Metabolism of aromatic compounds

M. SUGUMARAN AND C. S. VAIDYANATHAN
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

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Abstract

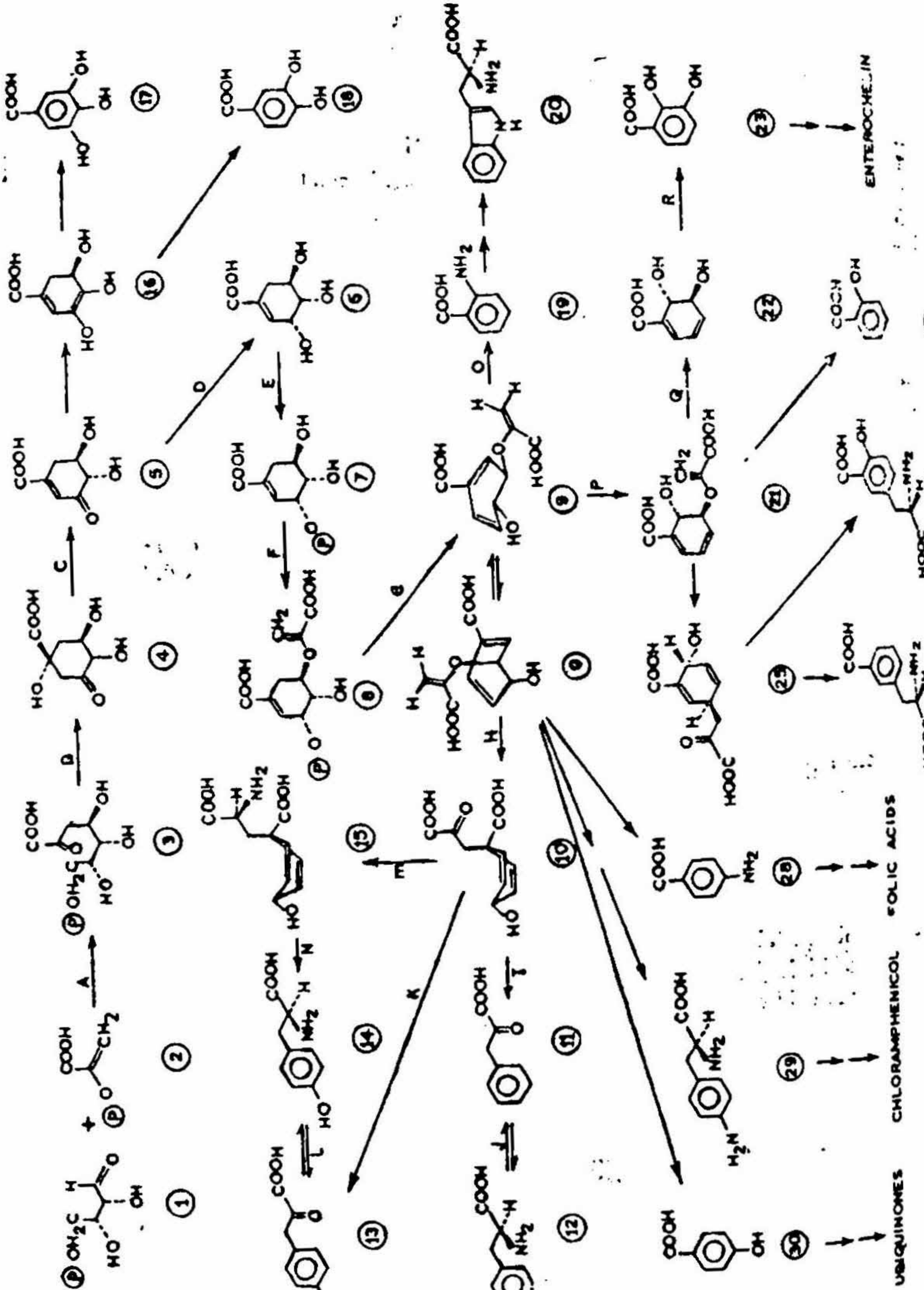
A brief account of the two major metabolic pathways which lead to the biosynthesis of a number of complex aromatic compounds is given. The metabolism of benzene and related aromatic hydrocarbons by microbes and mammals is compared. The microbial metabolism of phenol, cresols, xylenols, benzoic acid and its derivatives, phenylacetic and hydroxyphenylacetic acids, acetophenone, phenethylamines, phenylacetaldehyde, mandelic and hydroxymandelic acids, phenylpropionic and hydroxyphenylpropionic acids, phenylpyruvic acids, phenyllactic acids, coumarin, cinnamic acid and coumaric acid is reviewed. The initial reactions involved in the biotransformation of phenylalanine, tyrosine and dopa are given. A detailed survey of the various aromatic ring cleavage reactions of gentisaldehyde, gentisic acid, homogentisic acid, 3-hydroxyanthranilic acid, dopa, caffeic acid, 2, 3-dihydroxy phenylpropionic acid, homoprotocatechuic acid, protocatechuic acid, pyrocatechuic acid, methylcatechols, catechol, gallic acid and other compounds is made.

Key words : Aromatic compounds, biosynthesis, microbial degradation, unity and diversity in metabolism, dioxygenase reactions.

1. Introduction

Most of the naturally occurring organic compounds exhibit life cycles with strikingly similar characteristics. Encapsulated within the early part of their life cycle are a few brief moments of fame and glory—the possession of some novel structural feature, the display of unique biochemical reactivities and the occupation of a regulatory role over a versatile metabolic pathway—but once these have been assimilated into the main stream of biological chemistry, the life of the natural product almost invariably decays once more into gentle obscurity.

The diverse pathways by which complex organic compounds are synthesized and degraded in nature have long excited the curiosity of several biochemists and enzymologists. With the advent of labelling techniques, several natural products have been subjected to intensive experimental investigation and the dynamic relationships between the primary metabolic processes common to most of the organisms and the branching pathways leading to more complex, specialized secondary metabolities were established. The biochemical similarities of major metabolic pathways utilized by various forms of life have reinforced a long standing concept of the 'Biochemical Unity' in all forms of life[1]. Against this background, instances of unique diversities and subtle differences in the pathways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offer intriguing prospects for getting an insight into evolutionary relationships and ecological adaptations. This review attempts to give a brief account of the metabolism of aromatic compounds and the unities and diversities found in it in various forms of life.

2. The shikimate pathway

One of the most important and exciting achievements of modern biochemistry and enzymology has been the elucidation of the shikimate pathway by which a bewildering variety of aromatic compounds are synthesized. The biosynthetic pathway begins with the condensation of erythrose-4-phosphate and phosphoenol pyruvate, the first of the seven enzymatic reactions that culminate in the formation of chorismate (Fig. 1). Chorismate thus formed is utilized for the biosynthesis of a number of compounds[2]. Apart from the compounds depicted in Fig. 1, a plethora of characteristic natural products such as plastoquinones, plastochromanols, tocopherols, phylloquinones, menaquinones, phenazines, bacilysin, anticapsin, neoantimycin, mycobactins, etc., are synthesized by many forms of life employing this pathway[2]. The pathway seems to be of ubiquitous occurrence in nature with the exception of the animal kingdom.

The recently discovered pretyrosine pathway for the biogenesis of tyrosine deserves special mention. Although the 4-hydroxyphenylpyruvate pathway which is operated by prephenate dehydrogenase and 4-hydroxyphenylpyruvate transaminase seems to be the most widely distributed, blue green algae seem to possess exclusively the enzymes of pretyrosine pathway, *viz.*, prephenate transaminase and pretyrosine dehydrogenase, as judged by the comparative survey of dehydrogenase specificity in a number of microorganisms[3–5]. While the former pathway has a direct and refined control through the feedback inhibition of prephenate dehydrogenase by excess tyrosine, the latter pathway has a less refined and indirect control accomplished by the activating effect of excess tyrosine on prephenate dehydratase which favours the flow of metabolites towards phenylalanine and away from tyrosine.

FIG. 1. THE SHIKIMATE PATHWAY—1. D-Erythrose-4-phosphate. 2. Phosphoenol pyruvic acid. 3. 3-Deoxy-D-arabino heptulosonic acid-7-phosphate (DAHP). 4. 3-Dehydroquinic acid. 5. 3-Dehydroshikimic acid. 6. Shikimic acid. 7. Shikimic acid-3-phosphate. 8. 5-Enolpyruvyl shikimic acid-3-phosphate. 9. Chorismic acid. 10. Prephenic acid. 11. Phenylpyruvic acid. 12. L-Phenylalanine. 13. 4-Hydroxyphenylpyruvic acid. 14. L-Tyrosine. 15. Pretyrosine. 16. 3-Dehydroshikimic acid (enol form). 17. Gallic acid. 18. Protocatechuic acid. 19. Anthranilic acid 20. L-Tryptophan. 21. Isochorismic acid. 22. 2,3-Dihydro-2, 3-dihydroxybenzoic acid. 23. Pyrocatechuic acid. 24. Salicylic acid. 25. 3-(3-Carboxy-4-hydroxy cyclohexa-2, 5-dienyl. pyruvic acid. 26. *m*-Carboxyphenylalanine. 27. *m*-Carboxytyrosine. 28. 4-Aminobenzoic acid. 29. 4-Aminophenylalanine. 30. 4-Hydroxybenzoic acid.

A. DAHP synthetase; B. 3-Dehydroquinic acid synthetase; C. 3-Dehydroquinic acid dehydratase: D.. 3-Dehydroshikimic acid reductase: E. Shikimic acid kinase; F. 5-Enolpyruvyl shikimic acid-3-phosphate synthetase; G. Chorismic acid synthetase: H. Chorismic acid mutase: I. Prephenic acid dehydratase; J. Phenylalanine amino transferase: K. Prephenic acid dehydrogenase; L. Tyrosine amino transferase. M. Prephenic acid transaminase; N. Pretyrosine dehydrogenase: O. Anthranilic acid synthetase: P. Chorismic acid isomerase; Q. 2, 3-Dihydro-2, 3-dihydroxybenzoic acid synthetase; R. 2, 3-Dihydro-2, 3-dihydroxybenzoic acid dehydrogenase.

3. The acetate pathway

The implication of acetate in the biosynthesis of a wide variety of naturally occurring aromatic compounds, *viz.*, " the acetate hypothesis", which states that a linear polyketomethylene chain formed by head to tail self-condensation of acetate units could cyclize to give a remarkable array of complex aromatic compounds, was first deduced by Collie[4] and later established by Birch[7–8]. Several of the salicylic acid derivatives, phloroglucinols, resorcylic acid derivatives, benzophenones, xanthones, flavonoids, isoflavonoids, rotenoids, stilbenes, naphthalenes, naphthoquinones, anthraquinones, tropolones, pyrromycinones, tetracyclines and antibiotics such as griseofulvin, alternariol, penicillic acid and patulin arise from acetate and related compounds[9]. Though the acetate pathway is equally important in contributing a number of aromatic compounds to nature, its enzymological aspects have not been as clearly understood as those of the shikimate pathway, an exception to this being the recent studies on 6-methylsalicylic acid synthetase[10–12]. This enzyme has been purified as a multienzyme complex of molecular weight $1 \cdot 5 \times 10^6$ from *Penicillium patulum* and shown to be similar to the fatty acid synthetase in several of its properties[10–12]. The mechanism of the formation of 6-methylsalicylic acid proposed by Lynen[13] is shown in Fig. 2. A similar enzymatic reaction, without the intermediate reduction and dehydration reactions, would yield orsenillic acid.

4. Miscellaneous reactions

In addition to the above two major pathways, a number of minor reactions leading to the formation of aromatic compounds are also known. One such reaction is the simple aromatization of cyclohexane derivatives, the reduced counterpart of aromatic compounds. Mitoma *et al.*[14] showed the conversion of cyclohexane carboxyl CoA to benzoic acid probably *via* 1-cyclohexene carboxyl CoA[15] by guinea pig liver mitochondria in the absence of any added cofactors. Recently, it has been demonstrated that cell-free extracts of *Corynebacterium cyclohexanicum*, grown on cyclohexane carboxylic acid, could convert 4-ketocyclohexane carboxylic acid to 4-hydroxybenzoic acid, under both aerobic and anaerobic conditions[16]. Under anaerobic conditions, an electron acceptor such as potassium ferricyanide is essential for the reaction.

A number of aromatic derivatives of terpenes and steroids arise from desaturation reactions. These aromatic compounds, along with the polymeric materials such as lignins which are synthesized by plants, enter the soil ecosystem when the life of the organism ends. A range of aromatic hydrocarbons are also found in nature as constituents of fossil fuels[17]. Apart from these natural additions, a variety of synthetic aromatic compounds in the form of pesticides and industrial effluents are also added to the environment.

If such chemicals prove to be resistant to microbial decomposition, they could accumulate in the soil and cause serious ecological changes and disturbances in nature's 'carbon cycle'. However, microorganisms, endowed with amazing biochemical potential to utilize a vast array of compounds as sources of carbon and energy, degrade these products and convert them into useful biochemicals which will be accepted by every form of

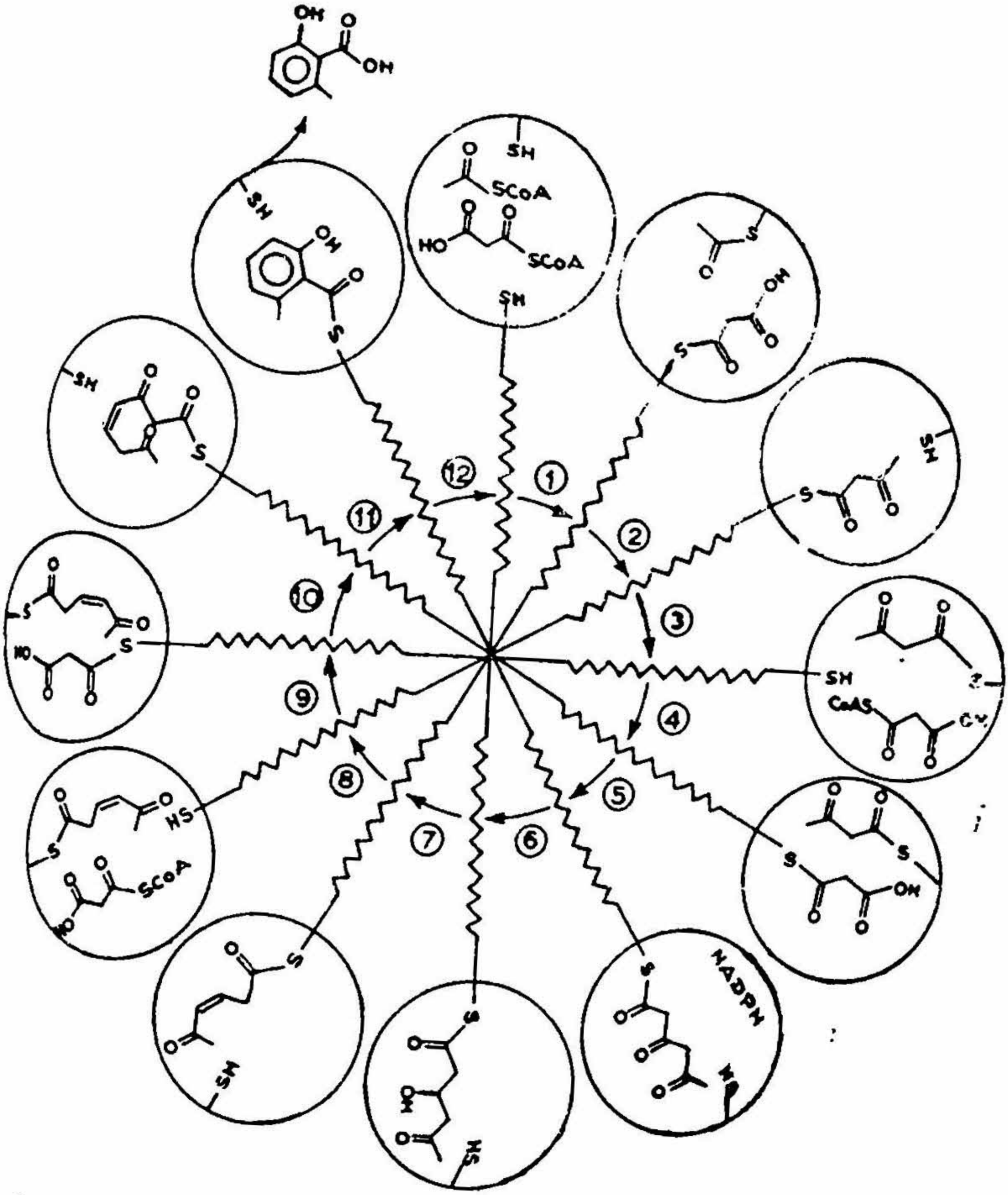


FIG. 2. HYPOTHETICAL MECHANISM FOR THE BIOSYNTHESIS OF 6-METHYLSALICYLIC ACID. The process is initiated by the transfer of acetyl group from acetyl CoA to the "peripheral" sulfhydryl group and malonyl CoA to the "central" sulfhydryl group (step 1) of the enzyme. Then condensation occurs to give acetoacetyl enzyme with the liberation of CO_2 (step 2). The acetoacetyl group is transacetylated to the peripheral SH (step 3), another malonyl CoA is loaded on to the central SH (step 4) and a second condensation occurs to give 3, 5-dioxohexanoyl enzyme (step 5). At this point, reduction of the 3-oxo group by NADPH to form 3-hydroxy-5-oxohexanoyl enzyme occurs (step 6). The reduced enzyme is then dehydrated to give *cis*-5-oxohex-3-enoylenzyme by a dehydrase (step 7). A third condensation with malonyl enzyme gives an 8-carbon enzyme bound intermediate, 3, 7-dioxo oct-5-enoyl enzyme (step 10), which undergoes an internal aldol type condensation between the C–2 and C–7 positions followed by dehydration to give enzyme bound 6-methylsalicylic acid (step 11). A thioesterase then releases the product (step 12).

life. In the following pages, the catabolism of some of the aromatic compounds is described.

