-cymene.

The utilization of 3-hydroxy cumic acid by cells grown on p-cymene, and the appearance of this compound during the fermentation of p-cymene indicated that 3-hydroxy but not 2-hydroxy cumic acid was an intermediate in the metabolism of this hydrocarbon. 3-Hydroxylation was then followed

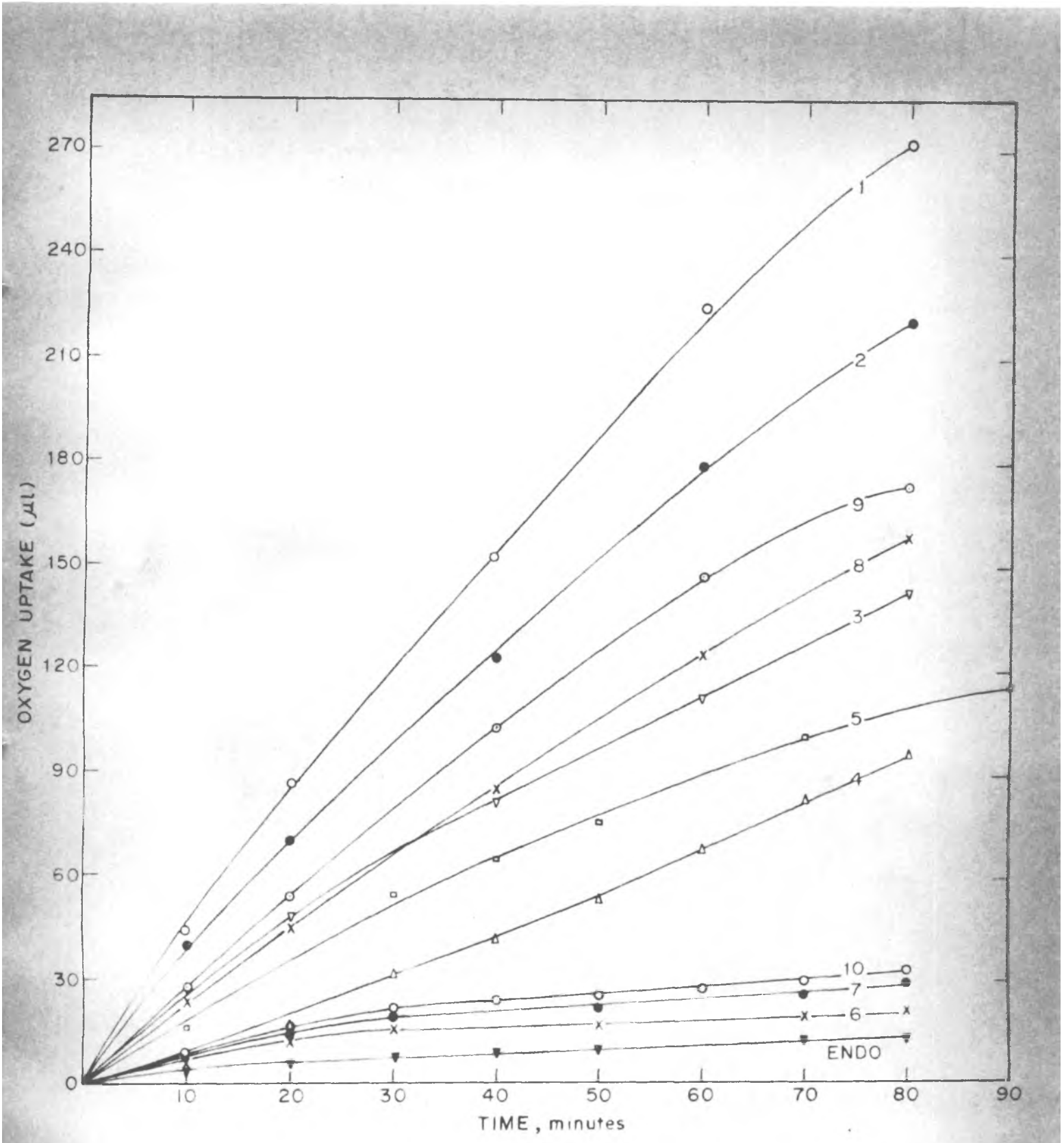


FIG XXX OXIDATION OF VARIOUS PROBABLE INTERMEDIATES BY p-CYMENE GROWN CELLS (1·44 mg DRY WEIGHT/FLASK)

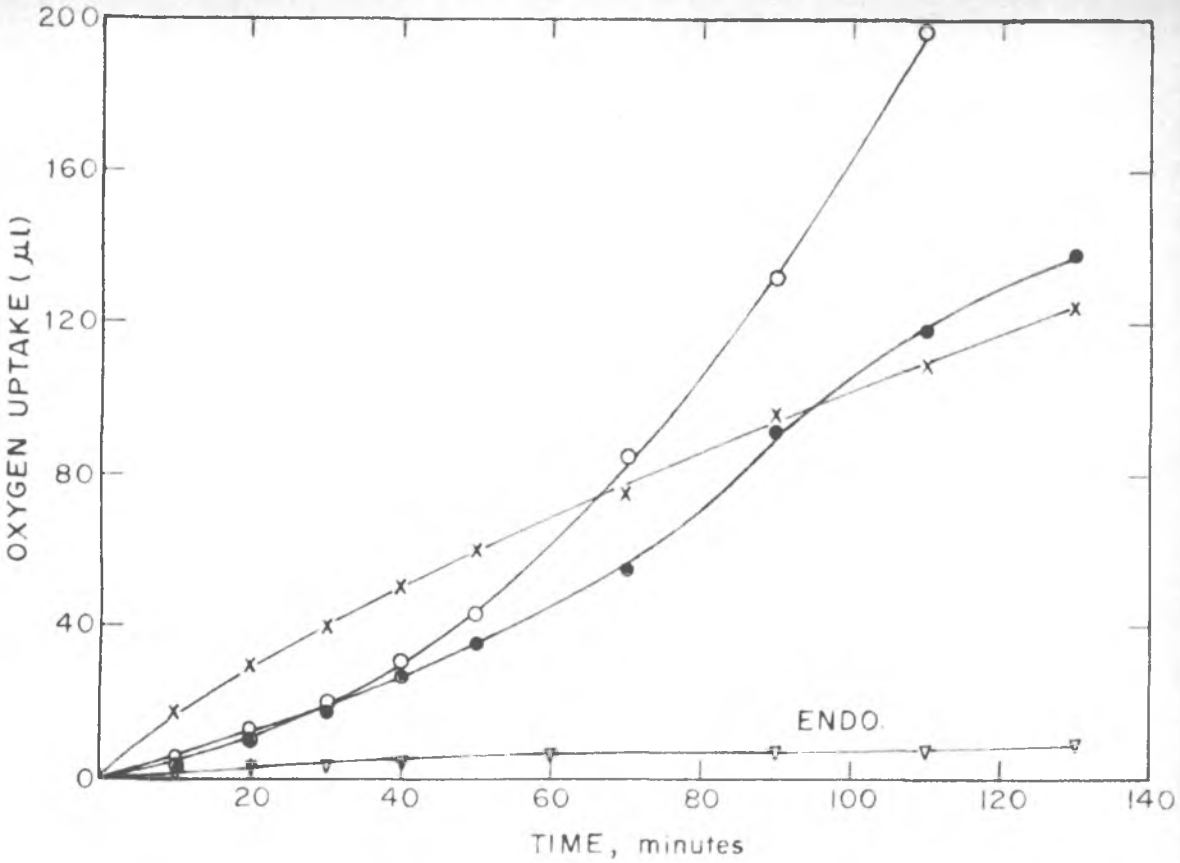
(1) p-CYMENE (2) CUMIC ALCOHOL (3) 2 3-DIHYDROXY CUMIC ACID
(4) 3-HYDROXY CUMIC ACID (5) ISOPROPYL PYRUVIC ACID
(6) 3-ISOPROPYL CATECHOL (7) 2-HYDROXY CUMIC ACID
(8) CUMIC ACID (9) 2,3-DIHYDROXY BENZOIC ACID
(10) α,P-TOLYL PROPYL ALCOHOL

by 2-hydroxylation giving 2,3-dihydroxy cumic acid prior to the ring cleavage. The possibility of decarboxylation of 2,3-dihydroxy cumic acid to 3-isopropyl catechol before the ring cleavage is not likely since the decarboxylated product was not further metabolized.

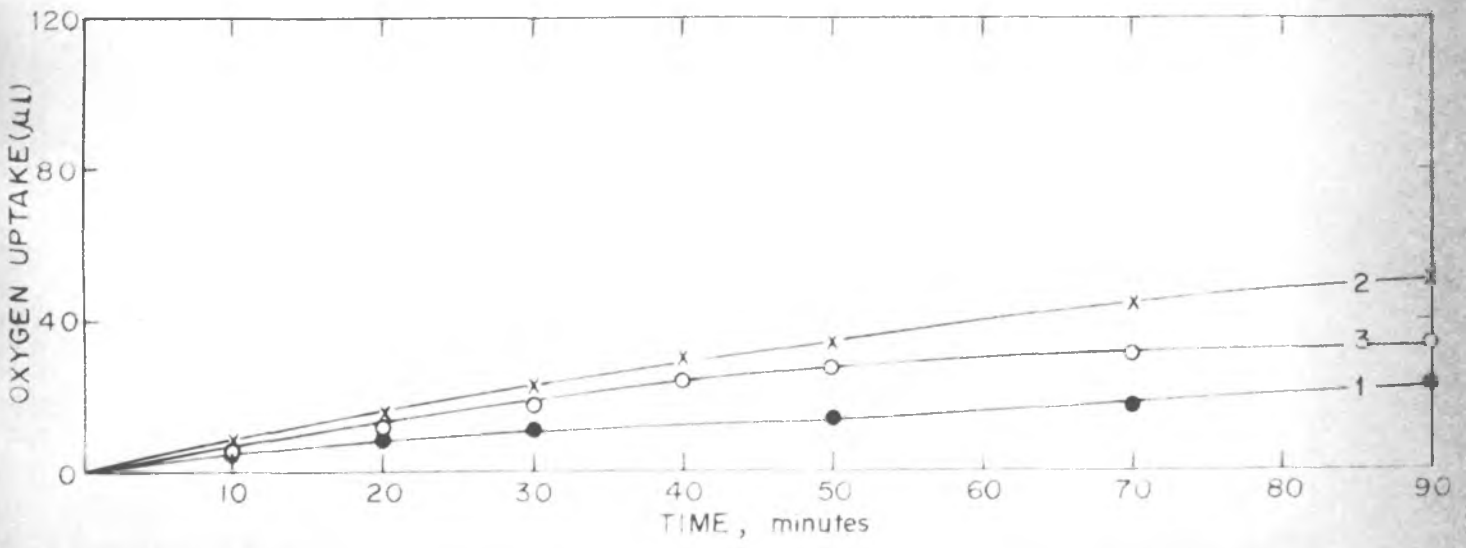
It is of interest to note that cells adapted to 2,3-dihydroxy cumic acid oxidized cumic acid and p-cymene only after a lag period of 50 and 70 min, respectively, and the rates of oxidation became extremely feeble in the presence of chloramphenicol (FigXXXIa,b). This clearly shows that the enzymes present in the cells are not constitutive but inducive in nature. These results lend additional support to the hypothesis that cumic and 2,3-dihydroxy cumic acids lie in the pathway for the metabolism of p-cymene.

The oxygen uptake by cells grown on glucose showed that p-cymene and cumic acid were oxidized in the absence of chloramphenicol after a lag period of 50 min (Fig.XXXIIa,b). The rate of oxygen consumption in the case of 2,3-dihydroxy cumic acid was very slow even in the absence of chloramphenicol. This to a certain extent explains the accumulation of 2,3-dihydroxy cumic acid in the broth when the fermentation of p-cymene was carried out with cells partially adapted to glucose. Glucose was oxidized by 'PL-strain' cells without any lag period with or without chloramphenicol.