5. Metabolism of aromatic hydrocarbons

Studies on the metabolism of benzene in *Pseudomonas aeruginosa* revealed its conversion to catechol without the intermediate formation of phenol[18]. Gibson *et al.*[19] demonstrated that *cis*-1, 2-dihydro-1, 2-dihydroxybenzene (*cis*-benzeneglycol) is an intermediate during the oxidation of benzene by *Pseudomonas putida*. Benzene-1, 2-epoxide can be excluded as the precursor of *cis*-benzeneglycol, as its hydration leads to *trans*- and not *cis*-benzeneglycol[20]. Gibson *et al.*[19] also established that at least two protein factors are involved in the conversion of benzene to *cis*-benzeneglycol. Experiments with $^{18}O_2$ revealed the dioxygenase nature of this reaction[21,22]. Further, it was also shown that *cis*- and not *trans*-benzeneglycol is dehydrogenated to give catechol by cell-free extracts of *P. putida* utilizing NAD^+[19]. The same pathway was found to operate in *Moraxella* also[22].

Benzene dioxygenase was purified recently from a *Pseudomonas* sp. grown on benzene by Axcell and Geary[23]. The enzyme has three protein components : two nonheme iron proteins of molecular weight 21,000 and 186,000 and a flavoprotein of molecular weight 60,000. Ferrous iron and NADH are absolutely essential for the reaction. These workers have also purified the *cis*-benzeneglycol dehydrogenase[24]. The homogeneous enzyme, which utilizes only the *cis*-isomer, requires NAD^+ and ferrous iron for its activity. It is made up of four subunits and has a molecular weight of 110,000.

It is interesting to compare the parallel reactions in mammalian metabolism of benzene. In this case, benzene is first epoxidized by a microsomal oxygenase. The epoxide is enzymatically hydrated to give *trans*-benzeneglycol[20], which then undergoes a dehydrogenation reaction to yield catechol (Fig. 3).

Alternate pathways exist for the catabolism of toluene. Katagawa[25] first demonstrated that a strain of *Pseudomonas aeruginosa* oxidized toluene through its side chain. Thus, benzylalcohol, benzaldehyde and benzoic acid are intermediates formed in the degradative pathway. Such a side chain oxidation also occurs in *Pseudomonas putida* (*arvilla*) mt-2[26]. Requirement of three protein fractions for the toluene hydroxylation has been demonstrated by Nozaka and Kusunose[27]. The alcohol and aldehyde dehydrogenases have been isolated and shown to require NAD^+.

On the contrary, Claus and Walker[28] showed that 3-methylcatechol, and not side chain oxidized products, to be the intermediate in the oxidation of toluene by *Pseudomonas* as well as an *Achromobacter* sp. Such a direct ring hydroxylation also occurs in *Pseudomonas mildenbergii*[29] and *Pseudomonas putida*[30,31]. A mutant strain of *P. putida*, which produced (+) *cis*-1, 2-dihydroxy 3-methylcyclohexa-3, 5-diene from toluene, was dehydrogenated by the parent strain to 3-methyl catechol[30, 31]. These results indicate that the initial reactions involved in toluene degradation by the above organisms are essentially the same as those employed for the metabolism of benzene.

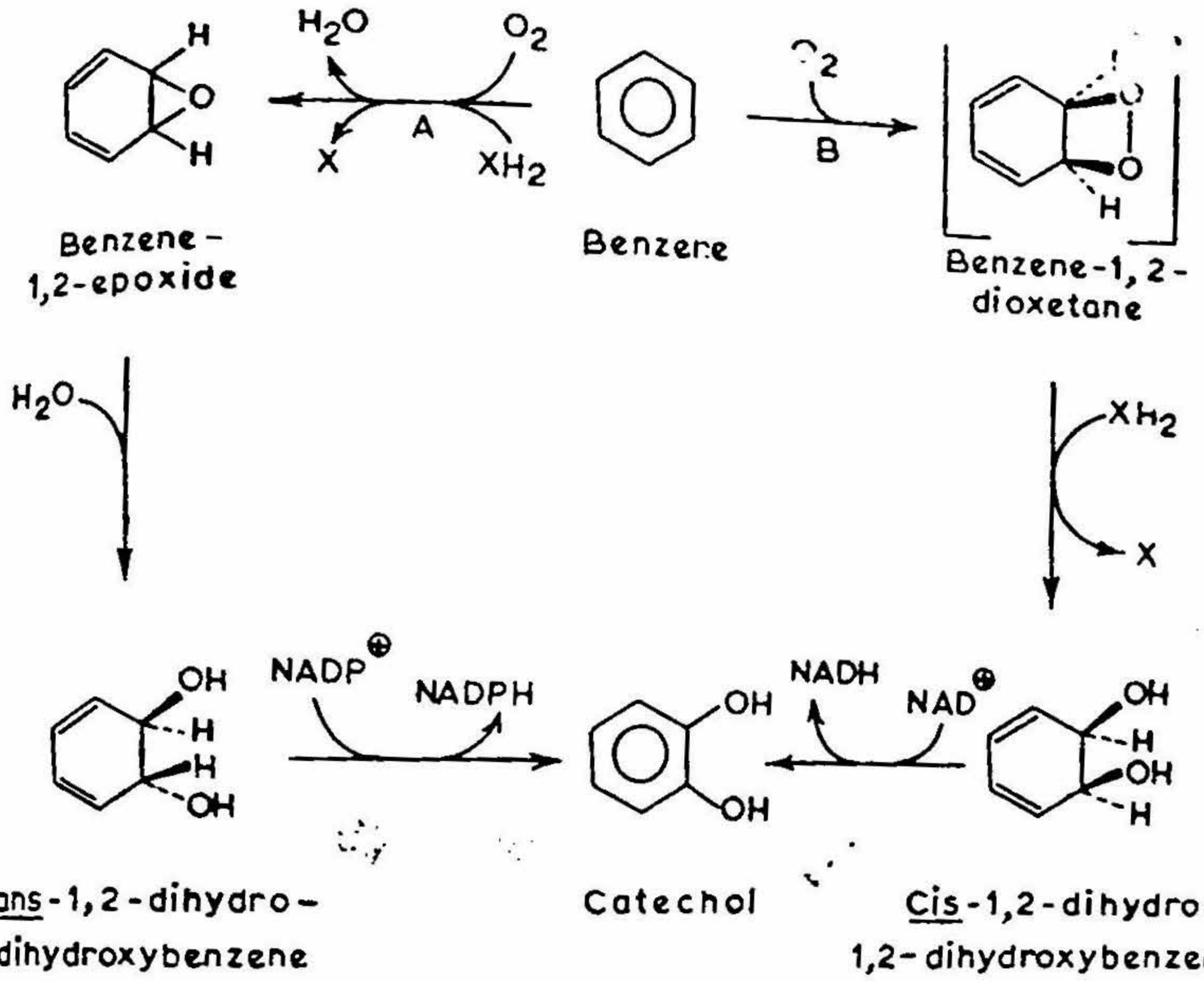


FIG. 3. Metabolism of benzene (A-mammalian; B-microbial).

However, with multiple substituents at the benzene ring, the initial reaction seems to be in favour of side chain oxidation and not ring oxygenation. Thus *m*-xylene and *p*-xylene are oxidized to their corresponding toluic acids[26, 32–36]. Diversity in the metabolism of *p*-xylene occurs at the level of *p*-toluic acid. While Davis *et al.*[34] observed the conversion of *p*-toluic acid to 4-methylcatechol, Omori and Yamada[37] demonstrated its conversion to *p*-cresol followed by side chain oxidation reactions leading to the formation of 4-hydroxybenzoic acid (Fig. 4). *p*-Cymene, an analog of *p*-xylene, is, however, initially oxidized to *p*-cumic acid and then to 2, 3-dihydroxy-*p*-cumic acid[38], *via* 2, 3-dihydroxy-2, 3-dihydro-*p*-cumic acid[39] by the PL-strain of *Pseudomonas putida*.

The microbial metabolism of phenyl-substituted alkanes has been reported, but detailed studies on the enzymological aspects have not been carried out so far. Webley *et al.*[40, 41] have shown with a *Nocardia* sp. that alkyl chains with an odd number of carbon atoms undergo β-oxidation leading to the formation of cinnamic and benzoic acids, while derivatives with an even number of carbon atoms give rise to phenylacetic acid. Such a side chain oxidation has also been shown to occur for the oxidation of 3-phenyldodecane by two *Nocardia* strains[42]. However, *Pseudomonas* strains seem to oxidize various alkylbenzenes by direct ring oxygenation reactions[42].

Oxidation of naphthalene, the binuclear aromatic hydrocarbon, by bacteria was initially reported to involve the intermediate formation of *trans*-1, 2-dihydro-1, 2-dihydroxy-

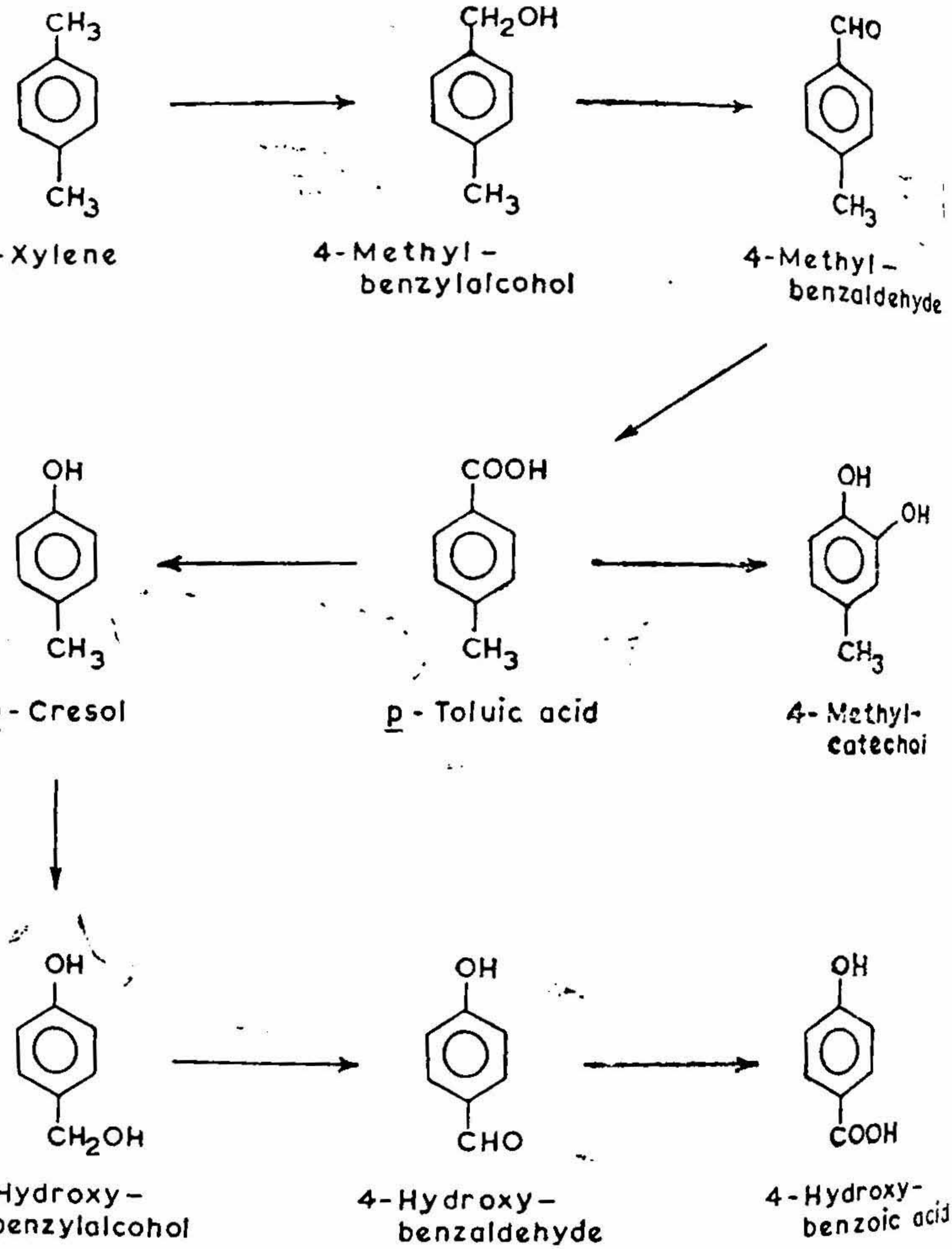


FIG. 4. Microbial metabolism of *p*-xylene.

naphthalene[43, 44]. Later, Jerina *et al.*[45] identified (+) *cis*-1 (R), 2 (S)-dihydroxy-1, 2-dihydronaphthalene as a metabolite of naphthalene with the use of a mutant strain of *Pseudomonas*. The *cis*-dihydrodiol formation has also been demonstrated by Williams' group[46].

Naphthalene dioxygenase utilized either NADH or NADPH to produce the dihydrodiol ; but the dehydrogenase specifically required NAD^+ and utilized only the (+)

isomer of *cis*-dihydrodiol[47]. When the dihydrodiol was substituted at C-2 position with deuterium, the dehydrogenation reaction showed a primary kinetic isotopic effect. Based on these results, a stepwise mechanism which involves an initial dehydrogenation of the secondary alcohol group at C-2 followed by a rapid aromatization reaction, was proposed for the formation of 1,2-dihydroxynaphthalene from *cis*-dihydrodihydroxynaphthalene[47]. The further metabolism of 1, 2-dihydroxynaphthalene appears to proceed by the pathway delineated by Davies and Evans[48].

The *cis*-dihydrodiol formation also occurs during the metabolism of biphenyl[49], anthracene, phenanthrene[17], benzo [a] anthracene and benzo [a] pyrene[50]. Thus, bacteria incorporate both the atoms of moelcular oxygen into the aromatic hydrocarbon employing a dioxygenase and produce *cis*-dihydrodiol, in sharp contrast to mammalian systems which produce *trans*-dihydrodiol *via* arene oxide employing a monooxygenase[17] (Fig. 3).

Metabolism of phenol, cresols and xylenols

Oxidation of phenol to catechol has been observed in bacteria, fungi and yeast[51–56]. The enzyme catalyzing this conversion has been purified to homogeneity from *Trichosporon* and shown to be a flavoprotein requiring NADPH for activity[56–58]. Although the requirement for NADPH as an external electron donor is absolute, the enzyme exhibits an unusually broad specificity towards the phenolic substrate[57]. A requirement for intact sulfhydryl groups is indicated by the susceptibility of the enzyme to inhibition by heavy metal ions and sulfhydryl reagents[58]. The phenol monooxygenase from *Brevibacterium fuscum* utilized either NADPH or NADH as the electron donor[59]. Interestingly, the enzyme from *Bacillus stearothermophilus* requires NADH and not NADPH[60]. While phenol monooxygenase from yeast was unaffected by iron and copper chelators[57], the enzyme from *Brevibacterium*[59] was inhibited by copper chelators and that of *Bacillus* by iron chelators[60].

Ribbons presented evidence consistent with a reaction sequence whereby *o*-cresol is oxidized to 3-methylcatechol by *Pseudomonas aeruginosa*[61]. The same pathway is operative in some *Pseudomonas* strains[62, 63], *Brevibacterium fuscum*[59], *Pseudomonas putida*[64], *Bacillus stearothermophilus*[60] and *Candida tropicalis*[65].

Diversities occur at the level of *m*- and *p*-cresol oxidation. Dagley and Patel[66] reported that the degradation of *p*-cresol by a nonfluorescent *Pseudomonas* strain proceeds by an initial attack on the methyl group. Such a side chain oxidation has also been shown to occur in *Pseudomonas putida*.[67] The side chain hydroxylating enzyme was recently isolated by Hopper[68] and shown to be an entirely new type of hydroxylase. Surprisingly, it did not utilize any electron donor and molecular oxygen for hydroxylation, but an electron acceptor like phenazine methosulfate and water. The reaction proceeds well even under anaerobic conditions. A mechanism involving dehydrogenation to a hetero-quinone followed by hydration to give 4-hydroxybenzyl alcohol was proposed[68]. The same enzyme fraction also converts 4-hydroxybenzyl alcohol to 4-hydroxybenzaldehyde[68].

In contrast, several species of fluorescent *Pseudomonas* introduced a new hydroxyl group adjacent to the hydroxyl group in *p*-cresol and oxidized the resultant 4-methyl

catechol[62–64, 69]. This type of ring oxygenation also occurs in *Bacillus stearothermophilus*[60], *Brevibacterium fuscum*[59] and *Candida tropicalis*[65, 70]. 4-Methylcatechol conversion to protocatechuic acid[66] is also known and may proceed by the same set of reactions as those employed for the conversion of *p*-cresol to 4-hydroxybenzoic acid.

Similar diversities occur during the metabolism of *m*-cresol also. Hopper *et al.*[71, 72] observed the oxidation of *m*-cresol and its derivatives to the corresponding 3-hydroxybenzoic acids and gentisic acids by nonfluorescent *Pseudomonas* sp. But hydroxylation at 2-position to give 3-methylcatechol was found to occur in fluorescent *Pseudomonas* strains[62–64, 67, 69]. Though the *m*-cresol grown cells of *P. putida* oxidized *m*-cresol by the catechol pathway, 3, 5-xylenol grown cells oxidized *m*-cresol by the gentisate pathway[67].

An alternate mode of hydroxylation occurs in *Brevibacterium fuscum*[59], *Bacillus stearothermophilus*[60] and *Candida tropicalis*[65]. These organisms convert *m*-cresol to 4-methylcatechol rather than to 3-methylcatechol. Furthermore, hydroxylation of *m*-cresol and *p*-cresol by purified phenol monooxygenase from *Trichosporon cutaneum*[57] yields only 4-methylcatechol in both cases. It is noteworthy that hydroxylation of *m*-cresol by gram negative bacteria yields 3-methylcatechol, while the gram positive species and yeast produce 4-methylcatechol.

Comparatively little work has been done on xylenol metabolism. Dagley and Patel[68], while studying the metabolism of *p*-cresol, observed the oxidation of 2, 4-xylenol and 3, 4-xylenol to 4-hydroxy-3-methyl benzoic acid and 4-hydroxy-2-methyl benzoic acid respectively. Oxidative metabolism of 2, 4-xylenol to 4-hydroxy-3-methylbenzoic acid has also been observed by Leibnitz *et al.*[73], and Chapman and Hopper[74]. The latter workers identified, in addition to the above acid, 4-hydroxy isophthalic acid and protocatechuic acid as metabolites of 2, 4-xylenol. They have also studied the oxidation of 2, 5- and 3, 5-xylenols and established their conversion to 4-methyl and 3-methyl gentisic acids *via* 3-hydroxy-4-methyl and 3-hydroxy-5-methyl benzoic acids, respectively[71].

Biosynthesis of patulin, an antibiotic produced by several species of *Penicillium* deserves special mention here. Several years of intensive investigation and brilliant experimentation led to the elucidation of the metabolic map for the biosynthesis of patulin which is depicted in Fig. 5. Radioactive tracer studies[75, 76] correctly paved the way for the recognition of the pathway starting from 6-methylsalicylic acid. This was further substantiated by enzymatic evidence for the individual reactions.

Light[77], and more recently Light and Vogel[78], purified and characterized 6-methylsalicylate decarboxylase from *Penicillium patulum*. A cytochrome P–450 dependent, NADPH requiring particulate monooxygenase was isolated from the same organism and shown to hydroxylate *m*-cresol to3-hydroxybenzyl alcohol and 2, 5-dihydroxytoluene[79]. The same fraction also hydroxylated 3-hydroxybenzyl alcohol to gentisyl alcohol with the consumption of NADPH[80]. 3-Hydroxybenzaldehyde was not ring hydroxylated, but oxidized to 3-hydroxybenzoic acid. 3-Hydroxybenzyl alcohol and gentisyl alcohol dehydrogenases, both requiring $NADP^+$, have also been characterized[81, 82]. A soluble

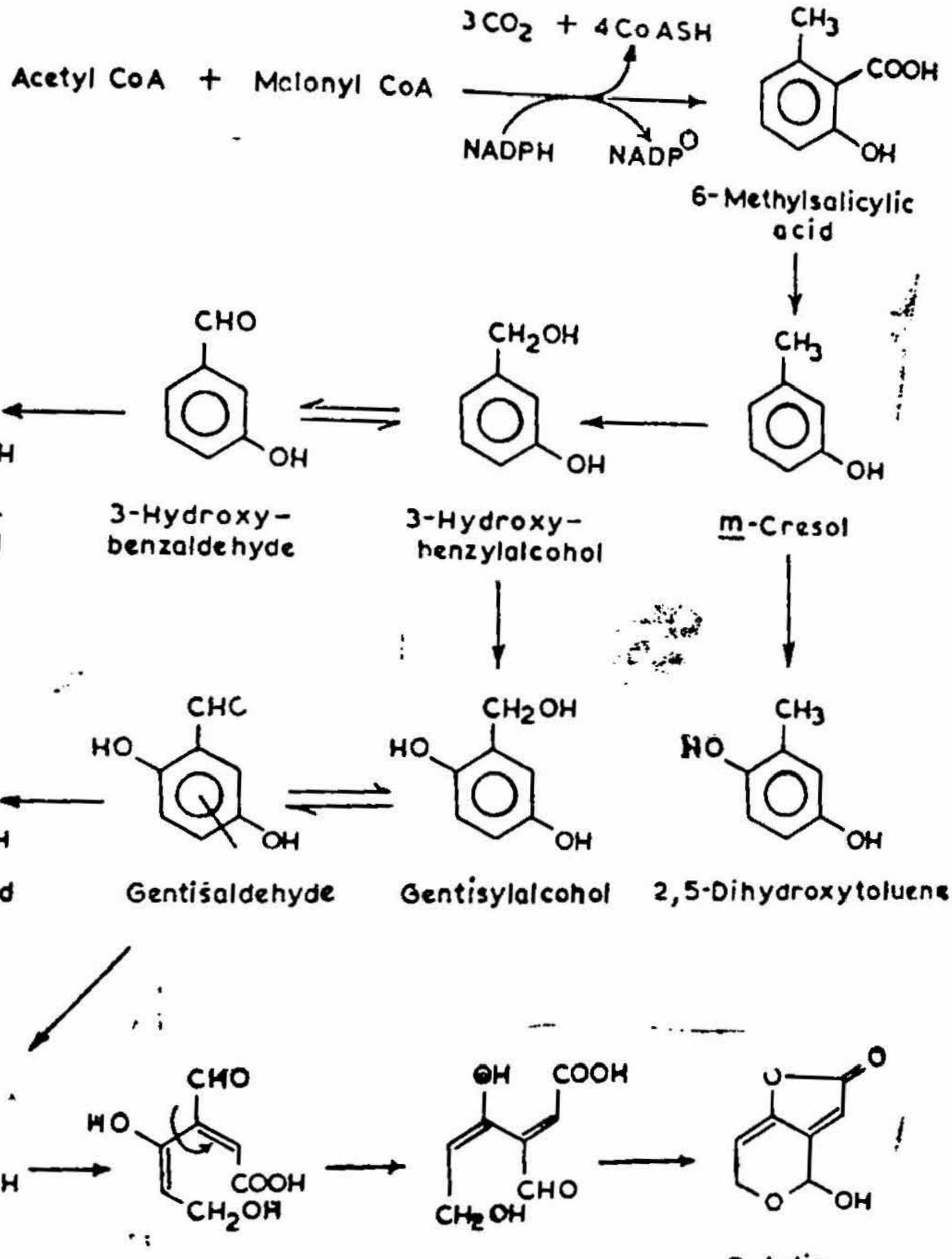


FIG. 5. Biosynthesis of patulin.

enzyme fraction catalyzing the conversion of gentisaldehyde to patulin, with the utilization of NADPH, was obtained by Scott and Beadling[82].

7. Metabolism of benzoic acid and its derivatives

Figure 6 gives various pathways for the metabolism of benzoic acid. Though the oxidative decarboxylation of benzoic acid to catechol was known for quite some time[83], the problem as to how it is initially attacked has been rather obscure, until Reiner[84, 85]

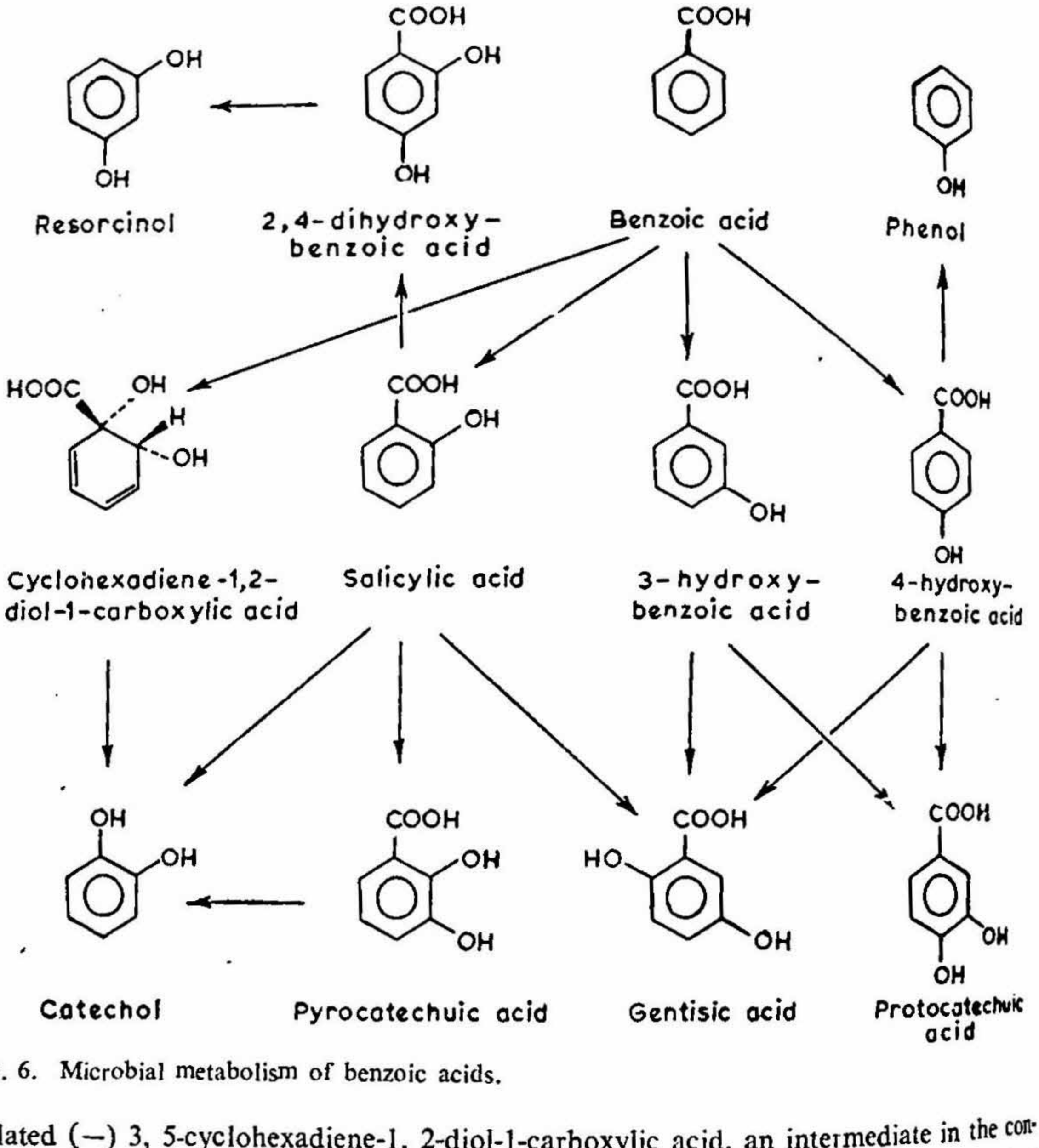


FIG. 6. Microbial metabolism of benzoic acids.

isolated (—) 3, 5-cyclohexadiene-1, 2-diol-1-carboxylic acid, an intermediate in the conversion of benzoic acid to catechol, and showed that it arises by a dioxygenase type of reaction. Subsequently, Reiner[86] purified (—) 3, 5-cylcohexadiene-1, 2-diol-1-carboxylate dehydrogenase to homogeneity. The dehydrogenase which has a molecular weight of 94,600 and four subunits of molecular weight 24,000 each, catalyzes both decarboxylation and dehydrogenation reactions utilizing NAD^+ as the cofactor.

Recently, Yamaguchi *et al.*[87] have purified benzoate-1,2-dioxygenase from *Pseudomonas arvilla*. The purified enzyme was found to consist of two protein components and it utilized NADH for oxygenation. They have also adduced evidence for the participation of NADH-cytochrome *c*-reductase in the double hydroxylation reaction.

Salicylic acid has also been implicated as the initial product of benzoate oxidation based on sequential induction experiments[88–90]. However, the lines of evidence pre-

sented by these workers are insufficient and the demonstration of benzoate-2-hydroxylation in the cell-free system is essential to substantiate this pathway.

Several species of bacteria belonging to the genus *Pseudomonas*[48, 88, 91], *Azotobacter vinelandii*[89], *Mycobacterium fortuitum*[92], *Acinetobacter* NCIB 8250[93] and *Pullularia pullulans*[94] oxidatively decarboxylate salicylate to catechol. Salicylate monooxygenase (decarboxylating) was the second flavoprotein monooxygenase to be discovered and the first example of the group of external flavoprotein monooxygenases[95]. It has one mole of FAD per mole of enzyme protein of molecular weight 57,000[96, 97]. The monooxygenase nature of the reaction has been established with the use of $^{18}O_2$ studies[98]. The mechanism of the hydroxylation reaction has been extensively studied by two groups of investigators headed by Katagiri and Kamin[99].

Hydroxylation of salicylic acid at the 3-position leads to the formation of pyrocatechuic acid. Though this pathway seems to be prevalent in fungi such as *Aspergillus niger*[100], *Aspergillus nidulans*[101] and *Trichoderma lignorum*[102], the enzyme catalyzing this reaction has not yet been isolated.

An unusual mode of hydroxylation occurs in *Aspergillus nidulans*[103] and *Trichosporon*[104]. In these organisms, salicylate seems to be hydroxylated to 2, 4-dihydroxybenzoate and degraded. However, it is converted to gentisate in *Pseudomonas*[105], *Trichoderma*[106] and *Trichosporon*[104]. Both these hydroxylase activities are yet to be demonstrated in cell-free systems.

3-Hydroxylation of benzoic acid occurs in *Pseudomonas testosteroni*[107], *Pseudomonas acidovorans*[107], *Arthrobacter*[108] and *Aspergillus niger*[109]. The enzyme effecting the hydroxylation is yet to be identified in cell-free systems.

Further metabolism of 3-hydroxybenzoic acid occurs in two ways. While *Pseudomonas fluorescens*[110], *Pseudomonas acidovorans*[107], an unidentified *Pseudomonas*[111], *Pseudomonas aeruginosa*[112] and several species of *Bacilli*[113] hydroxylate 3-hydroxybenzoate at 6-position, *Pseudomonas dacunhae*[105], *Pseudomonas testosteroni*[107, 114] and *Aspergillus niger*[115, 116] hydroxylate it at the 4-position.

3-Hydroxybenzoate-6-monooxygenase is a flavoprotein requiring either NADH or NADPH as electron donor for the conversion of 3-hydroxybenzoate to gentisate[112]. This enzyme, which has a molecular weight of 85,000, has been purified to near homogeneity and shown to have FAD as the prosthetic group[112]. 3-Hydroxybenzoate-4-monooxygenase has been purified both from fungal as well as bacterial sources[114–116]. The enzyme from *Aspergillus niger*[115, 116] and *Pseudomonas testosteroni*[114] is a flavoprotein, requiring either NADPH or NADH for activity, although the former is the preferred electron donor. The bacterial enzyme has a molecular weight of 145,000. Premakumar *et al.*[117] have shown that the hydroxylation can be completely inhibited by reasonably low levels of superoxide dismutase, implicating the involvement of O_2^- in the catalytic mechanism.

Fungi, in general, seem to oxidize benzoic acid to protocatechuic acid, *via* 4-hydroxy-benzoic acid[101, 102, 109, 118–125]. Occurrence of this pathway in a soil *Pseudomonas* has also been demonstrated[126]. Benzoate-4-monooxygenase has been purified from this laboratory both from fungal[127, 128] as well as bacterial[129] sources and shown to be a pteridine-dependent monooxygenase utilizing NADPH as the external electron donor.

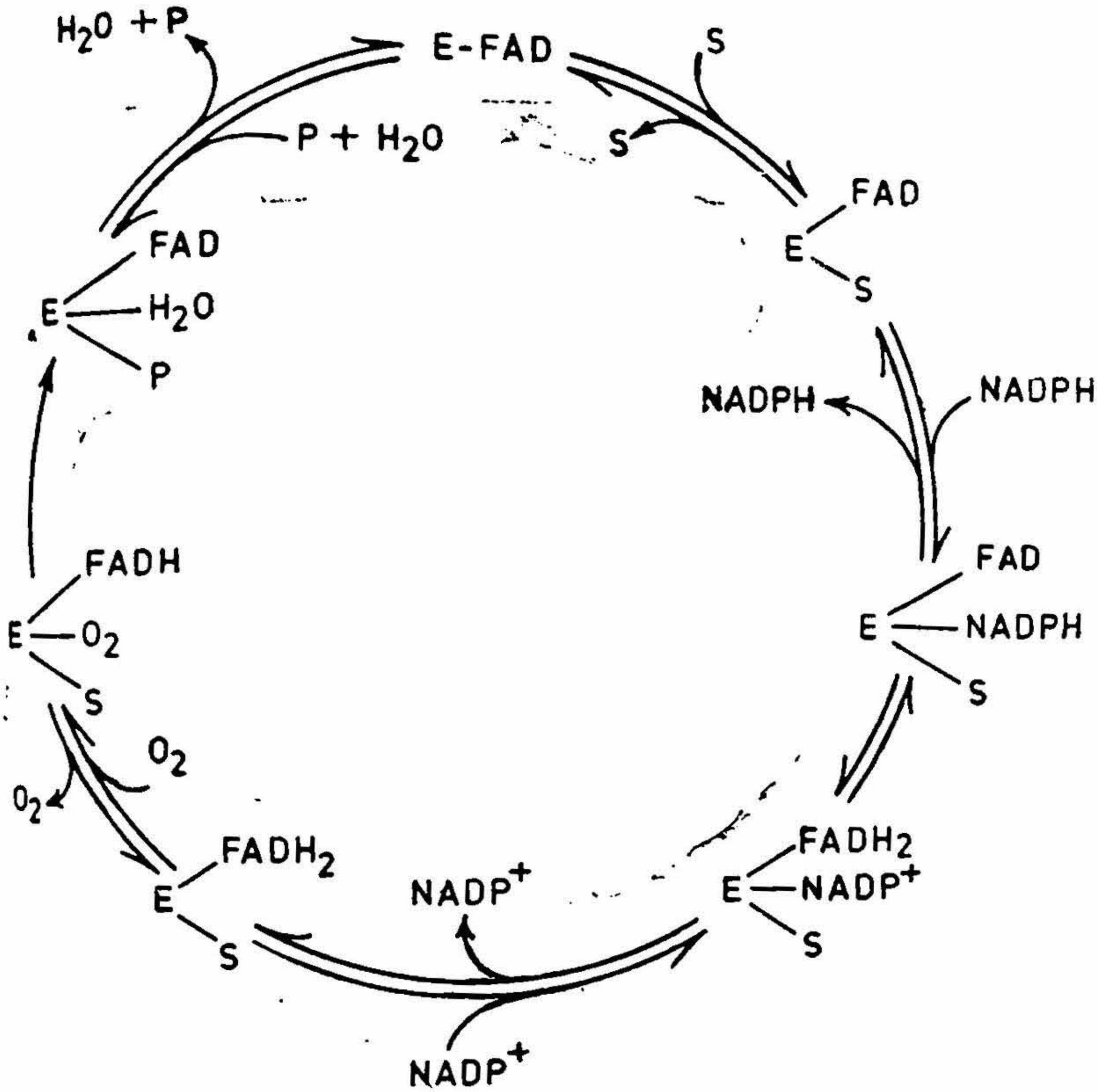
Further metabolism of 4-hydroxybenzoic acid proceeds by its conversion to protocatechuic acid. 4-Hydroxybenzoate-3-monooxygenase has been obtained in crystalline form from four different species of *Pseudomonas* grown on 4-hydroxybenzoate[130–133]. All of them show an absolute requirement for NADPH as the physiological external reductant and contain one mole of FAD per mole of enzyme. The molecular weight ranges from 65,000 to 93,000. While the enzyme accepts 4-aminobenzoate and 2, 4-dihydroxybenzoate as substrates and converts them to 3-hydroxy-4-aminobenzcate and 2, 3, 4-trihydroxybenzoate respectively, it uses 5-hydroxy picolinate and 6-hydroxy nicotinate as effectors only[134]. The catalytic mechanism, like other flavoprotein monooxygenases involves a Bi Uni Uni Uni Ping Pong mechanism[99] which is depicted in Scheme I. Perhaps this is the first monooxygenase subjected to X-ray crystallographic[135] and electron microscopic[136] studies.