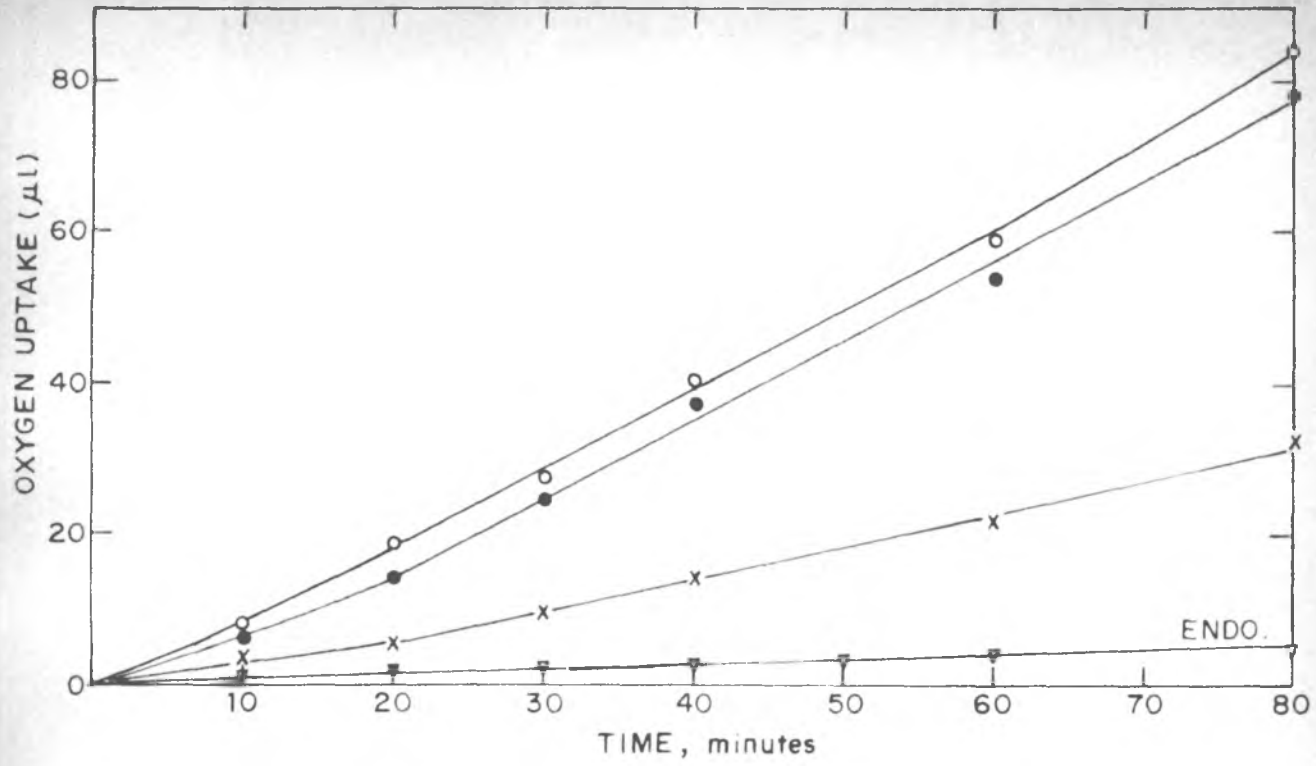
Oxygen uptake of this organism in presence of other acids structurally similar to the intermediates in p-cymene degradation, namely,



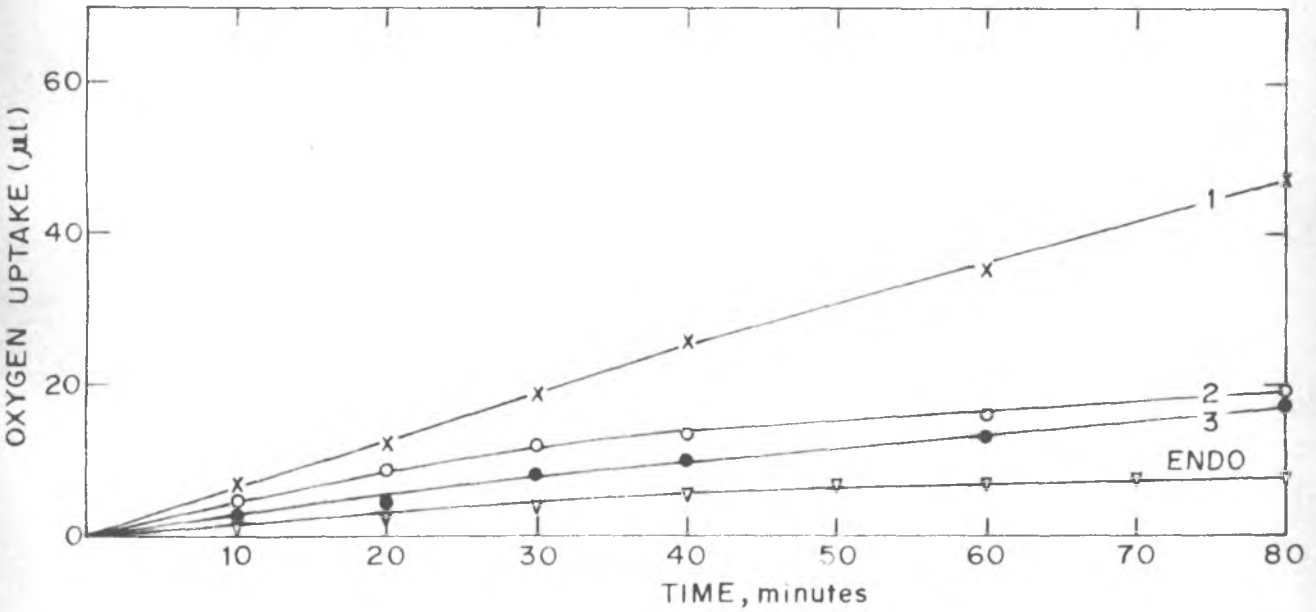
FIG·XXXI a. OXIDATION OF 2 3-DIHYDROXY CUMIC ACID (x——x) CUMIC ACID (o——o) AND P-CYMENE (●——●) BY 2 3--DIHYDROXY CUMIC ACID GROWN CELLS (1·46 mg DRY WEIGHT / FLASK).



FIG·XXXI b. OXIDATION OF P-CYMENE + CMP (1) CUMIC ACID + CMP(2) AND 2·3-DIHYDROXY CUMIC ACID + CMP(3) BY 2 3 DIHYDROXY CUMIC ACID GROWN CELLS (1 46 mg DRY WEIGHT / FLASK).



FIG·XXXII a. OXIDATION OF P-CYMENE (o——o), CUMIC ACID (●——●) AND 2·3-DIHYDROXY CUMIC ACID (x——x) BY GLUCOSE GROWN CELLS (1·54 mg. DRY WEIGHT / FLASK).



FIG·XXXII b. OXIDATION OF P-CYMENE + CMP(1), CUMIC ACID + CMP(2) AND 2·3 DIHYDROXY CUMIC ACID + CMP(3) BY GLUCOSE GROWN CELLS (1·54 mg. DRY WEIGHT/FLASK).

benzoic acid, p-hydroxy, 3,4-dihydroxy, 2,5-dihydroxy and 2,3-dihydroxy benzoic acids was studied. The rates of oxygen consumption in all these cases were significant and especially in the case of 2,3-dihydroxy, 3,4-dihydroxy and p-hydroxy benzoic acids, the rates were comparable to that observed with 2,3-dihydroxy cumic acid (Table viii, Fig. XXXIII).

As mentioned earlier, it is interesting to note that the organism can grow equally well on some hydroxy acids without the isopropyl side chain at C_4. Hence, as a probable intermediate, the presence of 2,3-dihydroxy benzoic acid was sought for in the culture medium. However, the failure to identify this compound from the fermented broth as well as from the incubations of 2,3 dihydroxy cumic acid with crude cell-free extract probably indicated that 2,3-dihydroxy benzoic acid may not be an intermediate in the biodegradation of p-cymene. It must be assumed, therefore, that this acid along with the other compounds that do not lie in the pathway have a close structural similarity with the parent compound to cause adaptation. Likewise, the possibility of catechol and isopropyl catechol as the intermediates were ruled out since neither of these compounds were oxidized in Warburg nor they were identified in the fermentation medium. The enzymatic studies as discussed later revealed that when 2,3-dihydroxy cumic acid was incubated with crude cell-free extract, isopropyl pyruvic acid and acetaldehyde were obtained.

Based on the above observation, the following plausible catabolic pathway of p-cymene has been postulated (Fig.XXXIV). The pathway for the

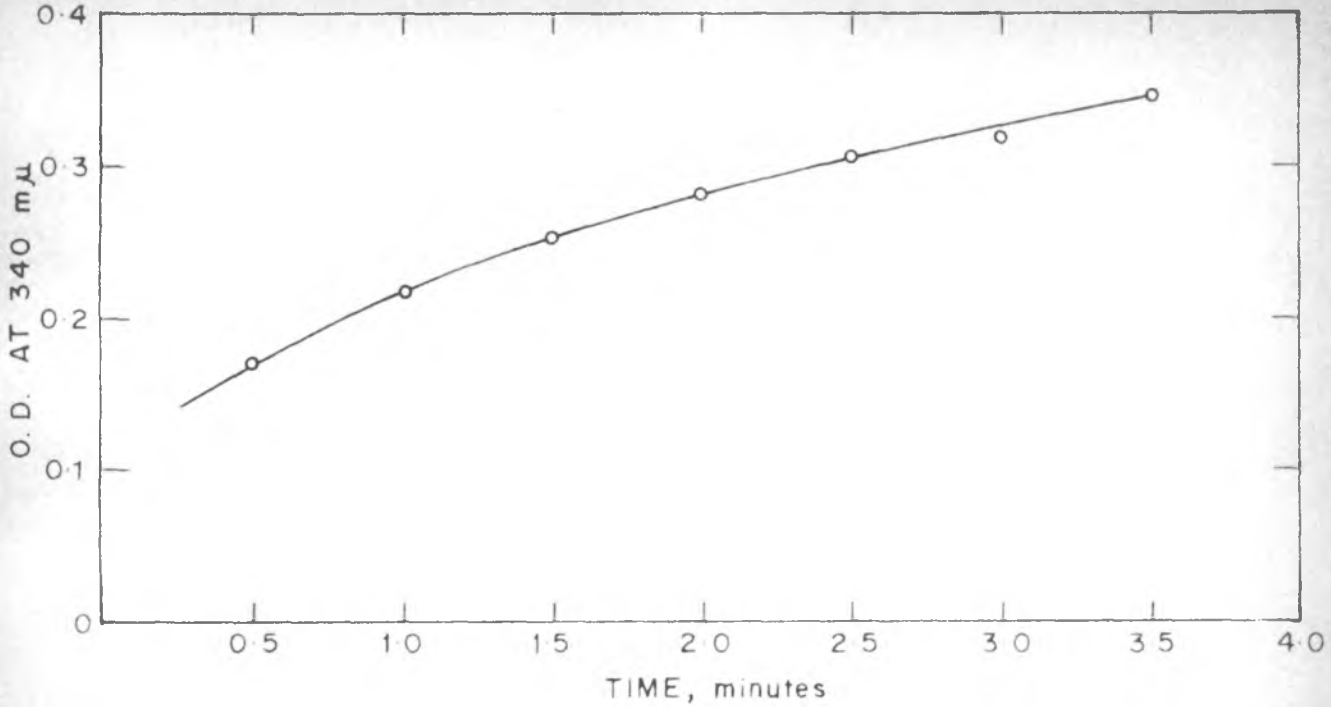


FIG. XL. ACTIVITY OF ALDEHYDE DEHYDROGENASE IN PRESENCE OF CUMIC ALDEDYDE.