Recently, an interesting reaction has been discovered in *Bacillus* sp.[137, 138]. In this organism 4-hydroxybenzoate is hydroxylated at the 1-position to give gentisate apparently involving a migration of the substituent carboxyl group to the 2-position.

Non-oxidative decarboxylation of benzoic acids appears to be a common reaction encountered in the metabolic transformations of these compounds. Table I summarizes the substrates and products, and the microorganisms which effect these decarboxylation reactions. Interestingly, studies on some of the decarboxylases reveal that these reactions do not require PALP or TPP, the common cofactors of the decarboxylation reactions[77, 78, 145, 150, 151]. Detailed studies on these enzymes are necessary to explore the nature of the cofactor and the mechanism of the reaction.

Pimelic acid was identified as the end product of photometabolism of benzoic acid under anaerobic conditions by Dutton and Evans[153] in *Rhodopseudomonas palustris*. Such a reductive pathway also occurs in *Pseudomonas* PN-1 strain when it grows anaerobically on benzoate[154]. Recently, a *Moraxella* sp. was found to metabolize benzoic acid through a reductive pathway involving anaerobic nitrite respiration, to adipic acid[155]. Cyclohexane carboxylate and 2-hydroxycyclohexane carboxylate are intermediates formed during this conversion[155] (Fig. 7). Methanogenic fermentation of benzoate by mixed cultures has also been reported (ref. 156 and the references cited therein).

Anthranilic acid, the end product of tryptophan catabolism, is oxidized by a *Pseudomonas* sp. to catechol[157] with the consumption of NADH. The purified enzyme system consists of two protein factors[158], and incorporates both the atoms of molecular oxygen into the substrate[159]. Fungal metabolism of anthranilic acid seems to be distinct. Terui *et al.*[144] reported the intermediate formation of pyrocatechuic acid in *Aspergillus niger*



SCHEME 1. Kinetic mechanism of the reaction catalyzed by 4-hydroxybenzoic acid-3-monooxygenase

Subsequent investigations proved the occurrence of such a pathway in *Claviceps paspali*[160] and *Aspergillus niger*[161]. Anthranilate hydroxylase which converts anthranilic acid to pyrocatechuic acid has been isolated in our laboratory and shown to require NADPH[162 163]. Evidence for the participation of iron[164] and O_2^-[117] in the double hydroxylation reaction has been provided. Floss *et al.*[165] have presented evidence for a monooxygenase type of reaction for the *Claviceps* enzyme with the use of $H_2{}^{18}O$.

Phthalic acid degradation was first studied by Ribbons and Evans[152] in *Pseudomonas*. They elucidated a pathway involving 4, 5-dihydroxyphthalic acid and protocatechuic acid

Table I

Decarboxylation of benzoic acid and its derivatives

Substrate	Product	Organism
Benzoic acid	Benzene	*Hypoxylum pruinatum*[139]
Salicylic acid	Phenol	*Glomerella cingulata*[140]
4-Hydroxybenzoic acid	Phenol	*Klebsiella aerogenes* (*Aerobacter aerogenes*) [141–143]
Pyrocatechuic acid	Catechol	*Glomerella cingulata*[140] *Aspergillus niger*[100, 144, 145] *Aspergillus nidulans*[101] *Trichoderma lignorum*[102]
2, 4-Dihydroxybenzoic acid	Resorcinol	*Aspergillus nidulans*[103, 106]
Gentisic acid	Quinol	*Klebsiella aerogenes* (*Aerobacter aerogenes*)[142]
Protocatechuic acid	Catechol	*Aspergillus*[147] *Klebsiella aerogenes* (*Aerobacter aerogenes*)[142, 143, 148] *Rhodopseudomonas*[149]
6-Methylsalicylic acid	*m*-Cresol	*Penicillium patulum*[77, 78]
Orsellinic acid	Orcinol	*Gliocladium roseum*[150] *Umbilicaria pustulata*[151]
4, 5-Dihydroxyphthalic acid	Protocatechuic acid	*Pseudomonas*[152]
Gallic acid	Pyrogallol	*Klebsiella aerogenes* (*Aerobacter aerogenes*)[142]

as intermediates. Such a pathway also occurs in *Nocardia*, *Arthrobacter*, some *Pseudomonas* sp.[166] and *Aeromonas*[167]. *Nocardia*[166] also converts terephthalic acid to protocatechuic acid. The initial conversions may involve a dicxygenase type of reaction. Experiments at enzymatic level are necessary to substantiate this.

Piperonylic acid [3, 4-methylenedioxybenzoic acid] was found to be metabolized to protocatechuic acid *via* vanillic acid[168, 169]. Though the normal metabolism of vanillate proceeds by its conversion to protocatechuate[170–173], *Polyporus dichrous*, a lignin destroying fungus, converts it to methoxyhydroquinone[174]. Such a type of conversion also occurs in the case of some aspergilli which oxidize 4-methoxybenzoate to

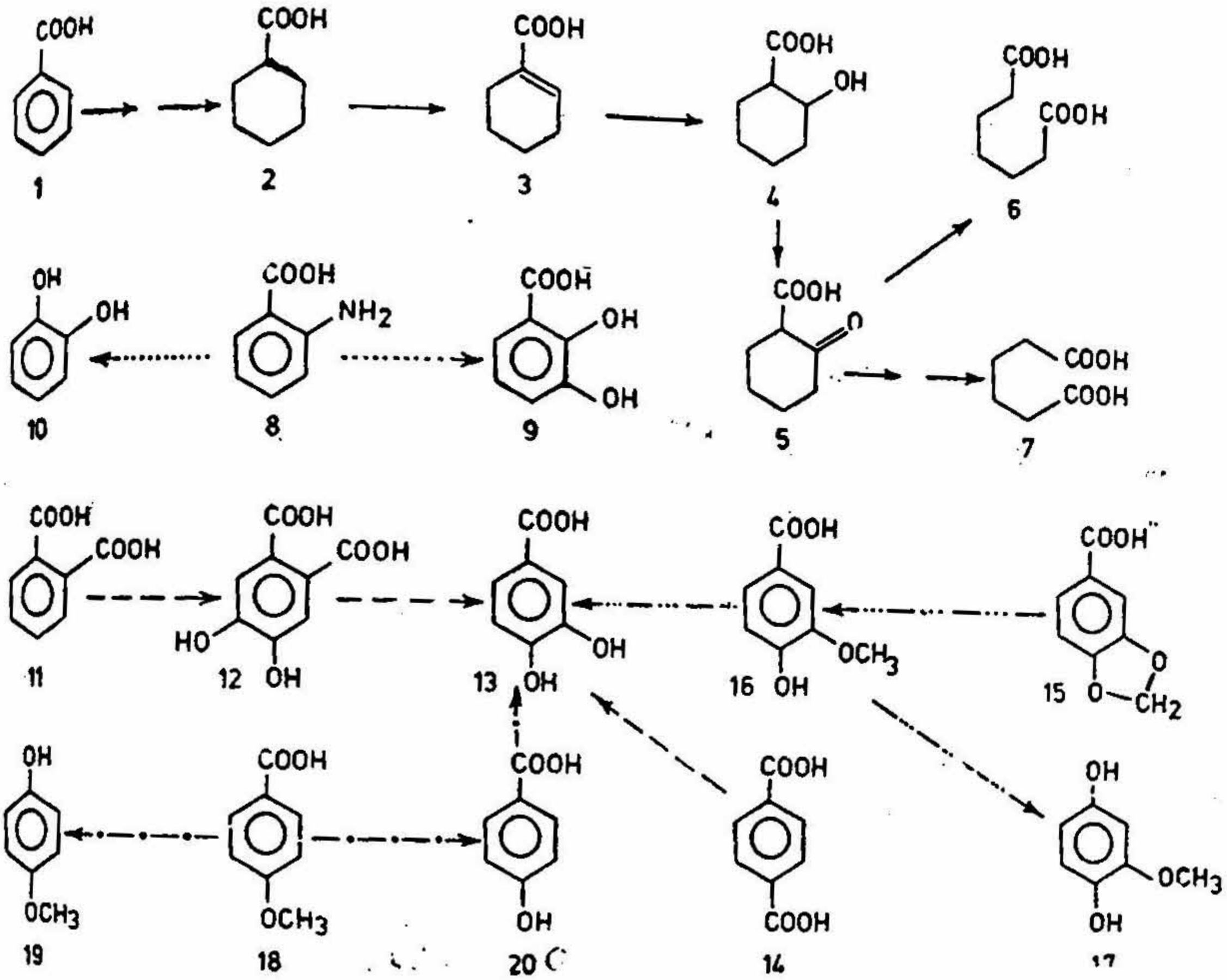


FIG. 7. METABOLISM OF BENZOIC ACID AND SUBSTITUTED BENZOIC ACIDS. 1. Benzoic acid. 2. Cyclohexane carboxylic acid. 3. Cyclohexene-1-carboxylic acid. 4. 2-Hydroxycyclohexane carboxylic acid. 5. 2-Oxocyclo hexane carboxylic acid. 6. Pimelic acid. 7. Adipic acid. 8. Anthranilic acid. 9. Pyrocatechuic acid. 10. Catechol. 11. Phthalic acid. 12. 4, 5-Dihydroxyphthalic acid. 13. Protocatechuic acid. 14. Terephthalic acid. 15. Piperonylic acid (3, 4-methylenedioxybenzoic acid). 16. Vanillic acid. 17. Methoxyhydroquinone. 18. 4-Methoxybenzoic acid. 19. 4-Methoxyphenol. 20. 4-Hydroxybenzoic acid.

4-methoxyphenol[175], while in other organisms, 4-methoxybenzoate is oxidized to 4-hydroxybenzoate by an O-demethylase. The enzyme has two components, an iron-sulfur protein and an iron containing flavoprotein[176]. Both O_2 and NADH are stoichiometrically consumed during the reaction.

8. Metabolism of phenylacetic acids

Phenylacetic acid is excreted mainly as the glutamine conjugate by man and higher primates, as the glycine conjugate by most rodent species, as the ornithine di-conjugate by the hen and as the taurine conjugate by the pigeon, some carnivores and marine species[177–179]. It is hydroxylated to 2- and 4-hydroxyphenylacetic acids in the rabbit

liver[180]. The conversion of 2- and 3-hydroxyphenylacetic acids to homogentisic acid and of 4-hydroxyphenylacetic acid to homoprotocatechuic acid also occur in the rabbit liver[180].

Fig. 8 depicts various routes for the microbial metabolism of phenylacetic acid. The carboxylation of phenylacetic acid to form phenylalanine presumably *via* phenylpyruvic acid has been observed in fermentations of mixed ruminal microbial populations as well as in pure cultures of *Ruminicoccus flavefaciens*, *Bacterioides succinogenes*, *Chromatium* and *Rhodospirillum rubrum*[181, 182]. Formation of benzaldehyde, probably *via* mandelic acid in *Penicillium chrysogenum*[183], reduction of phenethyl alcohol in *Mycobacterium phlei*[184] and oxidation to 4-hydroxybenzoic acid in *Poria weirii*[185] are instances of unusual metabolism of phenylacetic acid.

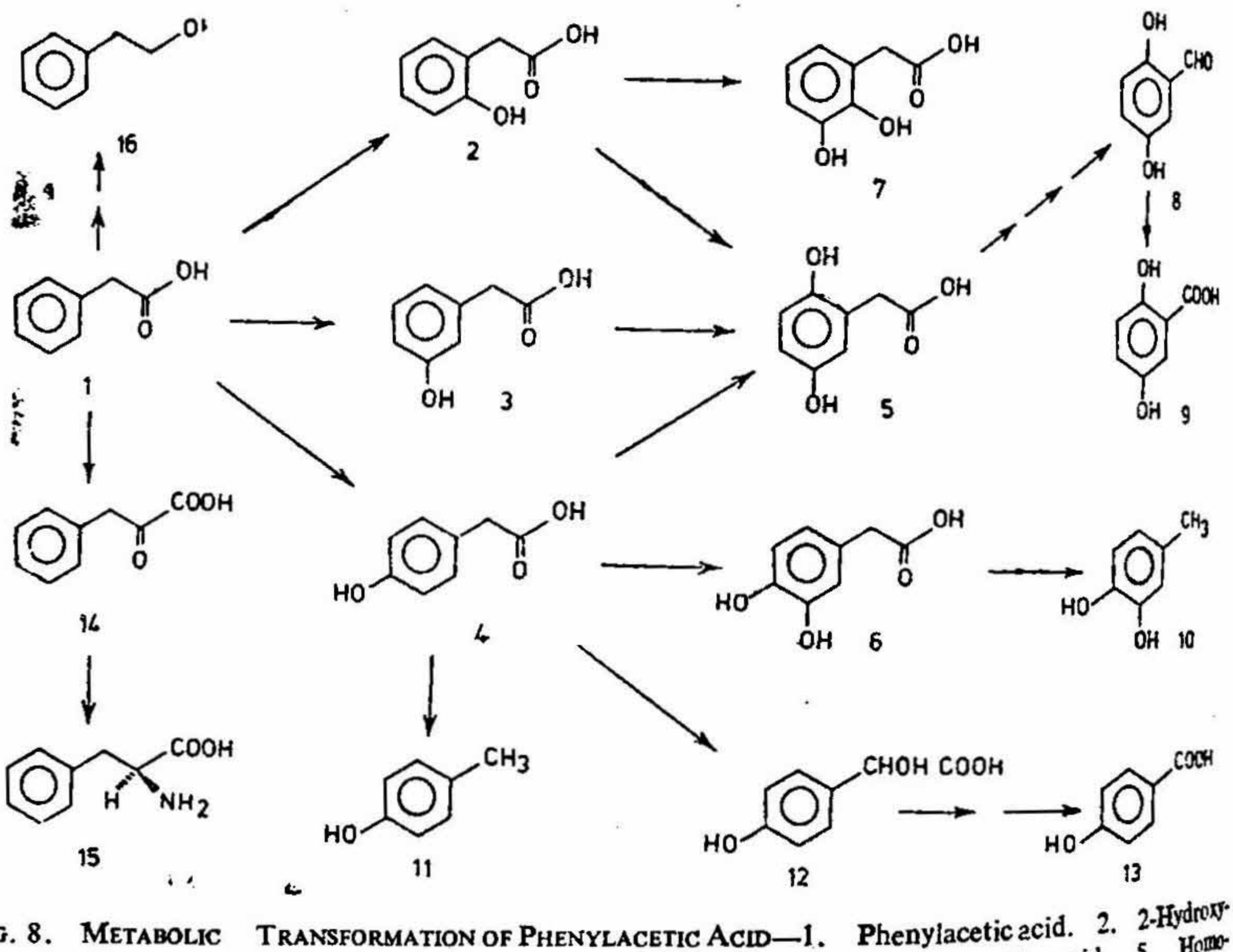


FIG. 8. METABOLIC TRANSFORMATION OF PHENYLACETIC ACID—1. Phenylacetic acid. 2. 2-Hydroxyphenylacetic acid. 3. 3-Hydroxyphenylacetic acid. 4. 4-Hydroxyphenylacetic acid. 5. Homogentisic acid. 6. Homoprotocatechuic acid. 7. 2, 3-Dihydroxyphenylacetic acid. 8. Gentisaldehyde. 9. Gentisic acid. 10. 4-Methylcatechol. 11. *p*-Cresol. 12. 4-Hydroxymandelic acid. 13. 4-Hydroxybenzoic acid. 14. Phenylpyruvic acid. 15. L-Phenylalanine. 16. Phenethyl alcohol.

Hydroxylation of phenylacetic acid at 2-position seems to be a common reaction encountered in its metabolism. Occurrence of 2-hydroxyphenylacetic acid as a metabolite of phenylacetic acid has been demonstrated in *Penicillium chrysogenum*[186], *Aspergillus niger*[109, 187], *Aspergillus sojae*[125], *Schizophyllum commune*[119], *Alternaria*[188], *Cladosporium*[44],

Fusarium[188], *Pseudomonas fluorescens*[189] and even in plants belonging to the genus *Astilbe*[109]. Further metabolism of 2-hydroxyphenylacetate proceeds *via* homogentisate[109, 125, 189]. A unique reaction occurs in the case of *Astilbe*, where it is transformed to 2-hydroxy-3-methoxyphenylacetate *via* 2, 3-dihydroxyphenylacetate[190].

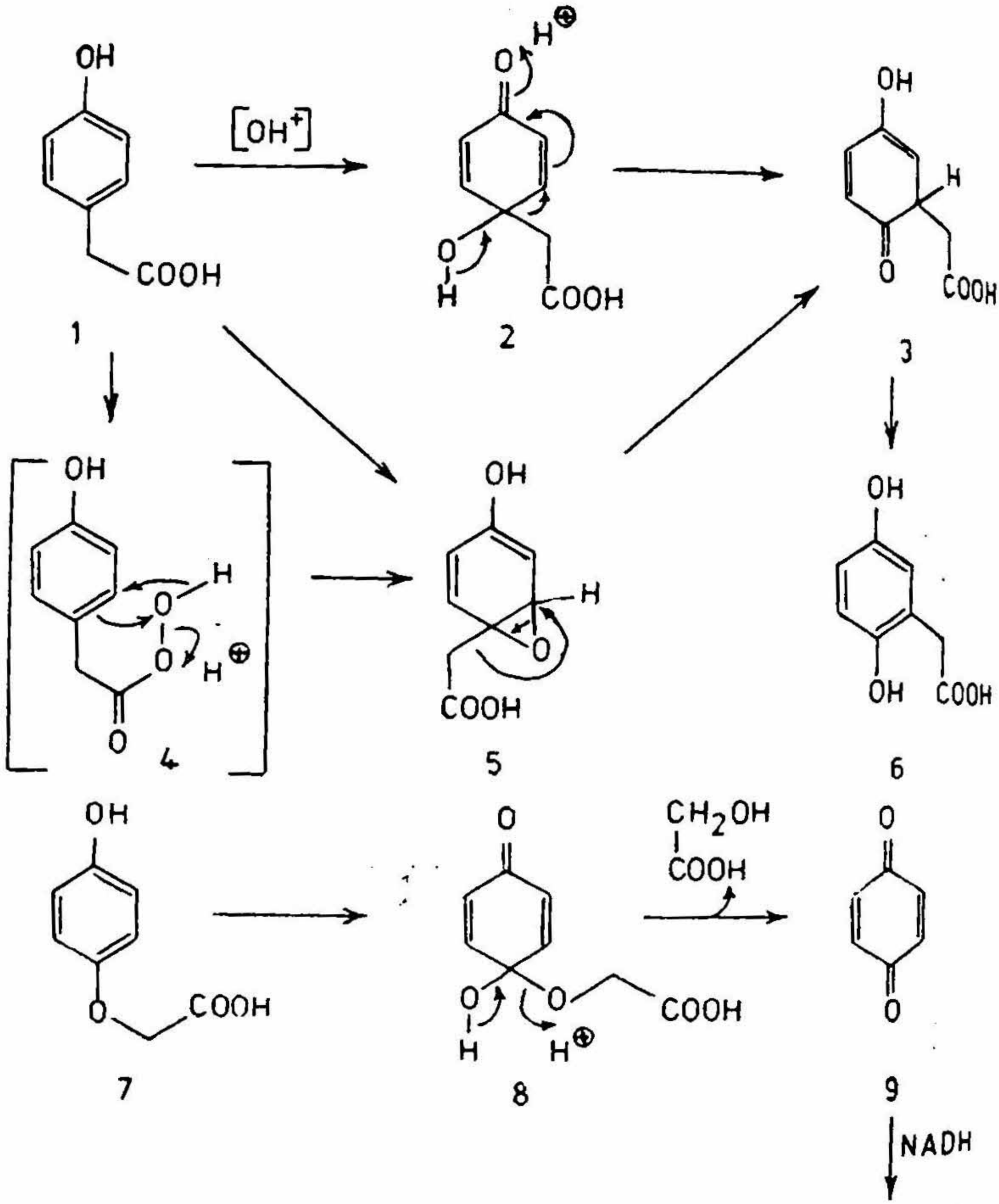
Phenylacetate-3-hydroxylation occurs in *Rhizoctonia solani*[188], *Aspergillus niger*[109, 187] and *Penicillium*[188]. *R. solani*, which is unique in producing 3-hydroxyphenylacetate in high yields, possesses an inducible enzyme to effect this reaction. Recently, this enzyme, which is highly specific for phenylacetate, has been partially purified and shown to be a pteridine-dependent monooxygenase requiring NADH as the external electron donor[191]. Though oxidation to homogentisate appears to be the major route for the catabolism of 3-hydroxyphenylacetate[189, 192], nothing is known about the enzyme catalyzing this conversion.

Pseudomonas fluorescens[193], an unidentified *Pseudomonas* strain[194] and *Penicillium chrysogenum*[195] appear to oxidize phenylacetic acid to the 4-hydroxylated product. However, the enzymatic evidence for this reaction is still lacking. Among the monohydroxylated products, only 4-hydroxyphenylacetate metabolism has been relatively well studied. Its conversion to homoprotocatechuic acid has been demonstrated to occur in *Penicillium chrysogenum*[195], *Pseudomonas fluorescens*[193], *Pseudomonas ovalis*[196], *Psudomonas putida*[192], *Acinetobacter*[192], *Micrococcus*[197], *Bacillus*[197], *Aeromonas aerogenes*[198] and *Arthrobacter*[199]. In fact, 4-hydroxyphenylacetate 3-monooxygenase has been partially purified from *P. ovalis* and shown to require NADH specifically[196]. However, the enzyme from *Micrococcus* requires NADPH[197].

Alternately, Blakely[200] has presented evidence that an unidentified bacterium hydroxylates 4-hydroxyphenylacetate at 1-position to give homogentisate utilizing either NADH or NADPH. Subsequently the enzyme catalyzing this reaction was purified by affinity chromatography from the cell-free extracts of *Pseudomonas acidovorans* and shown to require FAD and NADH/NADPH[201]. Apart from 4-hydroxyphenylacetate, the enzyme attacks 4-hydroxyphenoxyacetate as well as 4-hydroxy-hydratropate producing quinol and α-methylhomogentisate respectively. A mechanism involving 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl) acetic acid as the intermediate has been proposed (Fig. 9). However, a peracid and/or an epoxide intermediate shown in the figure are also equally possible intermediates.

Decarboxylation of 4-hydroxyphenylacetic acid to *p*-cresol occurs in an anaerobic microorganism[202], *Proteus vulgaris*[203] and *Clostridium difficile*[204]. Similar conversion in the case of homoprotocatechuic acid is effected by the microbial activity present in the rat fecal extracts.[205]

Side chain hydroxylation seems to be yet another route for the metabolism of 4-hydroxyphenylacetic acid. On the basis of 2, 5-dihydroxybenzoylformate formation from both 4-hydroxymandelate and 4-hydroxyphenylacetate, Crowden[206] suggested that 4-hydroxymandelate is formed from 4-hydroxyphenylacetate. Perrin and Towers[154] detected radic-


OH
COOH
[OH+]
H⊕
O
H
1
2
3
4
5
6
7
8
9
10
11
12
CH2OH
NADH

active 4-hydroxymandelate as a metabolite of labelled tyrosine. Its formation from 4-hydroxyphenylacetate has also been shown in *Aspergillus niger*[207, 208]. However, the enzyme effecting this reaction has not yet been isolated and characterized.

Side chain oxidation of homogentisate to gentisate seems to proceed *via* 2, 5-dihydroxymandelate, 2, 5-dihydroxybenzoylformate and gentisaldehyde in *Polyporus tumulosus*[206]. However, in a strain of *Pseudomonas aeruginosa* lacking homogentisate dioxygenase, the first product detected during homogentisate oxidation is gentisaldehyde[209].

9. Metabolism of some C_6–C_2 compounds

Acetophenone is oxidized by an *Arthrobacter* sp. to phenylacetate (ester) with the consumption of oxygen and NADPH[210]. This is an example of the biological equivalent of the Baeyer-Villiger oxidation by which ketones are oxidized to esters by peracids.

Oxidative metabolism of phenethylamines has been studied in a number of microorganisms. As early as 1942, Gale[211] observed the oxidation of tyramine to 4-hydroxyphenylacetaldehyde in *Pseudomonas pyocyanea* and *Bacterium coli*. An amine oxidase, initiating the degradation of amines in *Aspergillus*, *Penicillium*, *Monascus* and *Fusarium*, has been purified, crystallized and shown to be a cuproprotein having pyridoxal phosphate as the prosthetic group[212–215]. Tyramine oxidation to 4-hydroxyphenylacetaldehyde, 4-hydroxyphenylacetic acid and homoprotocatechuic acid occurs in *Aerobacter aerogenes*[198]. A monoamine oxidase specific to tyramine has been obtained from *Sarcina lutea* in crystalline form[216–218].

The metabolism of synephrine [4-hydroxy-(methylaminomethyl) benzylalcohol] in an *Arthrobacter* sp. has been recently examined[199] and its enzymatic conversion to 4-hydroxyphenylacetaldehyde has been reported[219]. Conversion of phenethylamine to phenethyl alcohol seems to occur in *Saccharomyces rouxii*[220].

Phenylacetaldehydes, which arise from amine oxidation as well as phenylpyruvate decarboxylation reactions, are oxidized to the corresponding acids by aldehyde dehydrogenases utilizing NAD^+[197, 199, 221]. An alternate reaction occurs in systems where phenethyl alcohol is produced. *Saccharomyces cerevisiae* reduces 4-hydroxyphenylacetaldehyde to tyrosol[222] and *Candida guilliermondii* reduces phenylacetaldehyde to phenethylalcohol[223].

10. Metabolism of mandelic acids

Apart from its formation from phenylacetic acid, mandelic acid can also arise from styrene[224] and phenylglyoxal[225]. Back in 1924, Supniewski reported the oxidation of

FIG. 9. MECHANISM OF ACTION OF 4-HYDROXYPHENYLACETIC ACID-1-HYDROXYLASE ON ITS SUBSTRATES. 1. 4-Hydroxyphenylacetic acid. 2. Quinol intermediate. 3. Dienone intermediate. 4. Peracid intermediate. 5. Epoxide intermediate. 6. Homogentisic acid. 7. 4-Hydroxyphenoxy acetic acid. 8. Quinol intermediate. 9. Benzoquinone. 10. Quinol, 11. α-Methyl-4-hydroxyphenylacetic acid. 12. α-Methyl homogentisic acid.

mandelic acid to benzoic acid by *Bacillus pyocyaneous*[226]. While *Bacillus pyocyaneous*[226], *Pseudomonas putida*[83, 227], *Pseudomonas fluorescens*[227], *Pseudomonas convexa*[228], *Azotobacter beijerinckii*[227] and *Aspergillus niger*[122] utilize both D- and L-mandelic acids, *Pseudomonas aeruginosa*[227, 229], *Pseudomonas multivorans*[229], *Bacillus sphaericus*[227], *Acinetobacter calcoaceticus*[230] and an yeast[227] utilize only the L-enantiomer. Due to the presence of a racemase which catalyzes the conversion of either enantiomer to a racemic mixture by a carbanion mechanism[231], most bacteria utilize D- and L-mandelates, while *A. niger*[122, 123] and *P. putida*[227] possess a D-mandelate oxidase for the utilization of D-mandelate. Other organisms, which lack the racemase, utilize only the L-enantiomer.

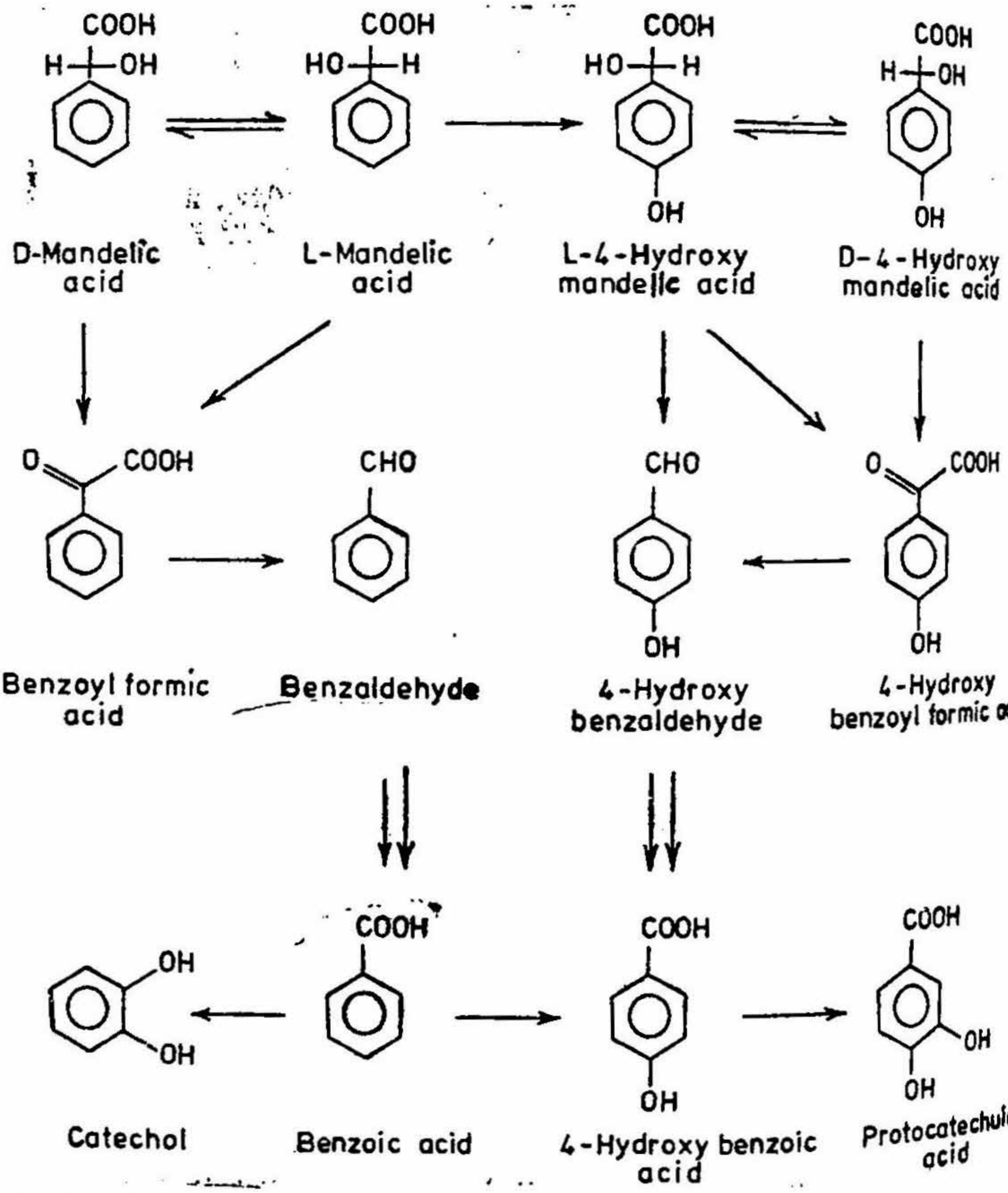


FIG. 10. Microbial metabolism of mandelic acid.

Mandelate racemase has been purified to homogeneity [227, 233] and shown to be a divalent metal ion-requiring enzyme with four identical subunits and a molecular weight of 2,78,000. L-Mandelate dehydrogenase, which is situated in the particulate fraction[234], has been partially purified and found to require 2, 6-dichlorophenol indophenol as the electron acceptor[235]. In *A. niger*, D-mandelate oxidase is a particulate enzyme requiring molecular oxygen[232] and L-mandelate dehydrogenase is a soluble enzyme requiring either molecular oxygen or 2, 6-dichlorophenol indophenol[122]. Benzoylformate decarboxylase converts the product thus formed into benzaldehyde. This enzyme which requires TPP as a cofactor has been purified from *P. putida*[236, 237].

With the use of two benzaldehyde dehydrogenases, one requiring NAD^+ and the other $NADP^+$, benzaldehyde is oxidized to benzoic acid by *P. putida*[236] and *A. niger*[122]. However, *Acinetobacter* seems to possess two NAD^+ specific dehydrogenases—one heat labile and the other heat stable[238 239]. But it lacks the $NADP^+$ specific enzyme. *Pseudomonas aeruginosa* lacks the NAD^+ specific enzyme and possesses the $NADP^+$ specific enzyme[240]. The point of divergence of mandelic acid metabolism in fungi and bacteria is at the level of benzoic acid. While bacteria oxidize benzoate to catechol, fungi convert it to 4-hydroxybenzoate (Fig. 10).

In an analogous way, 4-hydroxymandelic acid is oxidized to 4-hydroxybenzoic acid, via 4-hydroxybenzoylformic acid and 4-hydroxybenzaldehyde in *Pseudomonas putida*[241], *Acinetobacter* NCIB 8250[93], *Polyporus tumulosus*[206] and *Aspergillus niger*[122, 207, 208]. It is presumed, largely on the basis of kinetic evidence, that the same set of enzymes degrade mandelate as well as 4-hydroxymandelate to benzoate and 4-hydroxybenzoate, respectively[242]. *Acinetobacter*[93] also oxidizes 2- and 3-hydroxymandelate, 3, 4-dihydroxymandelate and vanillyl mandelate to the corresponding benzoate derivatives by parallel pathways. Similarly, *Polyporus tumulosus* oxidizes 2, 5- and 3, 4-dihydroxymandelic acids, which arise in this organism from 2, 5- and 3, 4-dihydroxyphenylacetic acids, to gentisic and protocatechuic acids respectively[206]. An interesting reaction employed by this organism is the conversion of 4-hydroxymandelic acid to 2, 5-dihydroxymandelic acid which is similar to the conversion of 4-hydroxyphenylacetic acid to homogentisic acid[200, 201] (Fig. 11).

A novel variant of the mandelate pathway occurs in *Pseudomonas convexa* (Fig. 10). This bacterium failed to oxidize both D- and L-mandelic acids directly, through the side chain, but hydroxylated the L-mandelic acid to 4-hydroxymandelic acid[228]. Mandelate-4-hydroxylase from this organism has been partially purified and shown to be a pteridine-dependent monooxygenase requiring NADPH as the electron donor[243, 244]. Even, the further metabolism of 4-hydroxymandelate is different in this organism. Employing FAD and Mn^{2+} as cofactors, a single enzyme seems to oxidatively decarboxylate 4-hydroxymandelate to 4-hydroxybenzaldehyde without the intermediate formation of 4-hydroxybenzoylformate[245]. This enzyme utilizes molecular oxygen and produces H_2O_2. The fate of 4-hydroxybenzaldehyde is the same as that observed in other organisms.