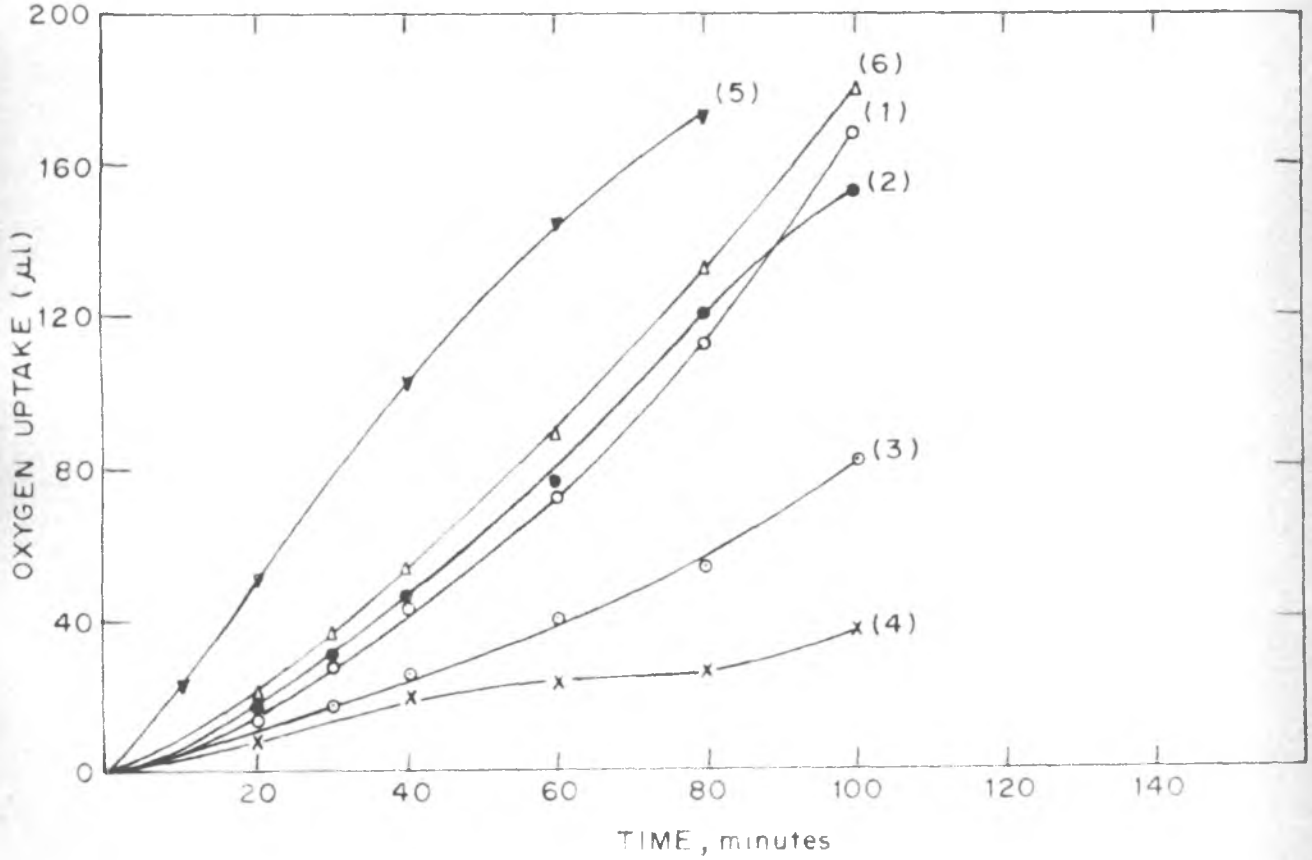
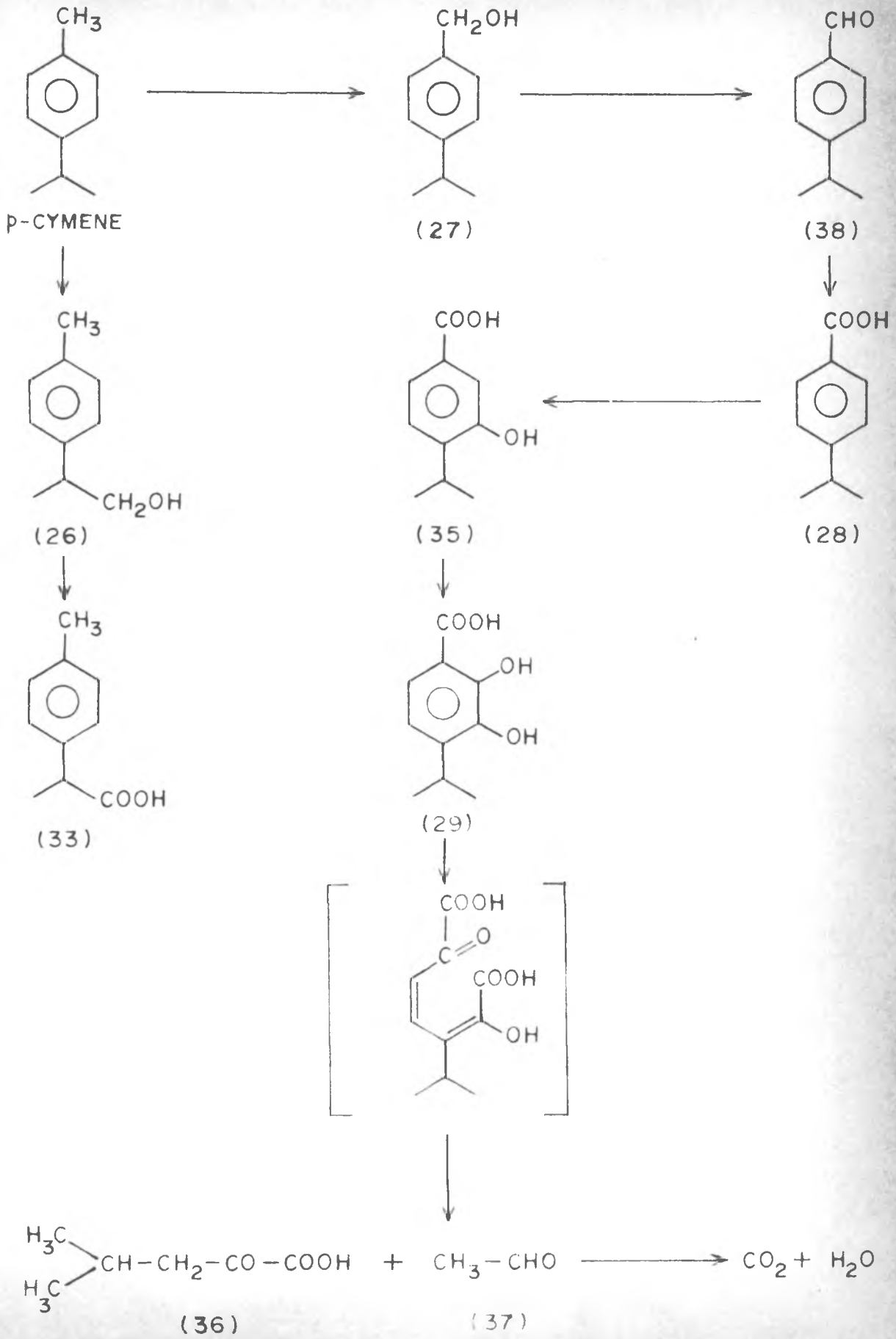


FIG. XXXIII OXIDATION OF VARIOUS AROMATIC ACIDS BY P-CYMENE GROWN CELLS (1·44 mg DRY WEIGHT / FLASK).

(1). p-HYDROXY BENZOIC ACID. (2) PROTOCATECHUIC ACID.
(3) 2,5-DIHYDROXY BENZOIC ACID. (4) SALICYLIC ACID
(5) 2,3-DIHYDROXY BENZOIC ACID. (6) BENZOIC ACID.

FIG. XXXIV. PROBABLE PATHWAY FOR THE DEGRADATION OF p-CYMENE



dissimilation of p-cymene was further confirmed by studying the different enzyme systems involved in this pathway which are discussed later in this chapter.

(B) STUDIES ON THE DIFFERENT ENZYME SYSTEMS

A general survey of the mechanisms of hydroxylation and ring cleavage of aromatic compounds has been dealt with before dealing individually the different enzymes which control the catabolic pathway of p-cymene.

All the studies on the aromatic ring degradation by bacteria have indicated that before the benzene nucleus is cleaved by oxygenases, two hydroxyl groups must be added to the benzene ring. The two hydroxyl groups usually should be ortho to each other, however, in some exceptional cases they are introduced across the nucleus in the 1,4-position. These groups are introduced into the benzene nucleus of the compounds usually by the action of a mixed function oxygenase. Sometimes the hydroxylation reactions are also brought about by dioxygenases (Kuno and Akaishi, 1961). The processes brought about by these two groups of oxygenases possess the following characteristics:

i) The source of the hydroxylic oxygen introduced is almost invariably the molecular oxygen. (Except in the case of hydroxylation of nicotinic acid to 6-hydroxy nicotinic acid by *Pseudomonas fluorescens* where water is the source of the hydroxylic oxygen (Hunt *et al.* 1947).

ii) Transition metal ions particularly ferrous ion have been implicated in most cases.

iii) An electron donor such as NADH or NADPH is required for the hydroxylation.

In hydroxylation reactions, it is probable that the function of the metal is to activate molecular oxygen, while in the ring opening, the phenolic substrates may simultaneously be activated through their ready capacity for electron donation.

Mono- and dioxygenases

Monooxygenases are bifunctional in nature catalysing the oxygen-fixation reaction on one hand and an oxidase type of reaction on the other. Mason (1957) has proposed the name "Mixed function oxidases" for these enzymes. They have recently been called "Hydroxylases" since many of them are concerned with hydroxylation of aromatic and steroidal compounds. Enzymatic hydroxylation of kynurenic acid (Taniuchi and Hayaishi, 1963) and the formation of catechol from salicylic acid (Hayaishi et al. 1963) fit into the class of monooxygenases.

Hayaishi et al. (1963) have shown that the formation of catechol from salicylic acid takes place in presence of NADH. One mole of FAD was found per mole of the enzyme protein. Assuming an active role for the ferrous ion in this reaction, the reaction sequence as depicted in Fig.XXXV may be suggested as a plausible mechanism. The activated oxygen

was visualized to react with the substrate as shown in Fig. XXXV. Here the substrate salicylic acid combines with the activated oxygen complex, presumably with a "perferryl ion" to form a ternary complex. One electron from the lone pair of the oxygen reduces the ferric ion. The anionic oxygen radical is simultaneously released from the complex and the subsequent electron shift enables this anionic oxygen radical to attack carbon 1 of the ring to form an oxygenated intermediate which is reduced by the electron donor, thus cleaving the bond between the two oxygen atoms and forming a mono oxygenated product and water.

In dioxygenase type reactions (Fig.XXXVI) as exemplified by the formation of catechol from anthranilic acid, the mechanism is more or less similar to that postulated for monooxygenase reactions. In this case, the substrate anthranilic acid combines with the activated oxygen complex to form a ternary complex. Once it is oxygenated, the enzyme protein will direct the flow of electrons to form a cyclic peroxide which is reductively cleaved to form catechol.

So in both mono- and dioxygenases the initial step of activation is effected by its divalent iron which forms a complex with oxygen and substrate. The flow of electrons through this complex activates both oxygen and the substrate to be oxygenated. Once the oxygenation of an organic substrate is brought about, further metabolism appears to be directed by the enzyme protein. The resultant oxygenated compound forms