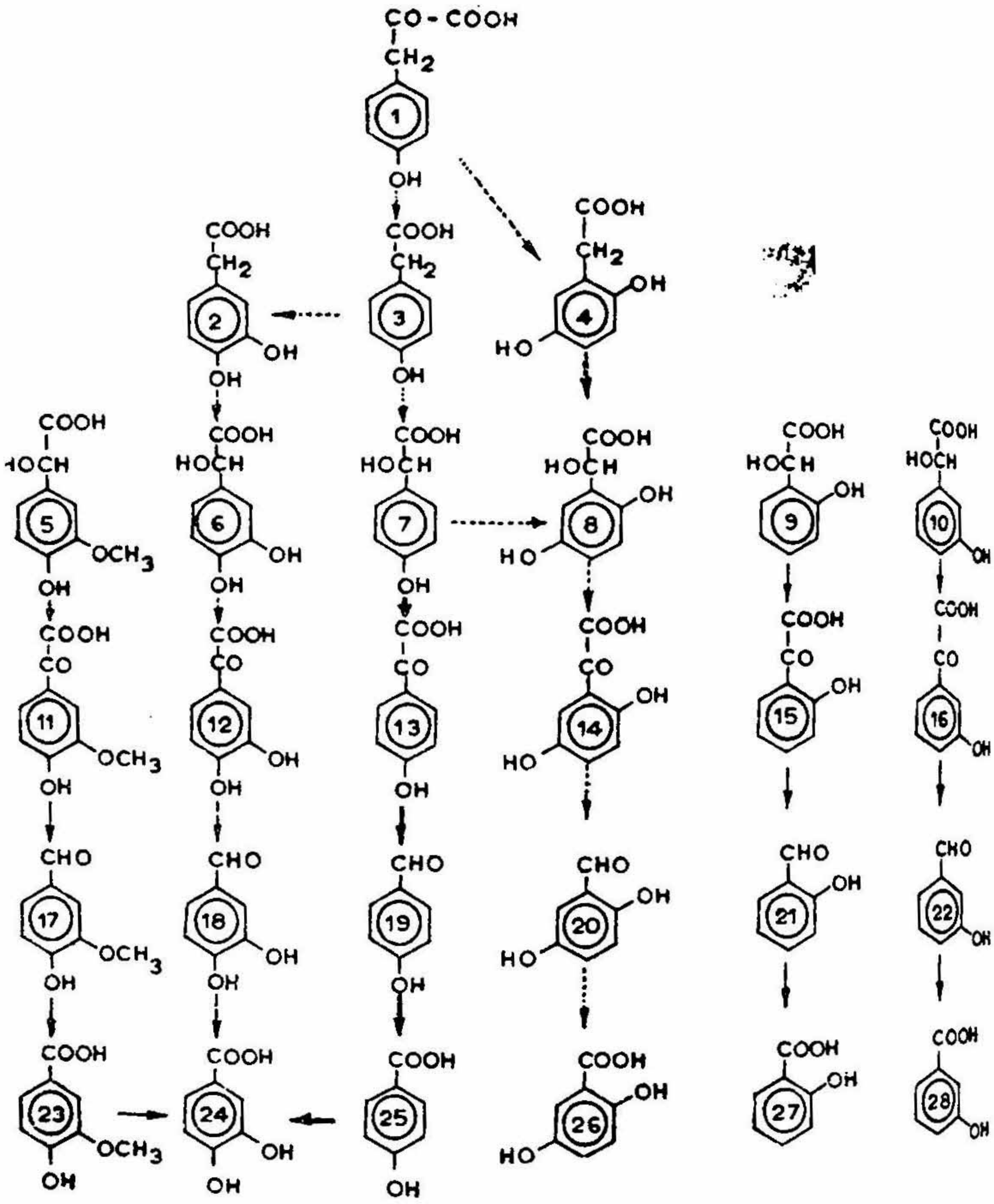


FIG. 11. METABOLISM OF HYDROXYMANDELIC ACIDS IN MICROORGANISMS

——→Pathways demonstrated in *Pseudomonas putida*, *Polyporus tumulosus*, *Acine tobacter* NCIB 8250 and *Aspergillus niger*.

——→Pathways possible in *Acinetobacter* NCIB 8250.

– – –→Pathway possible in *Acinetobacter* NCIB 8250 and *Polyporus tumulosus*.

. . . . .→Pathway demonstrated in *Polyporus tumulosus*.

1. 4-Hydroxyphenylpyruvic acid. 2. Homoprotocatechuic acid. 3. 4-Hydroxyphenylacetic acid. 4. Homogentisic acid. 5. 4-Hydroxy-3-methoxymandelic acid. 6. 3, 4-Dihydroxymandelic acid. 7. 4-Hydroxymandelic acid. 8. 2, 5-Dihydroxymandelic acid. 9. 2-Hydroxymandelic acid. 10. 3-Hydroxymandelic acid. 11. 4-Hydroxy-3-methoxybenzoylformic acid. 12. 3, 4-Dihydroxybenzoylformic acid. 13. 4-Hydroxybenzoylformic acid. 14. 2, 5-Dihydroxybenzoylformic acid. 15. 3-Hydroxybenzoylformic acid. 16. 3-Hydroxybenzoylformic acid. 17. Vanillin. 18. Protocatechualdehyde. 19. 4-Hydroxybenzaldehyde. 20. Gentisaldehyde. 21. Salicylaldehyde. 22. 3-Hydroxybenzaldehyde. 23. Vanillic acid. 24. Protocatechuic acid. 25. 4-Hydroxybenzoic acid. 26. Gentisic acid. 27. Salicylic acid. 28. 3-Hydroxybenzoic acid.

11. Metabolism of phenylpropionic acids

Coulson and Evans[246] demonstrated the direct hydroxylation of phenylpropionic acid to melilotic and 2, 3-dihydroxyphenylpropionic acids. Subsequently, this pathway was shown to be operative in a *Pseudomonas* strain[247] and an *Achromobacter* strain[248, 249]. The enzyme which converts melilotic acid to 2, 3-dihydroxyphenylpropionic acid has been isolated from *Arthrobacter* and shown to be a flavoprotein requiring NADH[250, 251]. This enzyme has also been isolated from a soil pseudomonad[252]. While the *Arthrobacter* enzyme is of monomeric nature with a molecular weight of 65,000, that of *Pseudomonas* has four subunits and a molecular weight of 250,000. Steady state kinetic analysis[252] revealed the operation of Bi Uni Uni Uni Ping Pong mechanism, which is similar to that shown in Scheme 1 for 4-hydroxybenzoate-3-monooxygenase reaction. With the use of [3, 5-^{2}H] melilotate, Strickland *et al.*[253] showed the occurrence of a primary kinetic isotope effect in the last reaction, *viz.*, the reaction of substrate bound, reduced melilotate monooxygenase with oxygen. There is no evidence for the existence of NIH shift during this reaction[253].

Dehydrogenation of phenylpropionic acid to cinnamic acid seems to occur in *Nocardia opaca*[40]. Decarboxylation of phenylpropionic acid occurs in some systems. Thus, the formation of 4-ethylphenol and 4-ethylcatechol from 4-hydroxyphenylpropionic[254] and 3, 4-dihydroxyphenylpropionic[255] acids are instances of decarboxylation of phenylpropionate moiety.

12. Metabolism of phenylpyruvic acids

Most of the microorganisms non-oxidatively decarboxylate phenylpyruvate to give phenylacetaldehyde. Thus a *Proteus* sp.[256], *Achromobacter eurydice*[221], *Schizophyllum commune*[119], some *Clostridia*[204], *Candida guilliermondii*[223] and *Aspergillus niger*[207, 208] seem to carry out this reaction. A similar conversion in the case of 4-hydroxyphenylpyruvate occurs in *Proteus vulgaris*[203], *Clostridia*[204], *Candida*[257], *Micrococcus*[197] and *Bacillus*[197]. The phenylpyruvate decarboxylase, which requires TPP and Mg^{2+}, has been purified from the cell-free extracts of *Achromobacter eurydice*[221].

Reduction of phenylpyruvate to phenyllactate seems to occur in *Exobasidium*,[258] *Schizophyllum commune*[119], *Clostridia*[204] and *Candida guilliermondii*[223]. The reaction

requires reduced pyridine nucleotide[222]. A similar conversion of 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate occurs in *Proteus vulgaris*[203], *Clostridia*[204] and *Candida* species[257]. The aromatic α-keto acid reductase has been purified from dog heart[258]. The fate of phenyllactate thus formed is not clearly understood. Though Tanaka[203] has postulated the formation of tyrosol from 4-hydroxyphenyllactate in *Proteus vulgaris*, Gopalakrishna *et al.*[223] have failed to observe such a decarboxylation reaction. Similarly, *p*-coumarate formation from 4-hydroxyphenyllactate reported by Tanaka[203], awaits confirmation by enzymatic evidence. Based on labelled studies, Weatherston and Percy proposed the conversion of cinnamic acid to phenethyl alcohol, *via* phenyllactic acid in the male bertha armyworm, *Mamestra configurata*[260].

However, mammalian system prefers carrying out an interesting reaction which involves hydroxylation at 1-position followed by an oxidative migration of the side chain to 2-position using the enzyme 4-hydroxyphenylpyruvate dioxygenase[261, 262]. The peracid intermediate 4 depicted in Fig. 9 seems to be a probable intermediate during the conversion of 4-hydroxyphenylpyruvate to homogentisate. At least in this case, there is evidence against the involvement of quinol as a free intermediate[263, 264]. *Pseudomonas*[265–267] and certain fungi[268] also seem to effect this reaction. Recently, 4-hydroxyphenylpyruvate dioxygenase has been purified to homogeneity from a *Pseudomonas* sp. and shown to be a ferrous iron requiring enzyme with a molecular weight of 150,000 and four subunits.[267]

13. Metabolism of cinnamic acids

Hydroxylation of cinnamate at 2- as well as 4-positions occurs in *Aspergillus niger*[269] and *Rhizoctonia solani*[270], while *Polystictus verisicolor*[271] and *Lentinus lepideus*[272] oxidize it to *p*-coumaric acid only. Though cinnamate-4-hydroxylase has not yet been isolated from any microorganism, it has been isolated from plant sources and identified as a cytochrome P-450 linked NADPH requiring monooxygenase[273]. Conversion of *p*-coumaric acid to caffeic acid occurs in *Lentinus lepideus*[272], *Streptomyces nigrifaciens*[274] and *Pseudomonas fluorescens*[275]. *p*-Coumarate-3-monooxygenase from *Streptomyces* has been purified to homogeneity and shown to be a phenolase type of enzyme with a molecular weight of 18,000[276].

Side chain oxidations of cinnamic acid and its derivatives are well known. Cinnamic acid itself undergoes oxidation to form benzoic acid in *Nocardia opaca*[40], *Ustilago hordei*[118], *Schizophyllum commune*[119], *Sporobolomyces roseus*[120], and *Alternaria*[123]. Similarly, *p*-coumarate is oxidized to 4-hydroxybenzoate in *Sporobolomyces roseus*[120], *Alternaria*[123], *Rhizoctonia solani*[270] and *Polyporus hispidus*[277], while *m*-coumarate is transformed to 3-hydroxybenzoate in *Sporobolomyces roseus*[120] and *Alternaria*[123]. *Alternaria*[123] also oxidizes caffeic, ferulic and sinapic acids to the corresponding benzoic acids. Ferulic acid is oxidized to vanillic acid by *Pseudomonas acidovorans* also[278].

Zenk[279] proposed β-oxidation type of reactions for the conversion of cinnamic acids to benzoic acids. This suggestion is in accordance with the results of Alibert *et al.*[280].

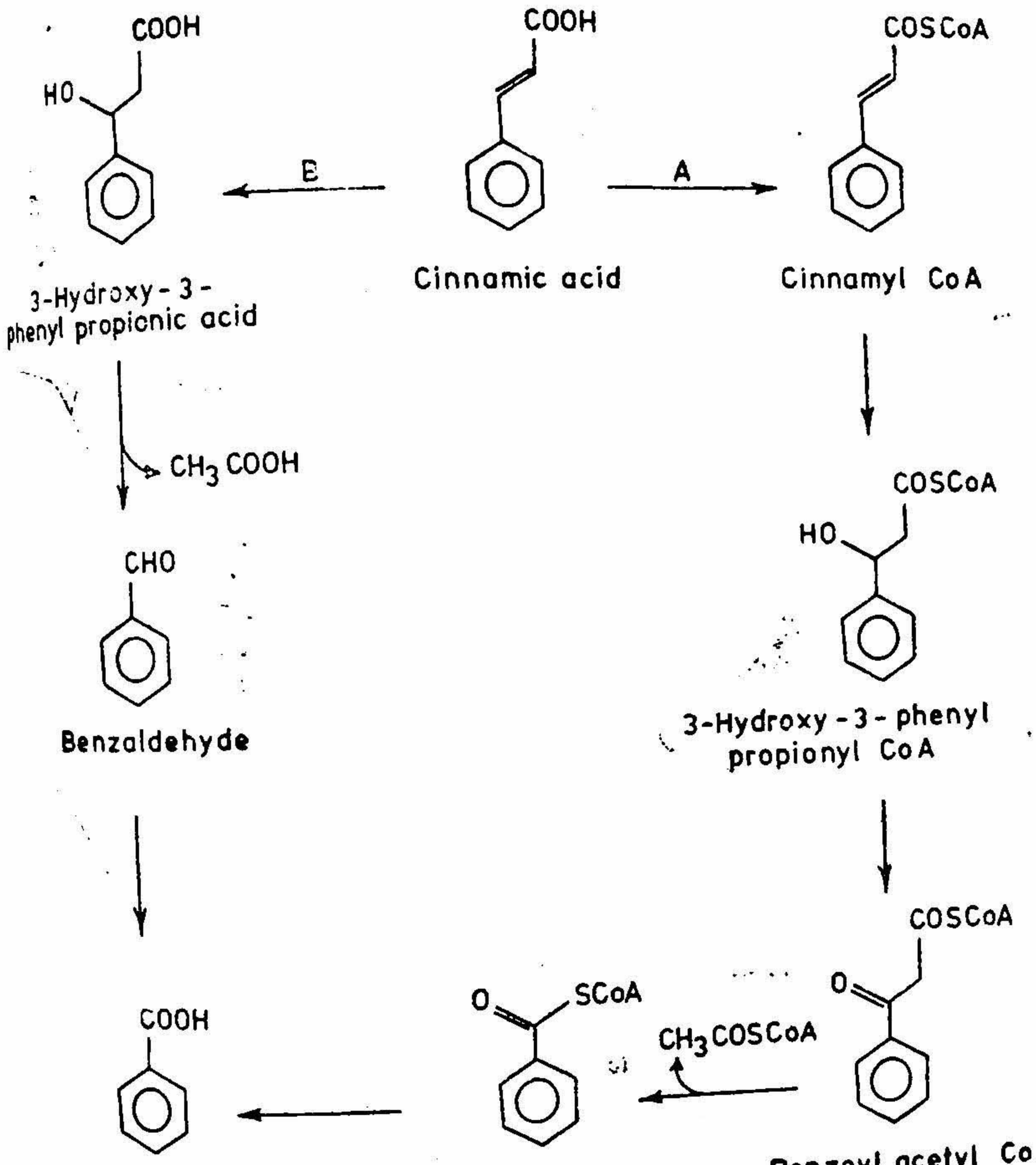


Fig. 12. Mechanism of conversion of cinnamic acid to benzoic acid. A : β-Oxidation type cleavage B : Non-oxidative aldolase type cleavage.

However, Toms and Wood[278], on the basis of their results, formulated a non-oxidative aldolase type cleavage as shown in Fig. 12. Such a type of reaction seems to occur in *Ricinus communis*[281], potato tubers and *Polyporus hispidus*[277].

Reduction of cinnamate occurs in a number of systems. *Lactobacillus pastorianus*, reduces the double bond of cinnamic, *p*-coumaric and caffeic acids[254, 255]. Cinnamate reduction also occurs in *Pseudomonas* sp[246, 247]. *Clostridia*[204] as well as *Proteus vulgaris*[203]

effect such reductions. The rat intestinal microflora reduce the double bond of *o*-, *m*-, and *p*-coumaric, ferulic and isoferulic acids *in vitro*[282].

Non-oxidative decarboxylation of cinnamic acids is yet another route for their metabolism. *Saccharomyces cerevisiae*[283] and *Aspergillus niger*[284] decarboxylate cinnamate itself. *Aerobacter aerogenes* produces a constitutive decarboxylase which decarboxylates *p*-coumaric acid, caffeic acid and even ferulic acid.[285] An enzyme with the same substrate specificity has also been obtained from the fungus *Cladosporium phlei*[286]. The decarboxylation proceeds by the retention of double bond geometry, probably by a two step addition-elimination reaction[287].

Coumarin is a good example for the occurrence of diverse pathways. While the mammalian system hydroxylates it either at 3- or 7-position[288], fungi convert in to 4-hydroxycoumarin[120, 269]. *Melilotus alba*, a higher plant[289] and a strain of *Pseudomonas*[290] reduce it to dihydrocoumarin. Coumarin reductase, which is specific for coumarin, has been partially purified from the latter source. *Arthrobacter*, however, hydrolyzes the lactone to *o*-coumaric acid and reduces the latter to melilotic acid [250, 291]. The *o*-coumarate reductase utilizes NADH and is highly specific for the aromatic substrate[292]. The metabolic transformations of cinnamic acid and its derivatives are shown in Fig. 13.

14. Metabolism of phenylalanine, tyrosine and dopa

(*a*) *Hydroxylation*

Phenylalanine-4-hydroxylase and tyrosine-3-hydroxylase are pteridine-dependent monooxygenases. They have been extensively studied from mammalian sources[293, 294]. The studies of Chandra and Vining[295] suggest that in addition to bacteria, the ability to hydroxylate phenylalanine is widespread in microfungi, but is rare or absent in actinomycetes, yeasts, and basidiomycetes. Phenylalanine-4-monooxygenase from *Pseudomonas* has been purified to near homogeneity and shown to require Fe^{2+} and NADH in addition to tetrahydropteridine[296–298]. It has a molecular weight of 27,000[298].

Attempts to develop a simple assay procedure, based on the release of tritium from 4-tritiated phenylalanine, led to the discovery of an unexpected phenomenon, best termed as hydroxylation induced migration ("the NIH shift"), occurring during the reaction[299]. The migration of deuterium[300, 301], tritium[301, 302], chlorine[303] and methyl[304] substituents from 4-position to the adjacent 3-position during hydroxylation at 4-position has been attributed to the formation of arene oxide intermediate.[305] The mechanism of the migration reaction is given in Fig. 14.