FIG. XXXV MECHANISM FOR THE CONVERSION OF SALICYLIC ACID TO CATECHOL

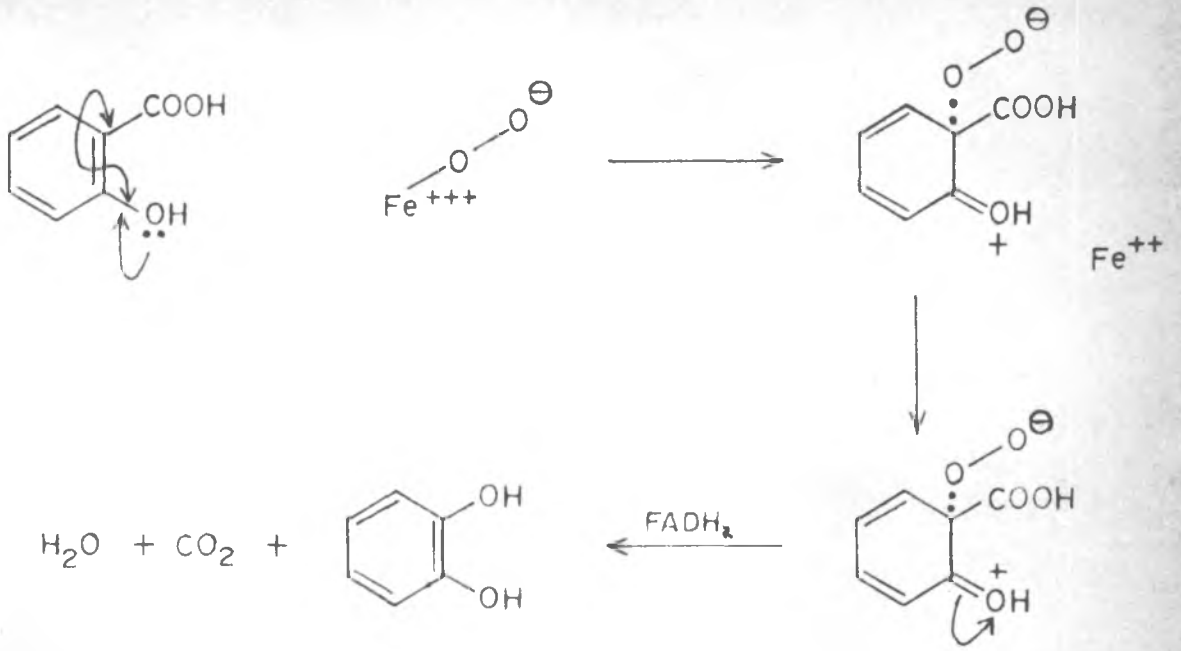
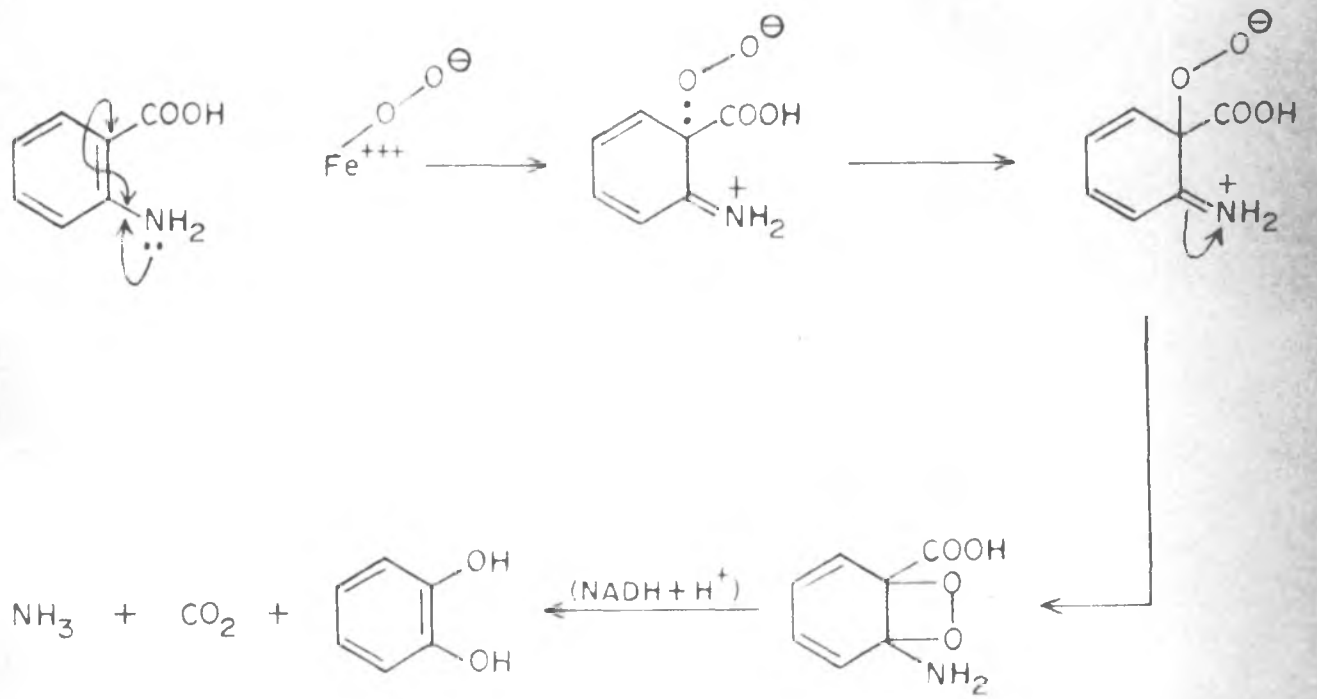
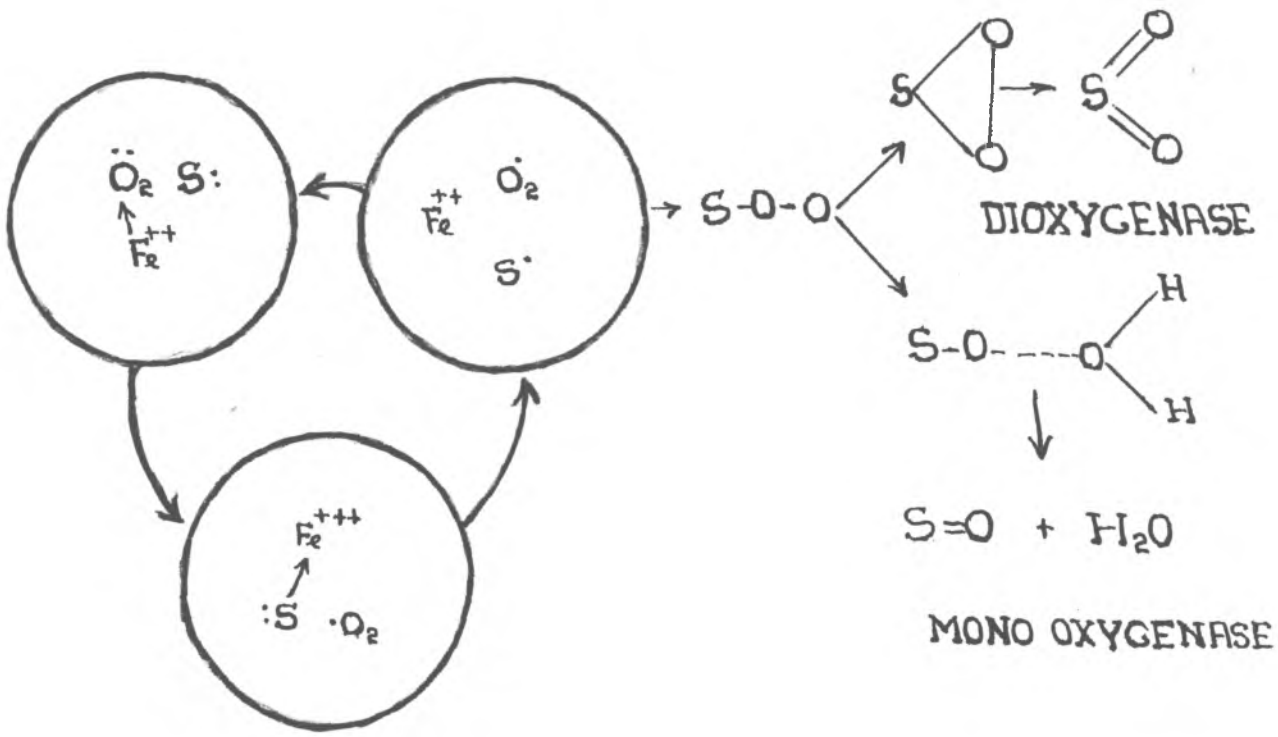


FIG. XXXVI. MECHANISM FOR THE CONVERSION OF ANTHRANILIC ACID TO CATECHOL



a cyclic peroxide intermediate, which is then either cleaved or reduced by an electron donor. According to Hayaishi (1964) a generalised mechanism for the two types of oxygenases can be represented as follows:



Aromatic ring-cleavage enzymes:

During the course of investigation on the metabolism of various aromatic compounds, a number of enzymes have been described which catalyze oxidative cleavage of the benzene nucleus of the dihydric phenol. This is accomplished by oxygenases which catalyze the incorporation of both atoms of molecular oxygen into the substrate. Pyrocatechase and metapyrocatechase are examples of such oxygenases, both of which act upon catechol. In some species of Pseudomonas, the benzene nucleus of catechol is cleaved by pyrocatechase to give cis-cis muconic acid (Evans et al. 1951; Hayaishi et al. 1957; Nakagawa et al. 1963) whereas, in other species catechol is oxidized

to 2-hydroxy muconic semialdehyde by an enzyme that has been designated as "catechol 2,3-oxygenase" by Dagley et al (1960) or "metapyrocatechase" by Kojima et al (1961).

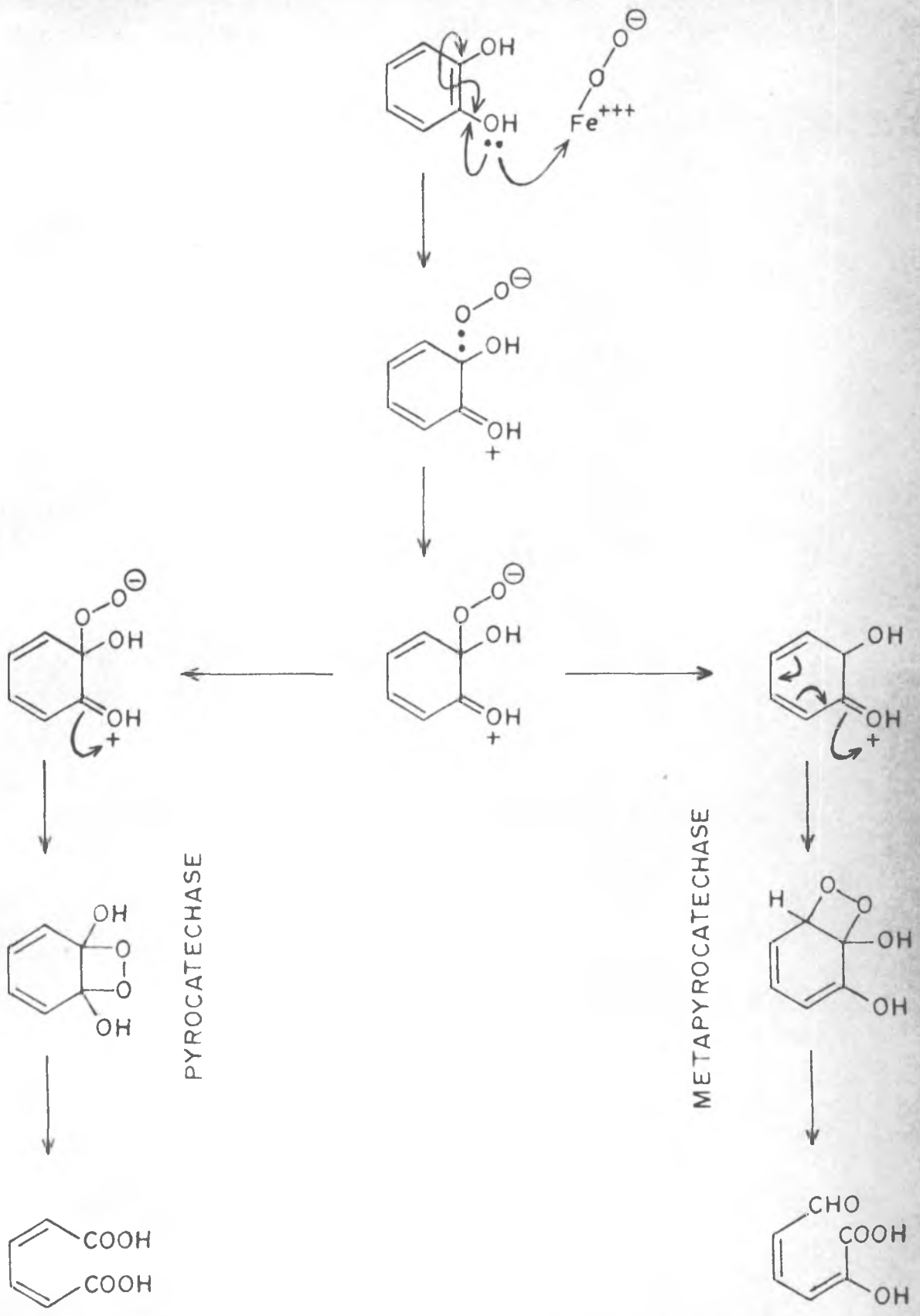
Proposed mechanism for metapyrocatechase and pyrocatechase type reactions

In this series of reactions the substrate catechol forms a complex with the activated oxygen (perferryl ion). An electron in the lone pair of the phenolic oxygen of catechol is transferred in order to reduce ferric ion. The anionic oxygen radical is simultaneously released from the complex attacking the α-carbon of catechol to form an oxygenated intermediate. The entire event is presumed to take place by a concerted mechanism in a ternary complex on the enzyme surface. Once the substrate is oxygenated the enzyme protein will direct the flow of the electrons and in the case of metapyrocatechase 1,6 cyclic peroxide is formed. On the other hand, pyrocatechase will facilitate the formation of a 1,2-cyclic peroxide which will be cleaved to form cis-cis muconic acid in the case of catechol (Fig.XXXVII).

Nature of pyrocatechase and metapyrocatechase

In an attempt to clarify the role of iron in pyrocatechase, Hayaishi et al (1955) studied the enzyme by electron spin resonance spectroscopy. Their results indicate that at least one atom of iron in native pyrocatechase is in the trivalent state (ferric state) and that the ferric ion undergoes reduction and reoxidation during the enzyme-catalyzed oxygenation of catechol. The amount of ferric ion bound to the enzyme is found

FIG. XXXVII PROPOSED MECHANISM FOR THE REACTION OF METAPYROCATECHASE AND PYROCATECHASE



to be one atom per molecule of the enzyme protein and it appears to be directly associated with the enzyme activity. When the trivalent iron in the enzyme combines with the substrate (S), the iron is reduced by the substrate and then it reacts with oxygen to form a complex. The transfer of an electron from iron to oxygen yields the highly reactive complex in which the two free radicals react to form an oxygenated product.

In the case of metapyrocatechase it has been shown that the iron is in the divalent state which then combines with oxygen to form $Fe^{++}O_2$ and is stabilized by the following ionic resonance forms:

(I) (II) (III)

It is known that ferric ion but not ferrous ion easily forms a complex with catechol. The existence of the species (III) has, however, been questioned from energy consideration (Bhattacharyya and Ganapathy, 1965). The species (II) combines with the substrate and forms a ternary complex.

At least one atom of iron in the trivalent state (ferric) is present in free pyrocatechase, while in metapyrocatechase it is in divalent state. Furthermore, metapyrocatechase is easily inactivated by oxidising agents while pyrocatechase is resistant to oxidizing agents and instead it is inactivated by reducing agents.

In the present work experiments were designed to demonstrate

qualitatively the existence of some of the enzymes which participate in the biodegradation of p-cymene. The enzymatic data obtained here fully substantiated the conclusions derived from the fermentation, growth and manometric studies. The first step in the degradation of p-cymene according to the scheme (Fig.XXXIV) is the oxidation of the C_7-methyl of p-cymene to a primary alcoholic group (CH_2OH) giving rise to cumic alcohol. A similar type of conversion was observed in the case of limonene (Dhavalikar and Bhattacharyya, 1966). The activity of the cell-free preparations was very feeble as far as this hydroxylation was concerned and no further attempts were made to study this system.

Alcohol dehydrogenase

The second step in the catabolism of p-cymene is the conversion of cumic alcohol to cumic aldehyde catalysed by a NAD linked alcohol dehydrogenase and has been demonstrated in the 15,000 X g supernatant of the cell-free extract. It is evident from the Table ix that cell-free extracts exhibited a slightly faster rate of reduction of NAD with cumic alcohol as compared to 9-hydroxy p-cymene and 2,3-dihydroxy cumic alcohol. Failure to get sufficient quantity of cumic alcohol from the fermented broth also suggested the presence of a strong cumic alcohol dehydrogenase in the system.

Aldehyde dehydrogenase

The third step in the degradation of p-cymene is the conversion of

cumic aldehyde to cumic acid which is brought about by an aldehyde dehydrogenase. The enzyme has been shown to be present in 15,000 X g supernatant. This dehydrogenase is also NAD dependent and showed greater activity in presence of cysteine. No reduction of NAD was observed in the presence of cumic aldehyde and boiled cell-free extract.

Hydroxylase

The oxidation of cumic aldehyde to cumic acid is followed by two successive hydroxylations of cumic acid to 3-hydroxy and 2,3-dihydroxy cumic acids, respectively. Major part of the 3-hydroxylating activity was present in 15,000 X g supernatant and very little residual activity was associated with the 15,000 X g sediment fraction. Data summarised in Table x show that C-3 hydroxylation of cumic acid is greatly increased in presence of NADH (generating system) as compared to NADPH (generating system). In both the incubations the formation of 3-hydroxy cumic and isopropyl pyruvic acids were taken as indices for the hydroxylating activity. All attempts to get 2,3-dihydroxy cumic acid from cumic acid when the latter was incubated with the cell-free extract in presence of a NADH generating system failed. Probably in this extract as soon as 2,3-dihydroxy cumic acid is formed it is cleaved further to isopropyl pyruvic acid, the presence of which was shown in the reaction mixture. Since this hydroxylase brings about the hydroxylation of cumic acid to 2,3-dihydroxy cumic acid in two stages and is probably NADH dependent, it may be classified as a mixed function oxidase or a mono oxygenase.

Mode of hydroxylation

It has been shown that cumic acid is initially hydroxylated at the position meta to the carboxylic group giving rise to 3-hydroxy cumic acid. The second hydroxylation takes place ortho to the one which is already present. However, since the 3-hydroxylation and the subsequent 2-hydroxylation take place at the electronegative sites of the ring, it is difficult to reconcile with the anion-radical mechanism proposed by Hayaishi (1964). It is not unlikely that these are brought about by a cationic or electron deficient oxygenating species which has been described by Mason (1965), and Ganapathy and Bhattacharyya (1965).

It was observed that the organism can use p-hydroxy benzoic acid as the carbon source. When this organism grew on p-hydroxy benzoic acid as the carbon source, 3,4-dihydroxy benzoic acid was formed in the growth medium. The presence of this acid was confirmed by the spectral comparisons of the authentic sample with the acid obtained from the culture medium (Fig.XXXVIII). In this instance the second hydroxylation also takes place ortho to the hydroxy group that is already present. This ortho diphenolic compound has got the necessary structural prerequisite to undergo an oxygenative ring cleavage which might follow one of the usual pathways that have been already worked out in the case of 3,4-dihydroxy benzoic acid (protocatechuic acid).

The organism is capable of growing on benzoic acid without any

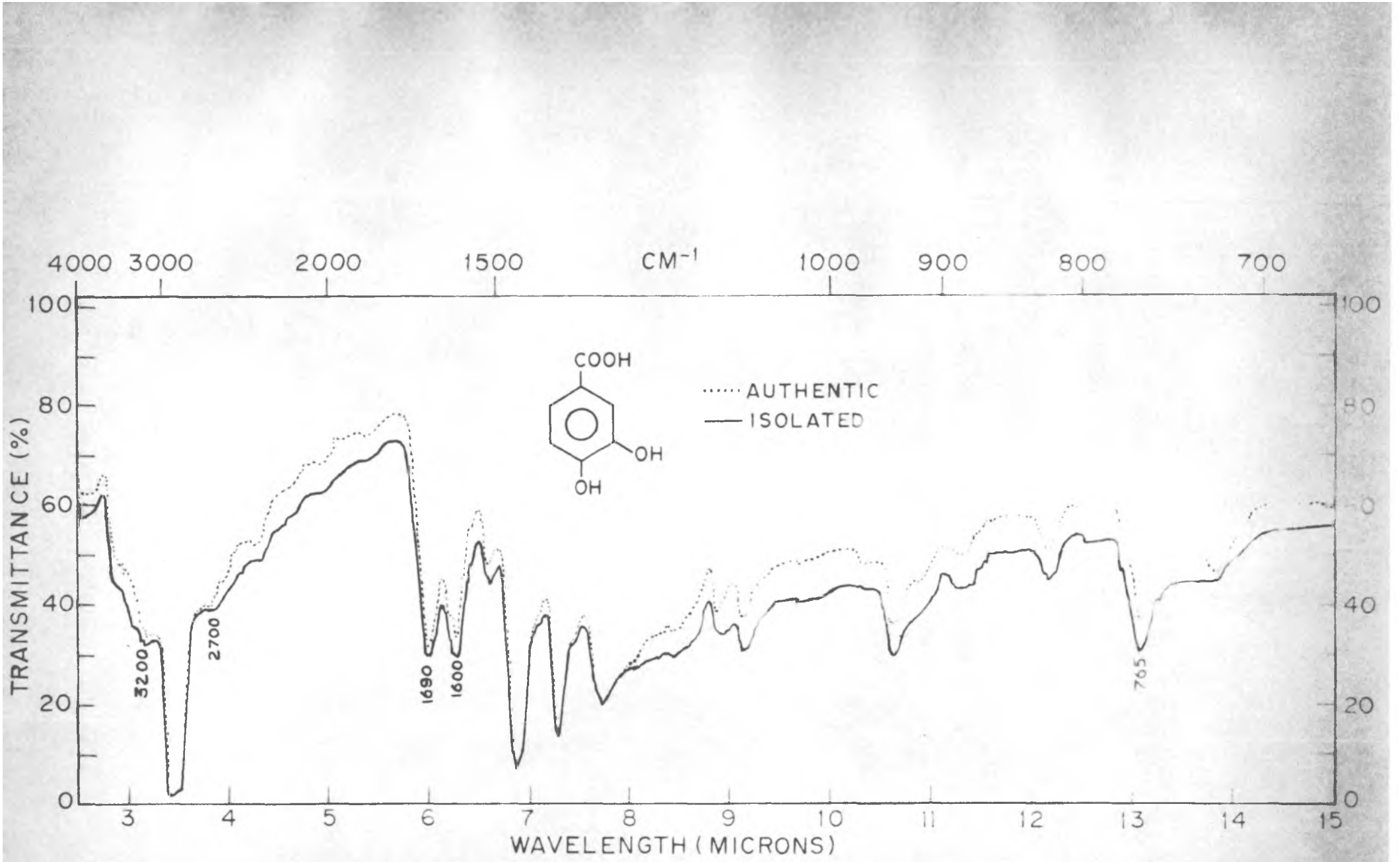

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
COOH
OH
OH
AUTHENTIC
ISOLATED
3200
2700
1690
1600
765
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG XXXVIII

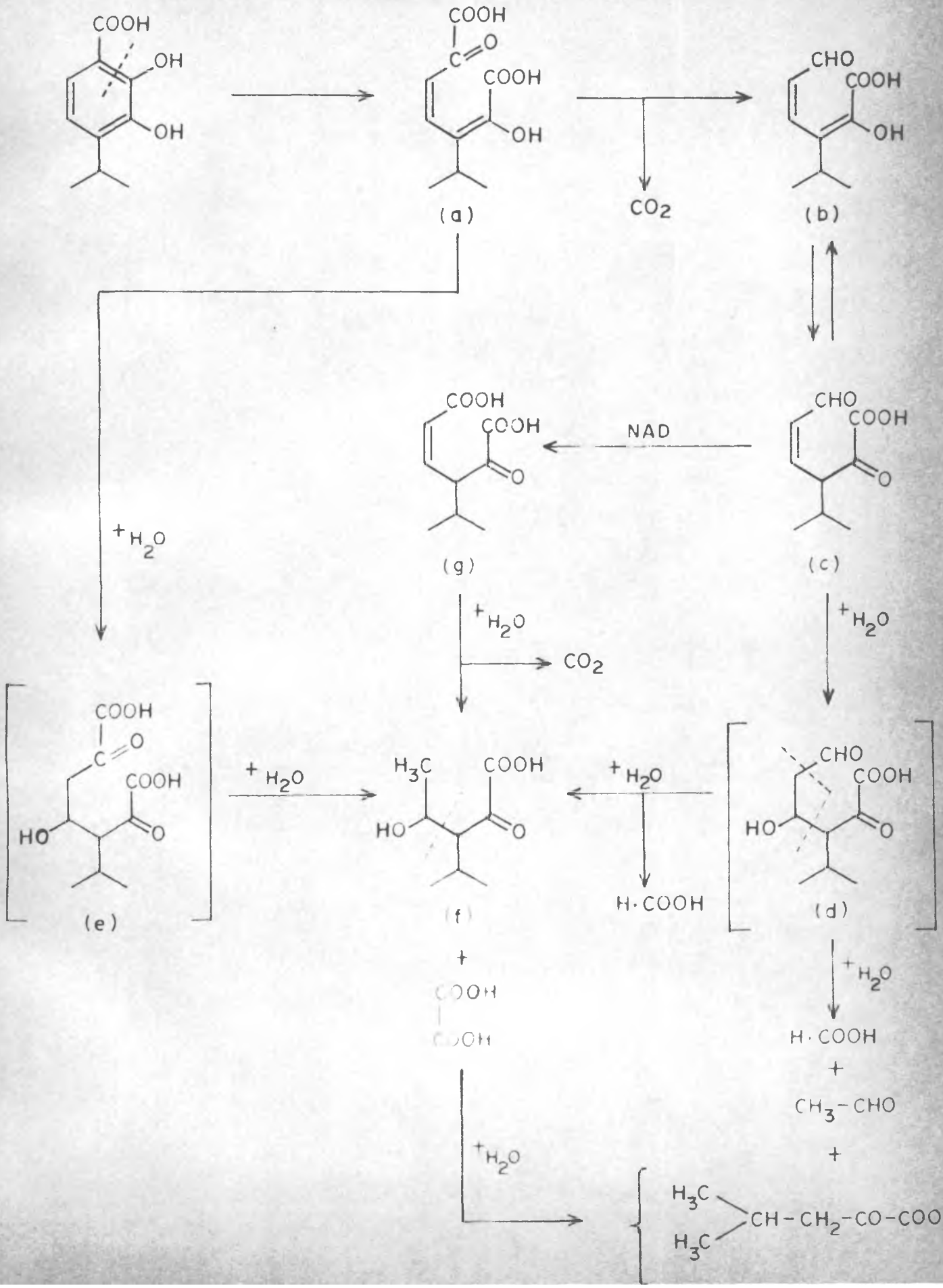
lag phase. When the washed suspension of the 'PL-strain' was incubated for 24 hr in a mineral salt medium containing benzoic acid, the incubation mixture contained a compound which in its mobility pattern corresponded to p-hydroxy benzoic acid. However, the presence of p-hydroxy benzoic acid in the reaction mixture could not be conclusively established. It was observed that p-cymene grown cells did not oxidize catechol and the rate of oxidation of salicylic acid with these cells was very slow. Failure to identify salicylic acid, or catechol from the fermented broth when benzoic acid was used as the substrate indicated that probably benzoic acid may not be metabolized via salicylic acid or catechol. This may be taken as an indirect indication that p-hydroxylation of benzoic acid molecule might take place in this system resulting in the formation of p-hydroxy benzoic acid. This is further converted to 3,4-dihydroxy benzoic acid. However, based on these isolated observations it is rather difficult to arrive at any precise conclusion regarding the pattern of hydroxylation of benzoic acid.