In addition to tyrosine-3-monooxygenase, tyrosine to dopa conversion is also effected by tyrosinase. But the latter enzyme catalyzes the subsequent oxidation of dopa to dopaquinone and further to melanin. This copper containing monooxygenase is widespread in bacteria, fungi, insects, marine animals, plants and mammals[306].

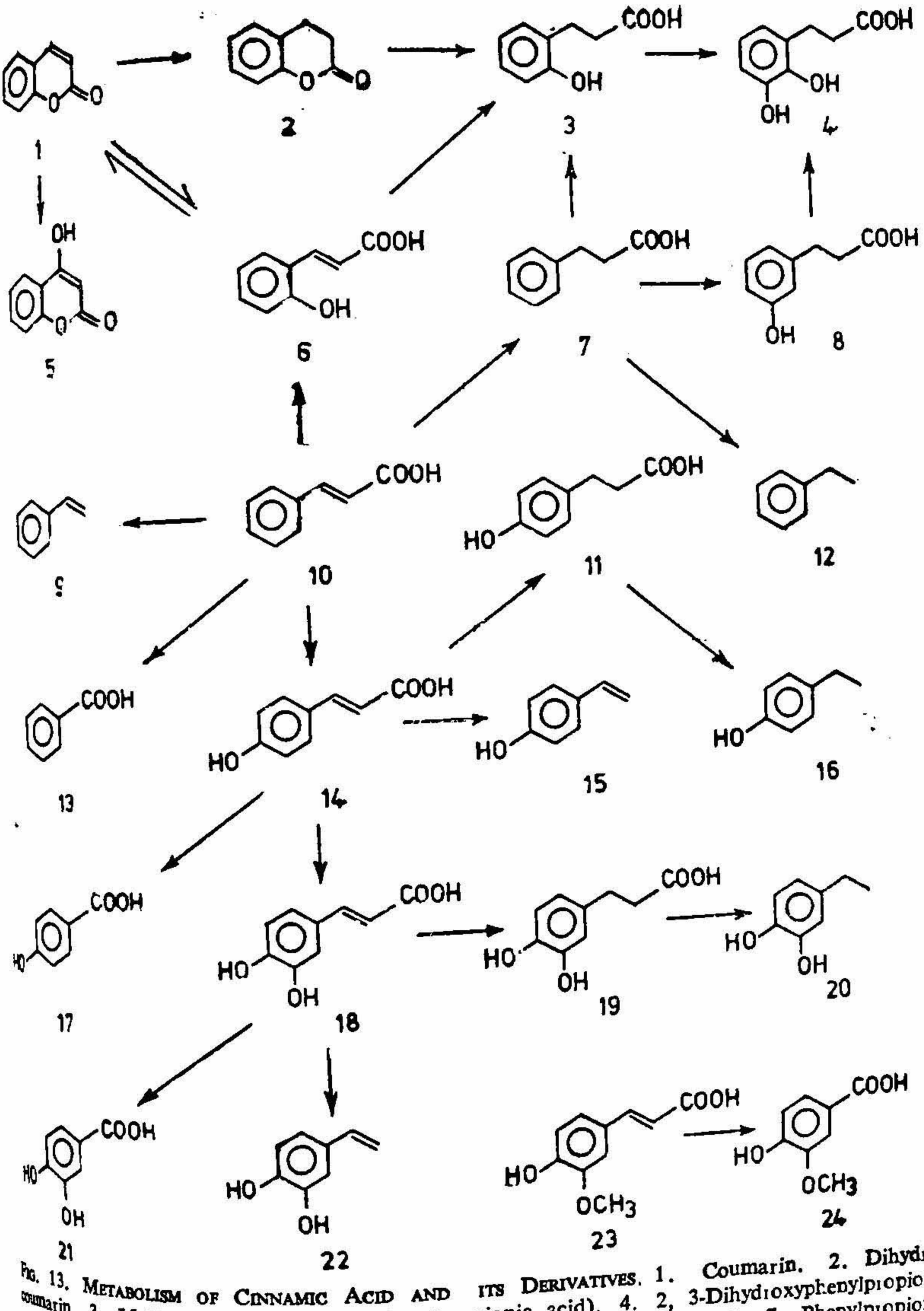


Fig. 13. Metabolism of Cinnamic Acid and its Derivatives. 1. Coumarin. 2. Dihydrocoumarin. 3. Melilotic acid (2-hydroxyphenylpropionic acid). 4. 2, 3-Dihydroxyphenylpropionic acid. 5. 4-Hydroxycoumarin. 6. *o*-Coumaric acid (2-hydroxycinnamic acid). 7. Phenylpropionic acid. 8. 3-Hydroxyphenylpropionic acid. 9. Styrene. 10. Cinnamic acid. 11. 4-Hydroxyphenylpropionic acid. 12. Ethylbenzene. 13. Benzoic acid. 14. *p*-Coumaric acid (4-hydroxycinnamic acid). 15. 4-Hydroxystyrene. 16. 4-Ethylphenol. 17. 4-Hydroxybenzoic acid. 18. Caffeic acid. 19. Dihydrocaffeic acid (3, 4-dihydroxyphenylpropionic acid). 20. 4-Ethylcatechol. 21. Protocatechuic acid. 22. 3, 4-Dihydroxystyrene. 23. Ferulic acid. 24. Vanillic acid.

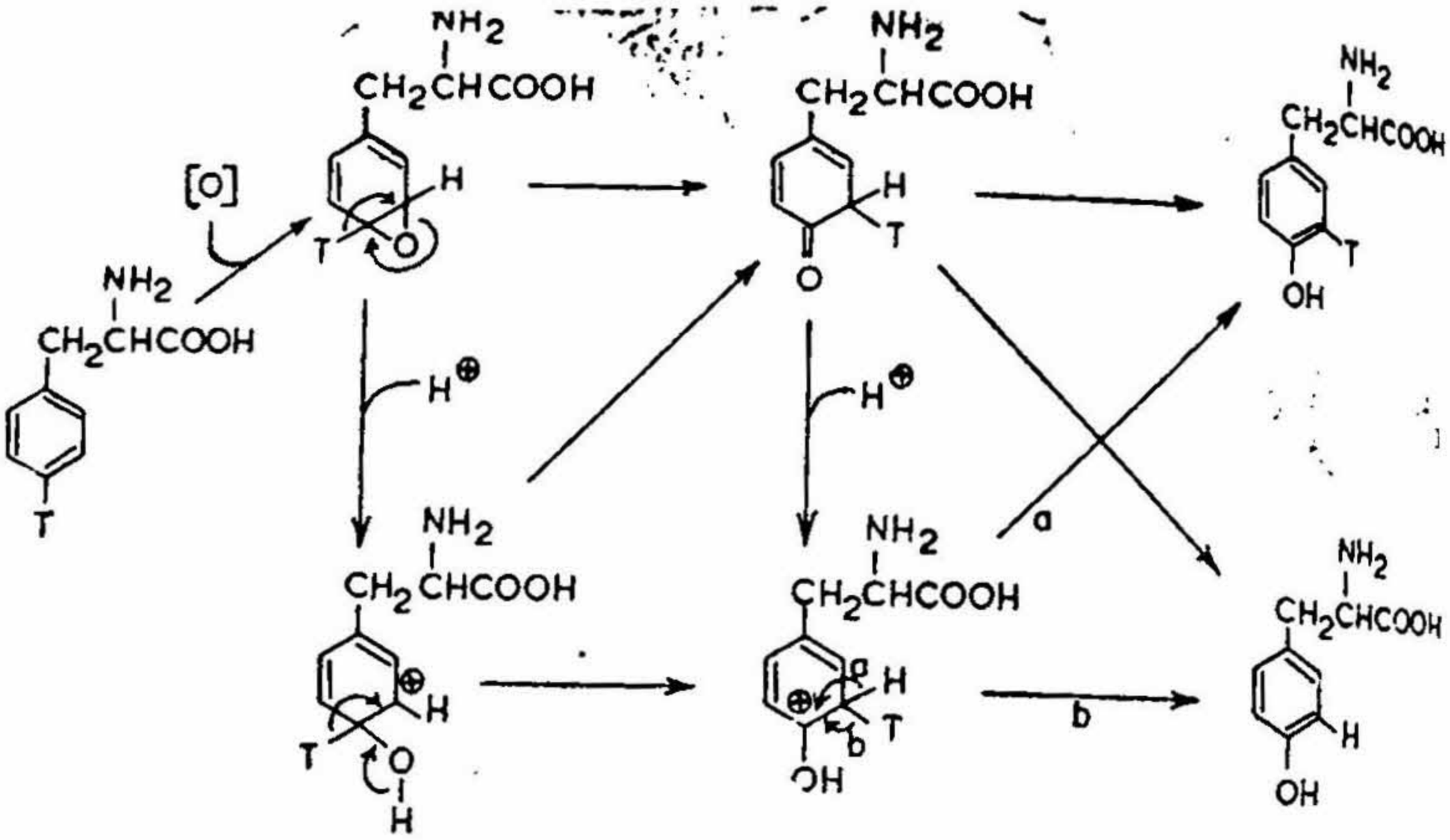


FIG. 14. Postulated mechanism for hydroxylation induced migration of a *para* substituent during phenylalanine hydroxylation.

(*b*) *Decarboxylation*

Decarboxylation of aromatic amino acids to form the corresponding phenethylamine derivatives is common in mammals, plants and bacteria[307]. By this process, the synthesis of a number of alkaloids in plants and neurohormones in mammals is initiated. Pyridoxal phosphate is essential for this reaction[307].

(*c*) *Cinnamic acid formation*

L-Phenylalanine ammonia-lyase, first characterized by Koukol and Conn[308], catalyzes the non-oxidative deamination of L-phenylalanine to form *trans*-cinnamic acid. It is widespread in plants, certain basidiomycetes and fungi imperfecta but is rare or absent in bacteria, algae and mammals[309]. While Havir *et al.*[310] have provided evidence for the deamination of both phenylalanine and tyrosine at the same active site of maize enzyme, there is evidence, at least in some systems, for the presence of two enzymes, one specific to phenylalanine and the other, to tyrosine and phenylalanine[311]. Phenylalanine ammonia lyase from different sources possesses very high molecular weight and in several instances shows non-Michaelian kinetics[309, 312, 313]. It follows an ordered Uni Bi mechanism with the release of cinnamate as the first product and the enzyme forms a dead end complex with cinnamate[309]. Studies on the stereochemistry of the reaction revealed the removal of Pro-3S hydrogen and the 2-amino group by a *trans*-elimination mechanism[314].

(d) Oxidative deamination

Both D- and L-amino acid oxidases which catalyze the conversion of phenylalanine and tyrosine to the respective phenylpyruvic acids are known to occur in a number of systems including mammalian liver and kidney, microorganisms, plants and insects. These flavoprotein oxidases utilize molecular oxygen and substrate, and produce pyruvic acid, ammonia and hydrogen peroxide[315].

(e) Transamination

Transaminases are of widespread occurrence in nature. Almost all transaminases follow a Ping Pong Bi Bi mechanism. They are all PALP-dependent enzymes.[316] An inducible phenylalanine amino-transferase with broad substrate specificity has been partially purified from *Achromobacter eurydice*[317].

Table II

List of reactions catalyzed by β-tyrosinase[321, 324]

	α-, β-elimination
A.	L(D)-Tyrosine + H_2O → Pyruvic acid + Phenol + NH_3
B.	L(D)-Serine → Pyruvic acid + NH_3
C.	S-Methyl-L-cysteine + H_2O → Pyruvic acid + CH_3SH + NH_3
D.	L-Cysteine + H_2O → Pyruvic acid + H_2S + NH_3
	β-replacement reaction
E.	L(D)-Tyrosine + Catechol → L-Dopa + Phenol
F.	L(D)-Serine + Phenol → L-Tyrosine + H_2O
G.	L(D)-Serine + Catechol → L-Dopa + H_2O
H.	S-Methyl-L--Cysteine + Resorcinol → 2, 4-Dihydroxy-L-Phenylalanine + CH_3SH
I.	S-Methyl-L-Cysteine + Pyrogallol → 2, 3, 4-Trihydroxy-L-Phenylalanine + CH_3SH
	Racemization
J.	L(D)-Alanine → DL-Alanine
	Reverse α, β-elimination
K.	Pyruvic acid + Phenol + NH_3 → L-Tyrosine + H_2O
L.	Pyruvic acid + Catechol + NH_3 → L-Dopa + H_2O

(f) N-Hydroxylation

In plants, occurrence of N-hydroxylation has been shown. Available evidence suggests that N-hydroxylation is the first step during the biogenesis of cyanohydrins, glucosinolates, and tyrosol derivatives[318, 319].

(g) Cleavage of the phenylpropane side chain

β-Tyrosinase which catalyzes the conversion of L-tyrosine into phenol, pyruvate and ammonia was first discovered by Kakihara and Ichihara[320] and later extensively studied by Yamada's group[321]. It is also called tyrosine phenol-lyase and has been obtained in crystalline form from *Escherichia intermedia*[322] and *Erwinia herbicola*[323]. It contains two moles of PALP per mole of enzyme[322] and catalyzes a range of α, β-elimination (A–D), β-replacement (E–I), racemization (J) and reverse α, β-elimination (K, L) reactions[321, 324] (Table II).

Out of 646 strains of bacteria, 140 strains of yeast, 138 strains of fungi and 117 strains of actinomycetes tested, only bacterial strains, mostly belonging to the Enterobacteriaceae, especially to the genera *Escherichia*, *Proteus* and *Erwinia*, were found to possess β-tyrosinase activity[325]. The initial reactions involved in the transformation of phenyl alanine and tyrosine are depicted in Fig. 15.

15. Aromatic ring cleavage

From the foregoing discussion, it is apparent that the degradation of aromatic compounds leads to the formation of dihydroxyphenols as the terminal benzenoid compounds. Dioxygenases initiate the degradation of such compounds by cleaving the aromatic double bond which may be located either between the two hydroxylated carbon atoms, or adjacent to a hydroxylated carbon atom, or in an indole ring (Fig. 16*a*). With substituted catechols, multiple modes of cleavages depicted in Fig. 16*b* are possible. A variety of substrates are available for ring cleavage and some of the reactions will be summarized below.

(a) Gentisaldehyde

During the conversion of gentisaldehyde to patulin (Fig. 5) by *Penicillium patulum*, a dioxygenase has been encountered which converts gentisaldehyde into 3-formyl-4-hydroxy muconic semialdehyde.[82]

(b) Gentisic acid

Oxidation of gentisic acid by cell-free extracts of a *Pseudomonas* sp. was first shown by Lack[326] and later by Sugiyama *et al*[327]. Gentisate-1, 2-dioxygenase from *Pseudomonas* requires Fe^{2+} for activity and is highly specific towards its substrate. Recently, Crawford *et al*. have isolated the enzyme from *Moraxella osloensis*[328]. It is made up of 4 subunits of molecular weight 40,000 each. Ferrous iron is absolutely essential for the enzyme

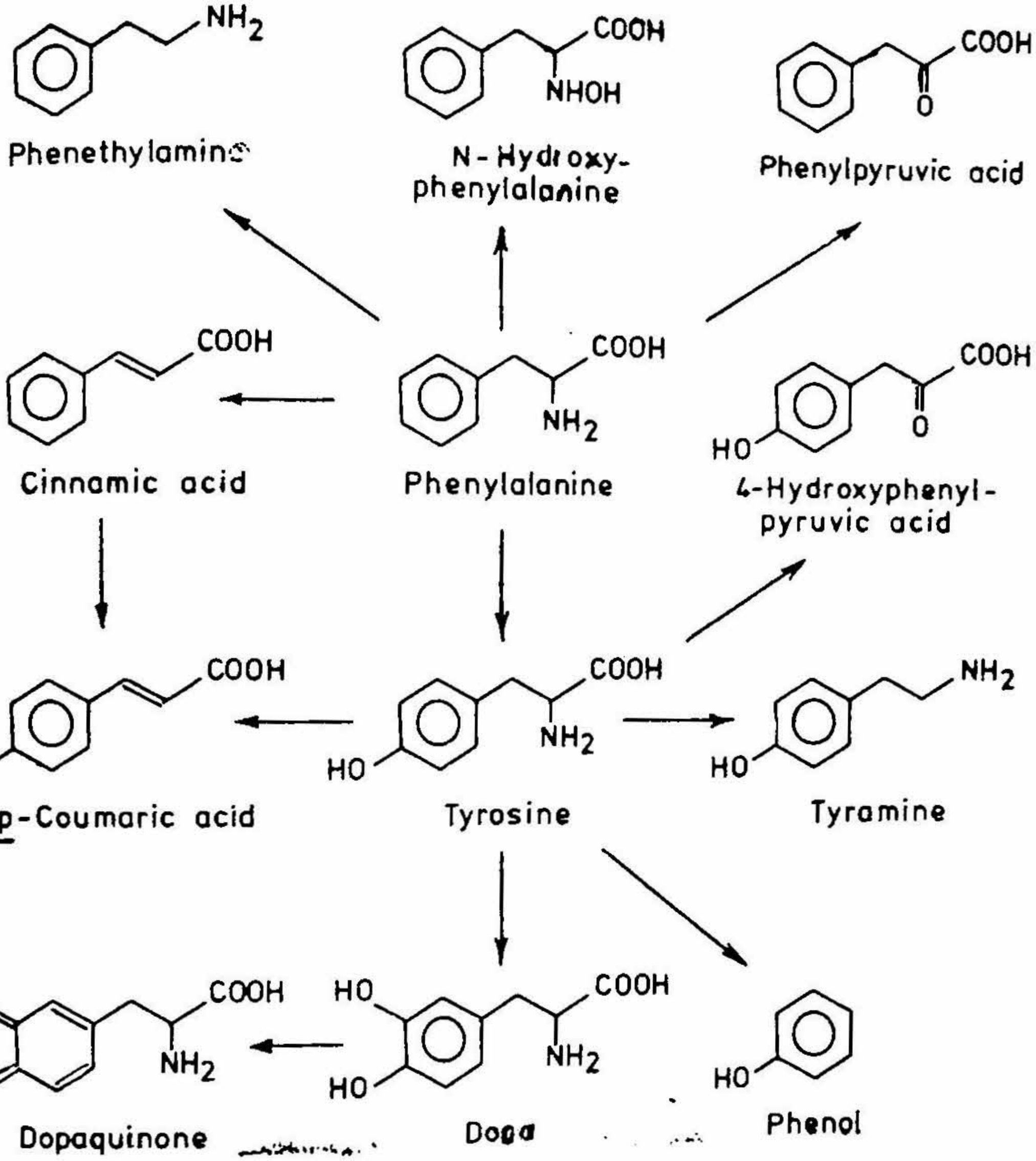


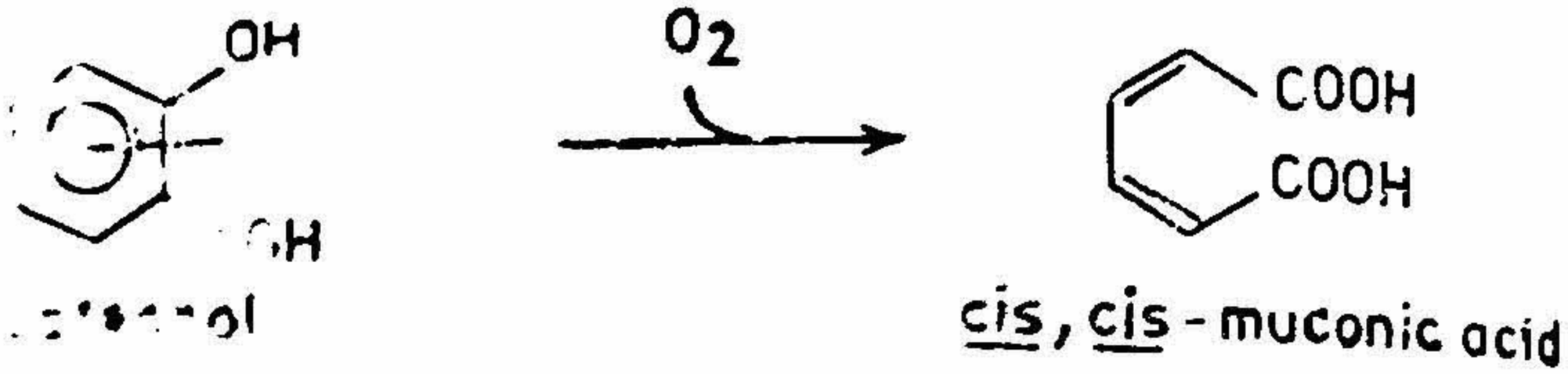
FIG. 15. Initial steps in the degradation of phenylalanine and tyrosine.