Evans in 1947 has reported the conversion of benzoic acid to 3,4-dihydroxy benzoic acid by a Vibrio 01. Both the possible intermediates in this pathway, namely, p-and m-hydroxy benzoic acids when employed as substrates yielded 3,4-dihydroxy benzoic acid. Salicylic acid was not utilized by this organism. On the basis of this data they have suggested that either p- or m-hydroxy benzoic acid might lie in the pathway of benzoic acid degradation. However, the isolation of either p- or m-hydroxy benzoic acids as metabolites of benzoic acid has not been reported so far in pseudomonads.

Cleavage enzymes

The next enzyme in this sequence brings about the cleavage of the benzene nucleus of 2,3-dihydroxy cumic acid. The dihydroxy acid was incubated without the addition of any cofactors with the cell-free extracts (15,000 X g supernatant) and from the incubation mixture isopropyl pyruvic acid and acetaldehyde were isolated. Identification of isopropyl pyruvic acid and acetaldehyde as the ring fission products of 2,3-dihydroxy cumic acid clearly indicated the position of the ring-cleavage in the oxidative metabolism of p-cymene by 'PL-strain'. It is reasonable, therefore, to assume that the cleavage of the benzene nucleus of 2,3-dihydroxy cumic acid might have taken place between the carboxyl and the hydroxyl functions giving rise to an hypothetical intermediate (a), the presence of which could not be detected, probably due to the unstable nature of the compound or else further enzymes in the pathway might have been more powerful than the cleavage enzyme. If the cleavage would have taken place between the two hydroxyls or in any other position, the formation of isopropyl pyruvic acid could not be explained. The formation of isopropyl pyruvic acid and acetaldehyde has been schematically shown in Fig. XXXIX. After the formation of the ring cleavage intermediate (a), there are two possibilities for the formation of isopropyl pyruvic acid and acetaldehyde. Accordingly, in one sequence compound (a) will be decarboxylated to compound (b) which will be in equilibrium with its tautomeric form (c). The next stage in the degradation will be the hydration of the double bond to give compound (d) followed

FIG. XXXIX GENERAL SCHEME FOR THE DEGRADATION OF 2,3-DIHYDROXY CUMIC ACID



by cleavage to formic acid, acetaldehyde and isopropyl pyruvic acid, of which the last two have been identified.

Alternatively, splitting of the compound (a) to isopropyl pyruvic acid and acetaldehyde can be explained by a postulated hydration of the compound (a) to (e) which on hydrolytic fission gives oxalic acid and compound (f). Compound (f) then splits to isopropyl pyruvic acid and acetaldehyde. Additional evidence for the above type of cleavage has been obtained in the case of ring cleavage of 2,3-dihydroxy benzoic acid. Cell-free extracts were incubated in presence of 2,3-dihydroxy benzoic acid and from the incubation mixture both pyruvic acid and acetaldehyde were identified. In this case also the formation of these products requires a cleavage between the carboxyl and the hydroxyl groups.

A similar type of cleavage for 2,3-dihydroxy benzoic acid has been suggested by Ribbons (1966). He has shown that extracts of cells catalyzed the oxidation of 2,3-dihydroxy benzoic acid with the consumption of 1 mole of oxygen and the evolution of 1 mole of carbon dioxide per mole of substrate. Here neither 2,3-dihydroxy benzoate was decarboxylated to catechol nor was catechol oxidized by extracts of this strain. In this case α-hydroxy muconic semialdehyde was identified as the ring cleavage product. On the basis of this it was suggested that the cleavage has taken place between the carboxyl and hydroxyl groups. Intermediates between 2,3-dihydroxy benzoate and α-hydroxy muconic semialdehyde have not been detected. The

enzymes responsible for α-hydroxy muconic semialdehyde degradation to pyruvate by *P.fluorescens 23 D-1* appear to catalyze reactions identical to those already established by Dagley *et al.* (1964).

It has already been mentioned in the growth and manometric studies that catechol and 3-isopropyl catechol were not utilized by the washed suspensions of 'PL-strain' which indicated, that carboxyl group present in 2,3-dihydroxy cumic and 2,3-dihydroxy benzoic acids were not decarboxylated before the cleavage of the benzene ring. It was also noticed that the incubation of catechol with 15,000 X g supernatant did not result in any cleavage product. In all probability, therefore, in the present organism decarboxylation takes place only after the cleavage of the benzene ring.

EXPERIMENTAL

GROWTH AND MANOMETRIC STUDIES

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 probable intermediates as well as on different structurally similar compounds are presented in Table vi and iv, respectively. The cells adapted to 2,3-dihydroxy cumic acid had a retarded growth on p-cymene and cumic acid in the initial stages of incubation (Table vi).

Manometric studies

Preparation of cells for manometry: Flasks containing 100 ml of mineral salt medium of the composition described earlier were inoculated as above with a 24-hr grown culture. After the addition of the respective substrates they were incubated at 28°±2 on a rotary shaker for 24-hr. At the end of this period the cells were centrifuged at 15,000 X g at 0-5°. The cells were washed twice with 0.05 M phosphate buffer (pH 7.0) and finally suspended in the same buffer to give a reading of 270-300 in a

Klett-Summerson colorimeter at 660 mμ (1.3-1.5 mg dry wt./ml). This cell suspension was used for manometric studies. Dry weights were calculated from a calibration curve relating the turbidity reading of the washed cell suspension to a known dry weight content of the organism.

Manometric methods

Oxygen uptake by the washed cell suspensions in presence of various substrates was followed manometrically in the Warburg apparatus (Umbreit et al. 1959). In these experiments the cell suspension usually 1.5 ml (1.5-2.5 mg of dry wt.) and phosphate buffer (1.0 ml, 0.05 M, pH 7.0) were placed in the main compartment of the Warburg flask and a solution of potassium hydroxide (0.2 ml, 20%) was added to the central well. Individual substrates (10-12 μM) were added from the side arm. When chloramphenicol was used, its concentration was 50 μg per ml. Distilled water was added and the final volume was adjusted to 3.0 ml. The incubations were carried out at 28°±0.2. The rates of oxidation are listed in Table vii and viii and graphically represented in Fig. XXX, XXXIa,b, XXXIIa,b and XXXIII.

TABLE viii*

OXYGEN UPTAKE BY p-CYMENE-GROWN CELLS IN PRESENCE OF VARIOUS RELATED AROMATIC COMPOUNDS

Substrates	Q_{O_2} (μl/mg dry wt./hr)
1) Benzoic acid	85
2) p-Hydroxy benzoic acid	102
3) 3,4-Dihydroxy benzoic acid	91
4) 2,5-Dihydroxy benzoic acid	50
5) Salicylic acid	45

*Legend as in Table vii

TABLE iv

GROWTH OF THE ORGANISMS ON PROBABLE INTERMEDIATES

Compound	Concentration in 100 ml (%)	Growth*		
		24 hr	48 hr	72 hr
1) p-Cymene	0.20	+++	+++	+++
2) 2-OH-p-Cymene	0.20	-	-	-
	0.10			
3) 3-OH p-Cymene	0.10	-	-	-
	0.20			
4) α-p-Tolyl propyl alcohol	0.10	-	-	-
5) 3-Isopropyl catechol	0.10	-	-	-
6) Catechol	0.10	-	-	-
7) Cumic alcohol	0.10	+++	+++	+++
8) Cumic aldehyde	0.10	++	+++	+++
9) Cumic acid	0.10	+++	+++	+++
10) α-p-Tolyl propionic acid	0.10	-	-	-
11) 2-OH Cumic acid	0.10	-	-	-
12) 3-OH Cumic acid	0.10	++	+++	+++
13) 2,3-Dihydroxy cumic acid	0.10	++	+++	+++
14) 2,3-Dihydroxy benzoic acid	0.10	++	+++	+++
15) 2,3-Dihydroxy terephthalic acid	0.10	-	-	-
16) Isopropyl pyruvic acid	0.10	+	++	++

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

Mineral salt medium (100 ml) containing the various substrates (0.1%) was inoculated with 2.0 ml of freshly washed cells (300 Klett reading) of a 24-hr grown culture and was incubated on a rotary shaker at 28°±0.2.

TABLE V

GROWTH AND CROSS-ADAPTATION STUDIES

Inoculum*	Time (hr)	Growth pattern on** Klett readings		
		p-Cymene	Cumic acid	2,3-Dihydroxy-cumic acid
1) p-Cymene grown cells	0	46	48	50
	24	390	290	200
	48	450	360	250
	72	550	400	300
2) Cumic acid grown	0	44	46	48
	24	320	285	230
	48	350	365	250
	72	425	410	285
3) 2,3-Dihydroxy cumic acid grown cells	0	42	44	47
	24	300	220	285
	48	360	250	325
	72	405	310	355

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

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

TABLE vi

GROWTH OF THE ORGANISM ON VARIOUS RELATED AROMATIC COMPOUNDS

Compound	Concentration in 100 ml (%)	Growth*			
		24 hr	48 hr	72 hr	96 hr
1) Benzoic acid	0.10	+	++	+++	+++
2) Benzaldehyde	0.10	+	++	+++	+++
3) p-Cresol	0.10	-	-	-	-
4) Salicylaldehyde	0.10	-	-	-	+
5) Salicylic acid	0.10	-	+	++	+++
6) m-Toluic acid	0.10	-	-	-	-
7) 2,5-Dihydroxy benzoic acid	0.10	++	+++	+++	+++
8) 3,4-Dihydroxy benzoic acid	0.10	++	+++	+++	+++
9) 3,5-Dihydroxy benzoic acid	0.10	-	-	++	++
10) 2,6-Dihydroxy benzoic acid	0.10	-	-	-	-
11) 2,4-Dihydroxy benzoic acid	0.10	-	-	+	++
12) 2,3-Dihydroxy benzoic acid	0.10	++	+++	+++	+++
13) 2,3-Dihydroxy terephthalic acid	0.10	-	-	-	-
14) Orcinol	0.10	-	-	-	-

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

Mineral salt medium (100 ml) containing the various substrates (0.1%) was inoculated with 2.0 ml of freshly washed cells (300 Klett reading) of a 24-hr grown culture and was incubated on a rotary shaker at 28° ± 0.2.

TABLE vii

OXYGEN UPTAKE BY p-CYMENE-GROWN CELLS IN PRESENCE OF NEUTRAL AND ACIDIC TRANSFORMATION PRODUCTS OF p-CYMENE AND OTHER PROBABLE INTERMEDIATES

Substrates	Q_{O_2} (µl/mg.dry wt./hr.
1) p-Cymene	210
2) 2-Hydroxy p-cymene	10
3) 3-Hydroxy p-cymene	11
4) ∝-p-Tolyl propyl alcohol	20
5) Cumic alcohol	170
6) Cumic acid	124
7) 2-Hydroxy cumic acid	17
8) 3-Hydroxy cumic acid	55
9) 2,3-Dihydroxy cumic acid	114
10) 2,3-Dihydroxy benzoic acid	129
11) ∝-p-Tolyl propionic acid	10
12) Isopropyl pyruvic acid	80
13) 3-Isopropyl catechol	12
14) Catechol	13
15) Endogenous	7.5

Each flask contained 1.5 ml cell suspension containing 1.44 mg (dry wt. of cells), 10-12 µM of substrate (side arm), 1.0 ml phosphate buffer (pH 7.0) and 0.2 ml of KOH (20%) in the central well. The total volume was made to 3.0 ml.

STUDIES ON THE DIFFERENT ENZYME SYSTEMS

Preparation of cell-free extracts from p-cymene grown cells

The cells grown on p-cymene (24 hr) were harvested, and washed as described under "Methods and Materials". Washed cells (wet weight 10 g) were suspended in phosphate buffer (50.5 ml, 0.05 M, pH 7.0), 5 ml of buffer was used for each gram of cell paste. It was homogenised and then disrupted by ultra sonic vibrations for 10-12 min. The sonicate (50 ml) was centrifuged for 45 min at 15,000 X g and the supernatant was used for alcohol and aldehyde dehydrogenase assays as well as to study the hydroxylating and the ring cleavage (2,3-dihydroxy cumic acid ⟶ isopropyl pyruvic acid) enzyme systems. All operations were carried out at 0-4°. The protein concentration in the extracts was estimated according to the method of Warburg and Christian (1942). These extracts usually contained 15-20 mg of protein/ml.

I. Alcohol dehydrogenase

Alcohol dehydrogenase activity in 15,000 X g supernatant of crude cell-free extract of p-cymene grown cells was estimated by NAD reduction in presence of suitable aliquots of enzyme and substrate. NAD reduction was followed by measuring the optical density change at 340 mμ in a Beckman model DU spectrophotometer, using a 3 ml cuvette of 1 cm light path. The data presented in Table ix indicated the presence

of an alcohol dehydrogenase capable of catalysing the reduction of NAD in presence of cumic alcohol, α-p-tolyl propyl alcohol and 2,3-dihydroxy cumic alcohol. No reduction of NAD was observed in presence of a boiled extract of 15,000 X g supernatant. In the absence of the substrate there was very little NADH oxidase activity which was measured by the decrease in optical density at 340 mμ.

TABLE ix

ALCOHOL DEHYDROGENASE ACTIVITY OF DIFFERENT SUBSTRATES IN CELL-FREE EXTRACT OF p-CYMENE GROWN CELLS

Substrate	Protein mg/ml	Activity units/ml	Specific activity
1) Cumic alcohol	10.4	8,400	819
2) 2,3-Dihydroxy cumic alcohol	10.4	3,800	363
3) α-p-Tolyl propyl alcohol	10.4	4,800	461

The assay system consisted of 150 μmoles TRIS, pH 8.6; 1 μmole NAD; 0.1 ml substrate suspension (0.1 ml substrate in 10 ml buffer) and enzyme (sufficient to give $\triangle$O.D. 0.01 to 0.02 per 30 sec) in a 3 ml cuvette.

The assay system in a 3 ml cuvette consisted of 150 umoles TRIS, pH 8.6; 1 μmole NAD; 0.1 ml of the substrate suspension and enzyme sufficient to give an optical density change of 0.01 to 0.02 per 30 sec. The reaction was started by adding the substrate suspended in TRIS and

the change in the optical density was recorded.

For activity determination only the initial linear rates were taken into account. A unit of enzyme is defined as that amount which produces an optical density change of 0.001 per min at 340 mμ. Specific activity is defined as units per mg.

II. Aldehyde dehydrogenase

The activity of the aldehyde dehydrogenase, which oxidizes cumic aldehyde to cumic acid was followed by measuring NAD reduction at 340 mμ in presence of the substrate. The results presented in Fig. XL showed the presence of an aldehyde dehydrogenase capable of catalysing the reduction of NAD in presence of cumic aldehyde. No reduction of NAD was observed in presence of cumic aldehyde and boiled cell-free extract. The assay system consisted in 1 ml TRIS (pH 8.6) 50 μmoles; NAD, 1 μmole; cysteine 10 μmoles; enzyme 0.1 ml of a diluted (ten times) sonicate; substrate 0.05 umoles in ethanol added to start the reaction.

III. Localization of hydroxylase activity and its cofactor requirements

Six grams of cells (wet) were suspended in 40 ml of phosphate buffer (0.05 M, pH 7.7) homogenised and then sonicated for 10-12 min. The sonicate (40 ml) was centrifuged at 15,000 X g for 45 min. The sediment (5 g) obtained on centrifugation was suspended in 50 ml of

phosphate buffer (0.05 M, pH 7.7). This suspension as well as the 15,000 X g supernatant were used separately to study the hydroxylase activity.

In order to find out the distribution of hydroxylase activity either in 15,000 X g supernatant or in sediment, and to determine its cofactor requirements, two sets of experiments were carried out with different time intervals. The first set of experiments were carried out with 15,000 X g supernatant at different time intervals, in presence of a NADH or a NADPH generating system. Similarly, the above set of experiments were carried out with 15,000 X g sediment. In both the experiments substrate as well as enzyme blanks were carried out. The incubation conditions for the two sets of experiments are presented in Table x.

The flasks containing the ingredients as mentioned in Table x were incubated on a rotary shaker (120 r.p.m.). After the desired incubation period, the reaction was terminated by adding 5 ml of 2N hydrochloric acid. The denatured protein was removed by centrifugation and the contents of the flasks were processed separately as follows.

The aqueous layer from each flask was extracted separately with ether (2 X 20 ml). Each fraction was then separated into acidic

TABLE X

HYDROXYLATING ACTIVITY IN THE SUPERNATANT					
Period of incubation (min)	90	60	90	90	20
Cumic acid (mg)	20	20	20	-	20
Phosphate buffer (0.05 M, pH 7.7) (ml)	18	18	18	18	25
Enzyme (ml) (12 mg protein/ml)	7	7	7	7	-
Cofactors	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	-
Hydroxylating activity	-	+	+++	-	-
HYDROXYLATING ACTIVITY IN THE SEDIMENT					
Period of incubation (min)	30	60	90	90	90
Cumic acid (mg)	20	20	20	-	20
Phosphate buffer (0.05 M, pH 7.7) (ml)	21	21	21	21	24.5
Enzyme (ml) (12 mg protein/ml)	3.5	3.5	3.5	3.5	-
Cofactors	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml, 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	NAD (0.2 ml 2 umoles) + 0.8 ml sod.lactate (0.5 M) + 0.4 ml LDH	-
Hydroxylating activity	-	-	+	-	-

+ feeble activity; +++ good activity; LDH - Lactate dehydrogenase

and neutral fractions by washing with 5% (w/v) sodium bicarbonate solution. The acidic fraction was then analysed by TLC (System III). It was noticed that the acidic fraction obtained from 'd' (i.e., 15,000 X g supernatant, 90 min incubation, Table x) was found to contain a compound having the same mobility pattern as that of 3-hydroxy cumic acid (R_F 0.35). It further showed the presence of a compound having the same R_F value (0.77) as that of isopropyl pyruvic acid. The aqueous layer after ether extraction was treated with 0.1% 2,4-dinitrophenyl hydrazine in 2N hydrochloric acid and kept for four hours at room temperature. The precipitated hydrazone was taken in ethylacetate and was then separated into acidic and neutral fractions by washing with 5% (w/v) sodium carbonate. The acidic hydrazones obtained were found to contain a compound having the same R_F value (0.48) as that of the hydrazone of isopropyl pyruvic acid in System V.

Negligible amounts of 3-hydroxy cumic acid and isopropyl pyruvic acid were obtained when the incubation periods were 30 and 60 min. Likewise, the 15,000 X g sediment contained a very feeble hydroxylating activity. The enzyme as well as the substrate blanks failed to show the presence of 3-hydroxy cumic and isopropyl pyruvic acids. Appreciable hydroxylating activities were not observed when NADPH (as generating system) instead of NADH was used as the cofactor.

These experiments led to the conclusion that the hydroxylase systems were found to be present in 15,000 X g supernatant and the enzyme is NADH dependent.

Mode of hydroxylation

(i) Cumic acid: Experimental details regarding the hydroxylation of cumic acid are already mentioned.

(ii) p-Hydroxy benzoic acid: In a typical experiment the cells from 3 flasks grown on p-cymene for 24 hr were centrifuged, washed and finally suspended in 20 ml of 0.05 M phosphate buffer, pH 7.0. A batch of 5 flasks containing sterilised medium and the substrate (p-hydroxy benzoic acid, 0.1%) were inoculated with 4 ml of the above cell suspension (Klett reading 400 at 660 mμ) and incubated on a rotary shaker (220 r.p.m.) for 24 hr. The contents from 5 flasks were then pooled together, acidified and extracted with ether (2 x 200 ml). The ether extract after concentration to 100 ml was separated into acidic and neutral fractions as mentioned earlier in "Methods and Materials".

The acidic fraction (100 mg) on TLC examination (System III) was found to contain a compound which had the same mobility pattern as that of 3,4-dihydroxy benzoic acid (R_F 0.29). The acidic fraction was further subjected to preparative TLC and the compound corresponding to 3,4-dihydroxy benzoic acid in mobility pattern was separated in a

pure form (15 mg). The IR spectrum (Fig.XXXVIII) of the isolated acid showed bands at 3200, 2700, 1690 and 1600 cm^{-1}. The spectrum was also found to be identical with that of an authentic sample of 3,4-dihydroxy benzoic acid. The hydroxylating enzyme present in the organism brings about the hydroxylation of p-hydroxy benzoic acid at the position meta to the carboxylic group and ortho to the hydroxylic group initially present, to give 3,4-dihydroxy benzoic acid.

(iii) Benzoic acid

Incubations were carried out with benzoic acid as the substrate. The experimental conditions were the same as described for p-hydroxy benzoic acid. TLC of the acidic fraction in System III showed a spot corresponding to that of p-hydroxy benzoic acid (R_F 0.50). However, the identity of p-hydroxy benzoic acid was not conclusively proved and hence it is difficult to conclude that benzoic acid is further metabolized via p-hydroxy benzoic acid.

Cleavage enzymes

i) 2,3 Dihydroxy cumic acid: 50 ml of the sonicate extract was diluted to 500 ml with phosphate buffer (0.05 M, pH 7.0) and was distributed into five flasks. 2,3-Dihydroxy cumic acid (50 mg) was added to each of the four flasks and the remaining flask was used as the enzyme blank. All the five flasks were incubated on a rotary shaker (120 r.p.m.) at 27° for a period of 2 hr. After the required

incubation period, the contents from four flasks were pooled together and the reaction was arrested by acidifying with hydrochloric acid (5N) to congo red. The denatured protein was removed by centrifugation. To the clear supernatant 0.1% 2,4-dinitrophenyl hydrazine solution in 2N hydrochloric acid was added in slight excess and was left to stand at 30° for 4 hr. The precipitated 2,4-dinitrophenyl hydrazone derivatives were taken in ethyl acetate and was separated into acidic (80 mg) and neutral (70 mg) derivatives using 10% sodium carbonate solution. The enzyme blank was also processed in a similar way, but failed to give any acidic 2,4-dinitrophenyl hydrazones.

The acidic 2,4-dinitrophenyl hydrazone fraction (80 mg) was found to contain three compounds on TLC examination (System V) and out of which one was found to be in major quantity. This fraction was further subjected to preparative TLC (System V) and the component which was present in major quantity was recovered (50 mg). It was further crystallized from ethanol to orange coloured needles, m.p.193-194°. When examined on TLC in System V it was found to have the mobility pattern same as that of 2,4-dinitrophenyl hydrazone of authentic isopropyl pyruvic acid (R_f 0.48). Depression in the melting point was not observed when admixed with the authentic sample. The presence of isopropyl pyruvic acid in the reaction mixture was then

conclusively proved by the spectral comparison (Fig. XLI) of the hydrazone of the authentic sample with that of the compound isolated from the experiment. Chromatography on paper also indicated identical mobility pattern (R_F 0.72) of the sample with the authentic compound in n-butanol:ethanol:water (7:1:2).

Two more acidic hydrazones present in the mixture could not be isolated and characterised due to the paucity of the material. But from the mobility pattern of the compounds it could be assumed that one of the two acidic hydrazones was more polar than the hydrazone of isopropyl pyruvic acid.

2,4-Dinitrophenyl hydrazone derivatives of neutral carbonyl compounds:

Neutral 2,4-dinitrophenyl hydrazones (70 mg) on TLC examination was found to contain three compounds (System VI) out of which one was present in 90%. It was crystallized from alcohol to pale yellow crystals, m.p. 160-61°. It analysed for $C_8H_8N_4O_4$. On TLC the purified hydrazone had an identical mobility pattern as that of 2,4-dinitrophenyl hydrazone of authentic acetaldehyde (System VI, R_F 0.90). The presence of acetaldehyde was finally proved by spectral comparisons of the hydrazones of authentic as well as the isolated compound from the reaction mixture (Fig. XLII).

When the incubation period was extended from 2 to 3 hr,

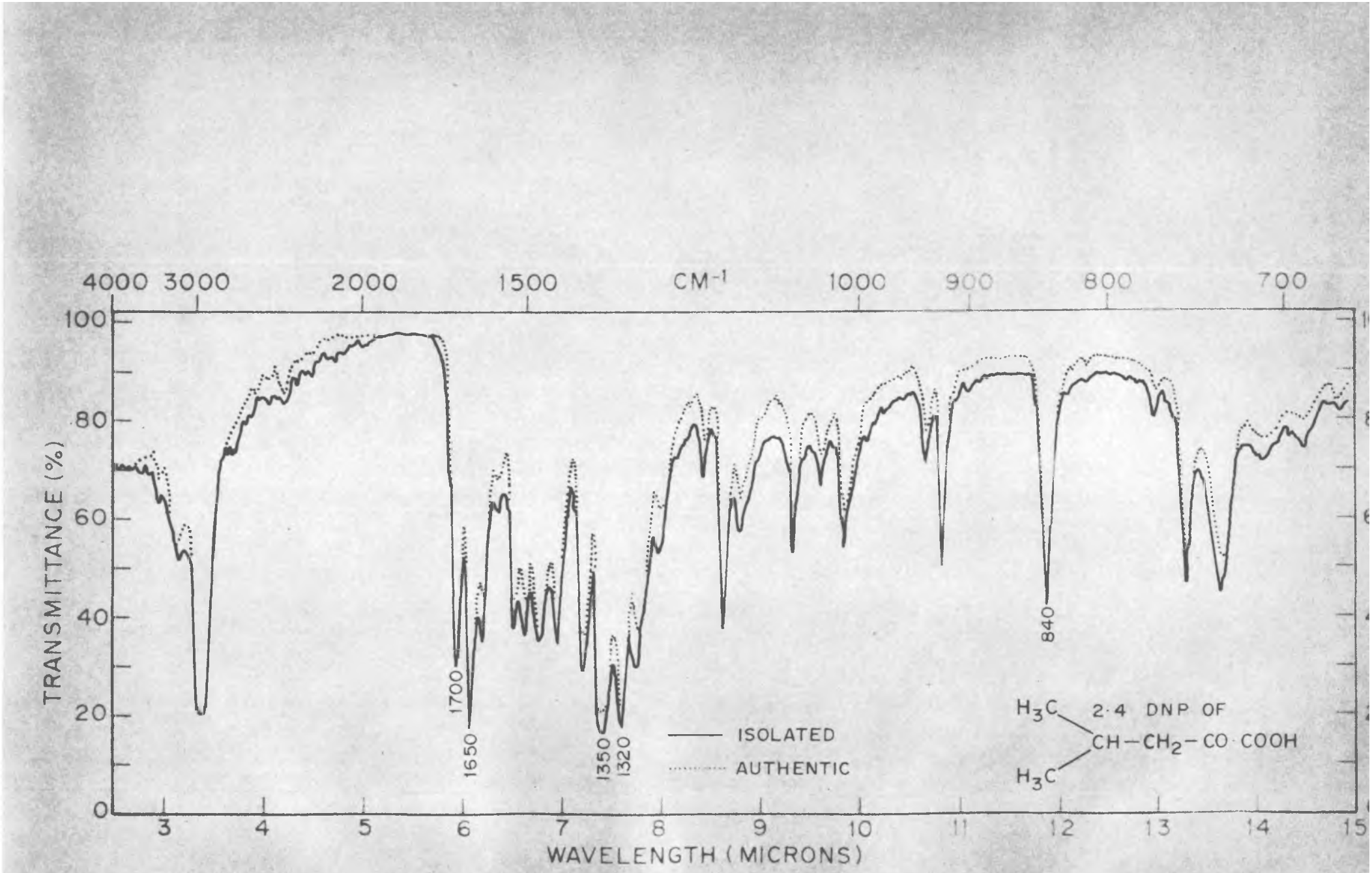
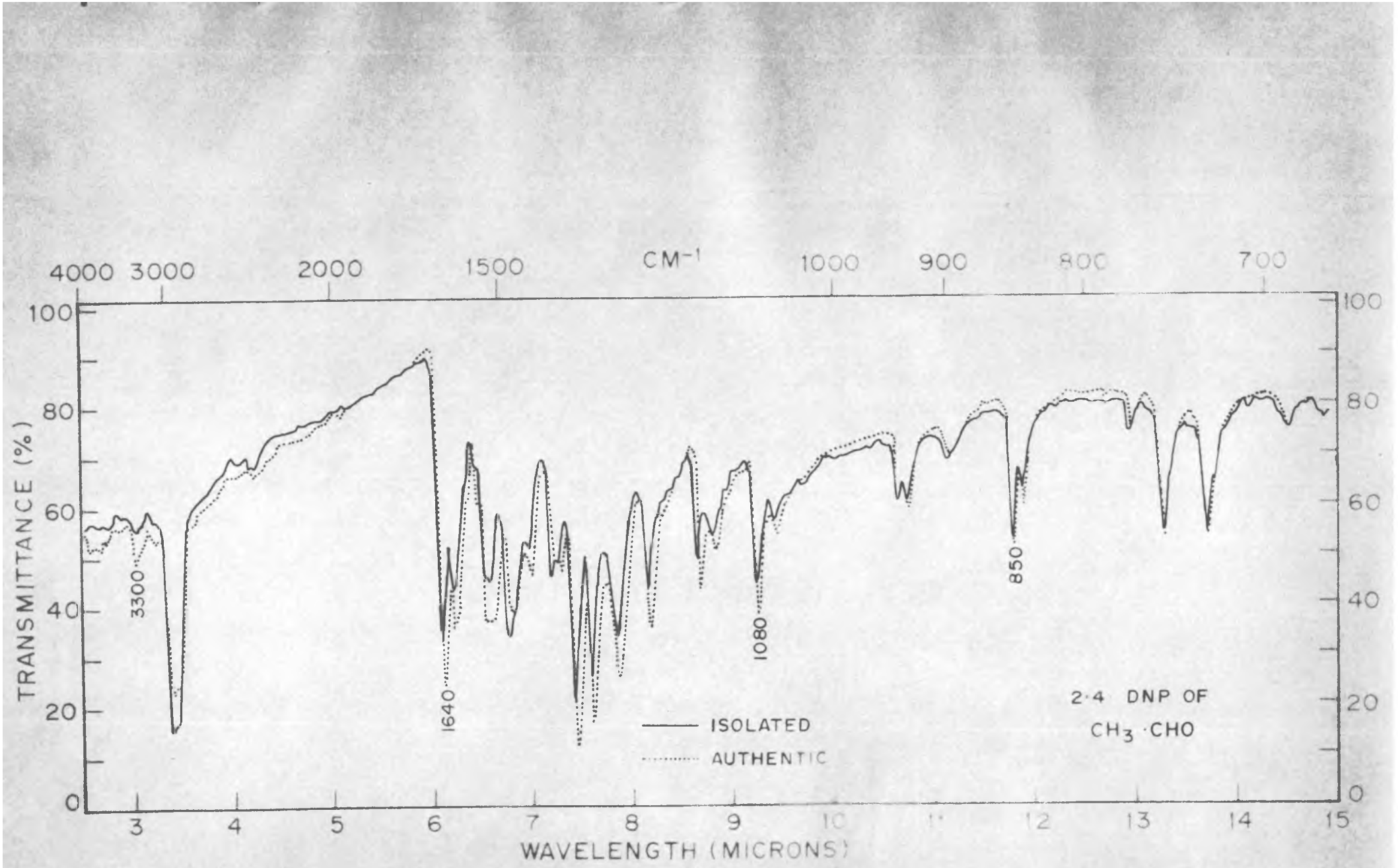

4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
1700
1650
1350
1320
840
ISOLATED
AUTHENTIC
H3C
2·4 DNP OF
CH—CH2—CO COOH
H3C
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG. XLI


4000 3000 2000 1500 CM-1 1000 900 800 700
100
80
60
40
20
0
TRANSMITTANCE (%)
3300
1640
1080
850
2·4 DNP OF
CH3·CHO
ISOLATED
AUTHENTIC
3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

FIG·XLII

accumulation of isopropyl pyruvic acid was not observed. Probably this acid when formed might have been degraded further on prolonged incubation.

(ii) 2,3-Dihydroxy benzoic acid

The above experiment was repeated in the same way as described earlier by incubating a mixture of 2,3-dihydroxy benzoic acid (50 mg) and 20 ml of crude cell-free extract (15 to 20 mg protein/ml) in 100 ml of phosphate buffer (0.05 M, pH 7.0) on a rotary shaker (120 r.p.m.) for 2 hr. The reaction was then stopped by the addition of 10 ml of 2N hydrochloric acid and the denatured protein was removed by centrifugation. The keto acids and neutral carbonyl compounds present in the aqueous layer was converted into their corresponding 2,4-dinitrophenyl hydrazones.

The acidic 2,4-dinitrophenyl hydrazones (20 mg) on TLC examination (System V) found to contain 3 to 4 compounds. The mobility pattern of one of the compounds corresponded to that of the 2,4-dinitrophenyl hydrazone of pyruvic acid (R_F 0.85). In paper chromatography the authentic hydrazone of pyruvic acid had the same R_F value (0.43) as that of the derivative isolated from the reaction mixture. Since the amount of the acidic hydrazone formed was very

little, it was not possible to separate them and hence further chemical and physical studies could not be carried out. In this experiment the enzyme blank did not yield any acidic hydrazones at all.

From the neutral hydrazone mixture (30 mg), 2,4-dinitrophenyl hydrazone of acetaldehyde was identified by TLC (System VI, R_f 0.90).

Chapter V

SUMMARY

SUMMARY

It has been known since long that the microorganisms can be utilized extensively for the production of various compounds of economic value, but less is known about the detailed nature of dissimilation 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 compounds especially that of aromatic 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 metabolized by 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, p-cymene. The present studies deal with the catabolism of this aromatic hydrocarbon by 'PL-strain'. In order to isolate and characterize the transformation products, bench scale fermentations of p-cymene were carried out which has enabled to find the pathways by which the bacterial enzymes manipulate and cleave the aromatic ring.

Among the acidic products cumic, 3-hydroxy cumic, 2,3-dihydroxy-cumic, α-p-tolyl propionic and isopropyl pyruvic acids were identified

by known methods including comparative infrared and n.m.r. spectra. From the neutral fraction, cumic alcohol, 9-hydroxy p-cymene and acetaldehyde were identified by the conventional methods. Growth and oxidation studies have shown that α-p-tolyl propyl alcohol (9-OH-p-cymene) and α-p-tolyl propionic acid are the side products and do not seem to be intermediates in the dissimilation pathway of p-cymene. Incubation of 2,3-dihydroxy cumic acid with cell-free extracts yielded considerable amounts of isopropyl pyruvic acid and acetaldehyde which were isolated and identified as their 2,4-dinitrophenyl hydrazones.

Induced enzyme studies as well as oxidation of different substrates showed that the p-cymene grown cells oxidise cumic alcohol, cumic acid, 3-hydroxy cumic acid and 2,3-dihydroxy cumic acid but not 2-hydroxy cumic acid, 3-isopropyl catechol, 2-hydroxy p-cymene, 3-hydroxy p-cymene and 9-hydroxy p-cymene. It was found that when 'PL-strain' was grown on p-cymene, the cells rapidly oxidized 2,3-dihydroxy cumic acid without any lag phase. On the other hand, cells adapted to 2,3-dihydroxy cumic acid utilized p-cymene and cumic acid only after a lag phase. It has also been noticed that the p-cymene grown cells grow equally well on cumic alcohol, cumic acid and 2,3-dihydroxy cumic acid without any lag.

This strain grown on p-cymene is capable of oxidising other acids such as benzoic, p-hydroxy, 2,3-, 3,4-, and 2,5-dihydroxy benzoic

acids, which have close structural similarities with the intermediates in the p-cymene degradation. Furthermore, the growth rate of this organism in the presence of 2,3-dihydroxy benzoic acid was comparable to that observed in the presence of 2,3-dihydroxy cumic acid. However, failure to identify 2,3-dihydroxy benzoic acid in the culture medium probably suggests that it may not be one of the intermediates in p-cymene dissimilation.

Although p-hydroxy benzoic acid served as a growth substrate for this organism, p-cresol failed to promote the growth. On salicylic acid the organism grew after a lag period of 48 hr. Likewise, the rate of growth was almost negligible in presence of catechol. When fermentations were carried out using p-hydroxy benzoic acid as the substrate, 3,4-dihydroxy benzoic acid was found to be present in the growth medium. Similarly when benzoic acid was used as the substrate, p-hydroxy benzoic acid was suspected to be present in the fermented broth. However, presence of this compound remains to be fully confirmed.

Studies with regard to the mode of hydroxylation of several benzenoid compounds and their cleavage pattern have been fully discussed.

Cell-free extracts from the bacterium contained an oxygenase of the metapyrocatechase type, which brings about the oxidative fission of 2,3-dihydroxy cumic acid between the Carbons 1 and 2 (between carboxyl and hydroxy groups) giving rise to a hypothetical keto acid intermediate

which then undergoes cleavage to isopropyl pyruvic acid and acetaldehyde. Under the same conditions, 2,3-dihydroxy benzoic acid yielded pyruvic acid and acetaldehyde.

The presence of two dehydrogenases, namely, cumic alcohol dehydrogenase and cumic aldehyde dehydrogenase have been shown in 15,000 X g supernatant of the sonicate. Both the dehydrogenases require NAD for the oxidation. The hydroxylating activity capable of hydroxylating cumic acid at position 3 to give 3-hydroxy cumic acid has also been shown to be present in this (15,000 X g) supernatant requiring NADH for its maximal activity. NADPH is very much less effective than NADH.

On the basis of the above observations a possible pathway for the degradation of p-cymene by 'PL-strain' has been formulated (Fig.XXXIV). From p-cymene to cumic acid the organism follows a pathway similar to that encountered with limonene and Δ^1-p-menthene which give rise to perillic and phellandric acids. But all evidences indicate that in the case of cumic acid instead of hydration as in the case of perillic and phellandric acids, a monooxygenation reaction takes place leading to 3-hydroxy cumic acid which is further oxygenated at C_2-position to yield a dioxygenated product, 2,3-dihydroxy cumic acid.

Chapter VI

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

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