activity. The homogeneous enzyme attacks a number of halogen and alkyl substituted gentisic acids. Such a non-specific oxidation also occurs in the case of a *Pseudomonas* sp.[72]

Maleylpyruvate, thus formed, undergoes isomerization to fumarylpyruvate which is then hydrolyzed to fumarate and pyruvate[329]. However, in some organisms it undergoes direct hydrolysis to give maleate and pyruvate[72, 330].

(*c*) *Homogentisic acid*

The occurrence of homogentisate-1, 2-dioxygenase has been demonstrated in *Pseudomonas*[201, 265, 331], *Vibrio*[332], *Bacillus*[333], *Moraxella*[333], plants[334] and mammalian systems[335–338].



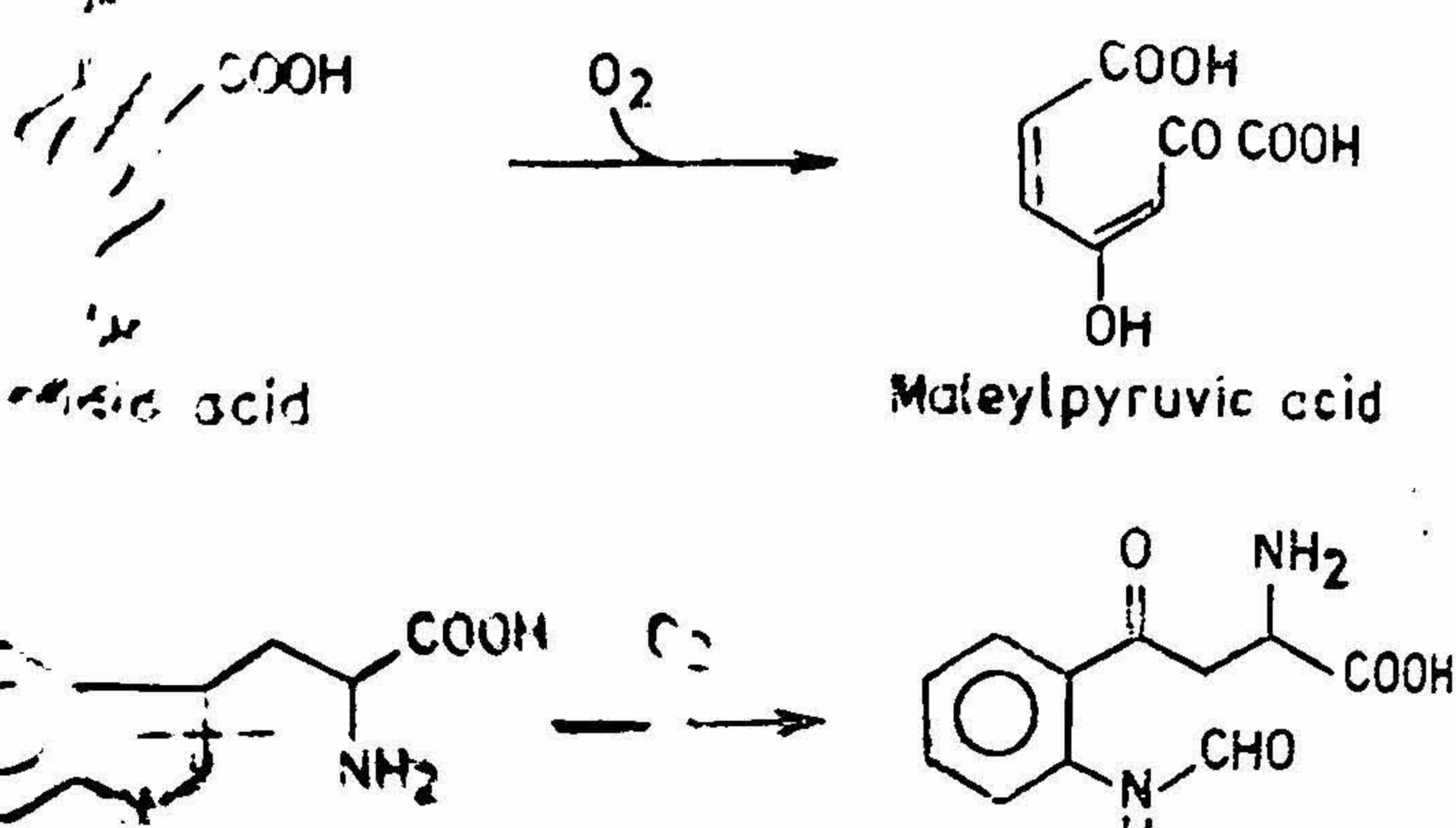
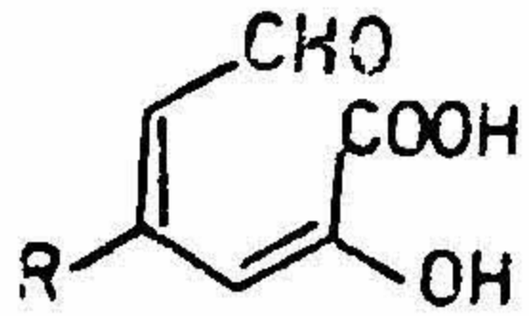
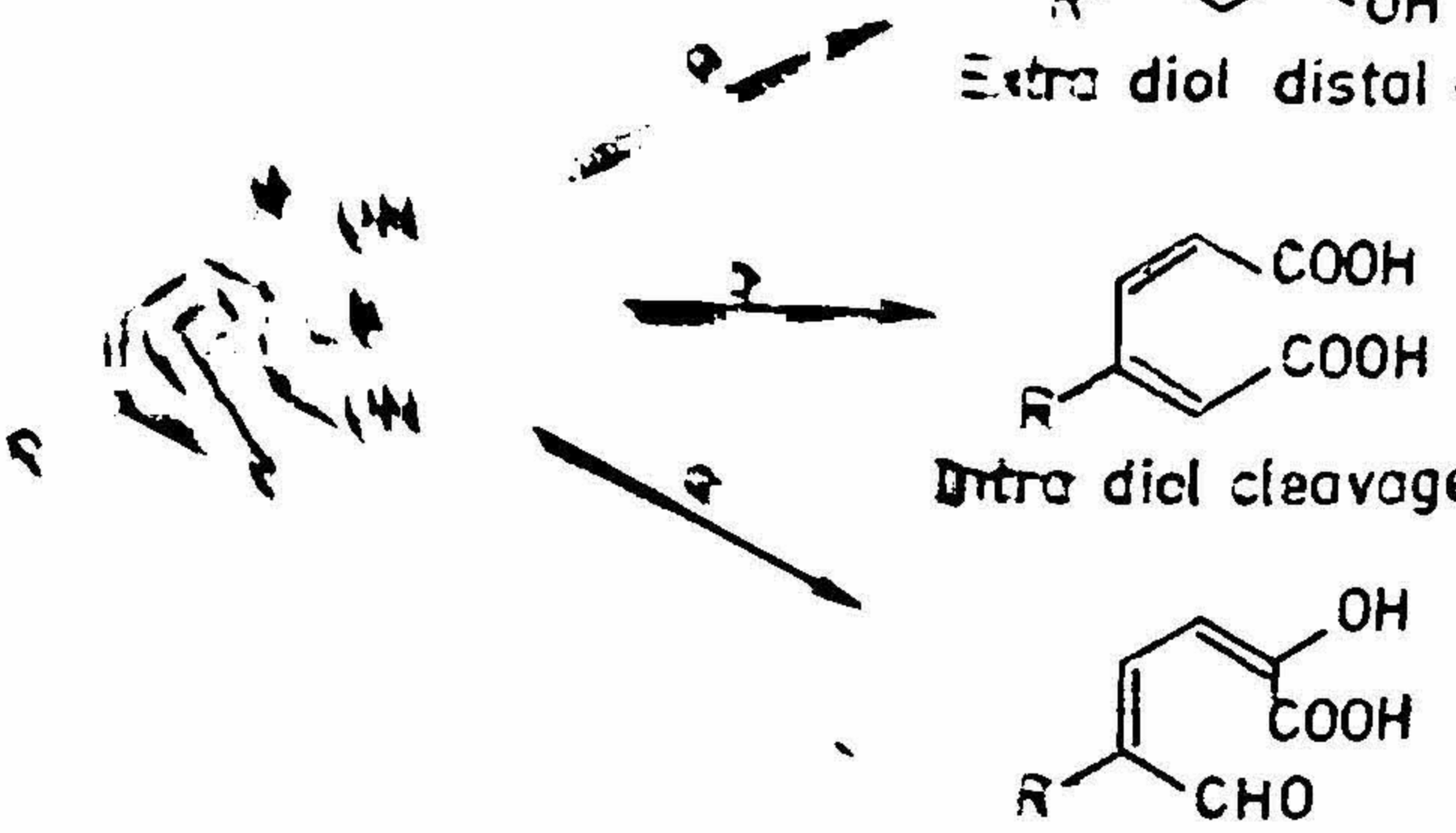


Fig. 16 a



Extra diol distal cleavage



Intra diol cleavage

Extra diol proximal cleavage

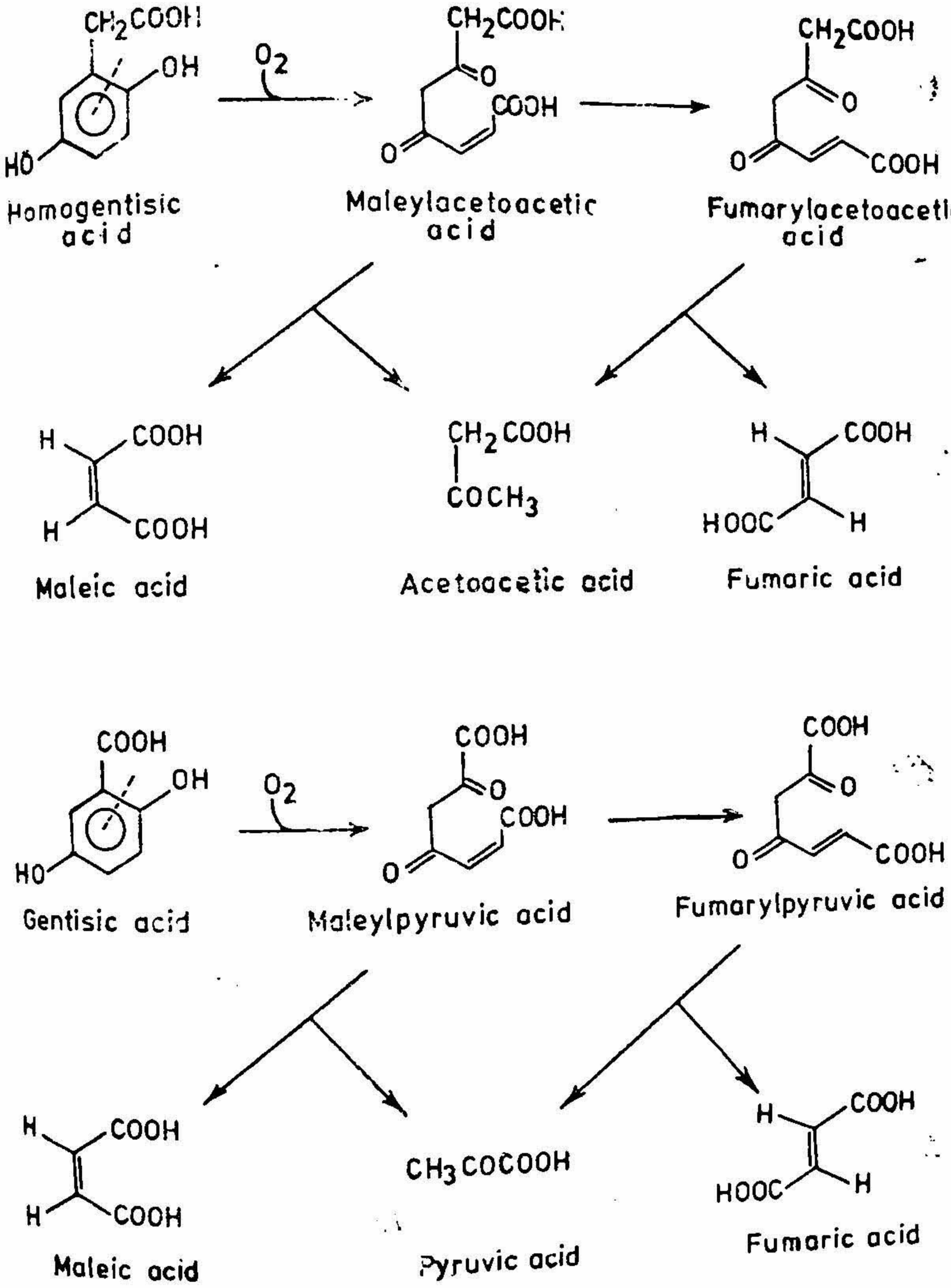


FIG. 17. Metabolism of homogentisic and gentisic acids.

It requires Fe^{2+} and intact sulfhydryl groups for activity. The dioxygenase nature of the enzyme has been established by $^{18}O_2$ and $H_2{}^{18}O$ studies[339]. The enzyme from *Pseudomonas fluorescens* has been purified to homogeneity and crystallized[331]. While the bacterial enzyme has a molecular weight of 380,000[331], that of mammalian, has 254,000[339].

Most organisms convert maleylacetoacetate formed by the action of homogentisate dioxygenase into fumarylacetoacetate and hydrolyze it to fumarate and acetoacetate. However, a novel variant of this pathway occurs in *Bacillus* sp. where maleylacetoacetate is directly hydrolyzed to maleate and acetoacetate[333]. The metabolic transformations of gentisic and homogentisic acids are given in Fig. 17.

(*d*) 3-*Hydroxyanthranilic acid*

Oxidation of 3-hydroxyanthranilic acid to 2-amino-3-carboxymuconic semialdehyde is an important reaction leading to the biogenesis of pyridine nucleotides. Most of the earlier studies on 3-hydroxyanthranilate-3, 4-dioxygenase were carried out with partially purified preparations. Recently, it has been purified from beef kidney[340]. It is a single subunit protein with a molecular weight of 34,000 and readily aggregates to form apparently inactive, high molecular weight oligomers. Fe^{2+}, which is required for the enzyme activity, gets equilibrated with the enzyme freely, albeit slowly, during the course of the reaction, even in the presence of saturating concentrations of the substrate.

(*e*) *Dihydroxyphenylalanine* (*Dopa*)

Dopa ring cleavage seems to occur during the biosynthesis of antitumor antibiotic 11-demethyltomamycin in *Streptomyces achromogenes*[341]. Occurrence of dopa dioxygenases seems to be frequent in the case of higher plants. The betalamic moiety of the brightly coloured betalain pigments, which are widespread in *Centrospermae*, seems to arise from dopa by an extradiol ring cleavage followed by ring closure reactions as shown in Fig. 18[342, 343]. Stizolobinic acid and stizolobic acid, new type of heterocyclic non-protein amino acids found in *Stizolobium hassjoo* and related plants, arise from dopa by extradiol cleavage reactions (Fig. 19)[344]. Recently, the enzyme system converting dopa to stizolobinic and stizolobic acids has been obtained from the cell-free extracts of the etiolated seedlings of *Stizolobium hassjoo*[345] and shown to utilize molecular oxygen and reduced pyridine nucleotides for the reaction.

(*f*) *Caffeic acid*

Seidman *et al.*[275] have isolated a dioxygenase from *Pseudomonas fluorescens* grown on *p*-coumaric acid, which cleaves caffeic acid by an intradiol fashion (Fig. 20).

(*g*) 2, 3-*Dihydroxyphenylpropionic acid*

Dagley *et al.*[248, 249] have partially purified a ferrous iron requiring dioxygenase from *Achromobacter* grown on phenylpropionate, which cleaves 2, 3-dihydroxyphenylpropio-

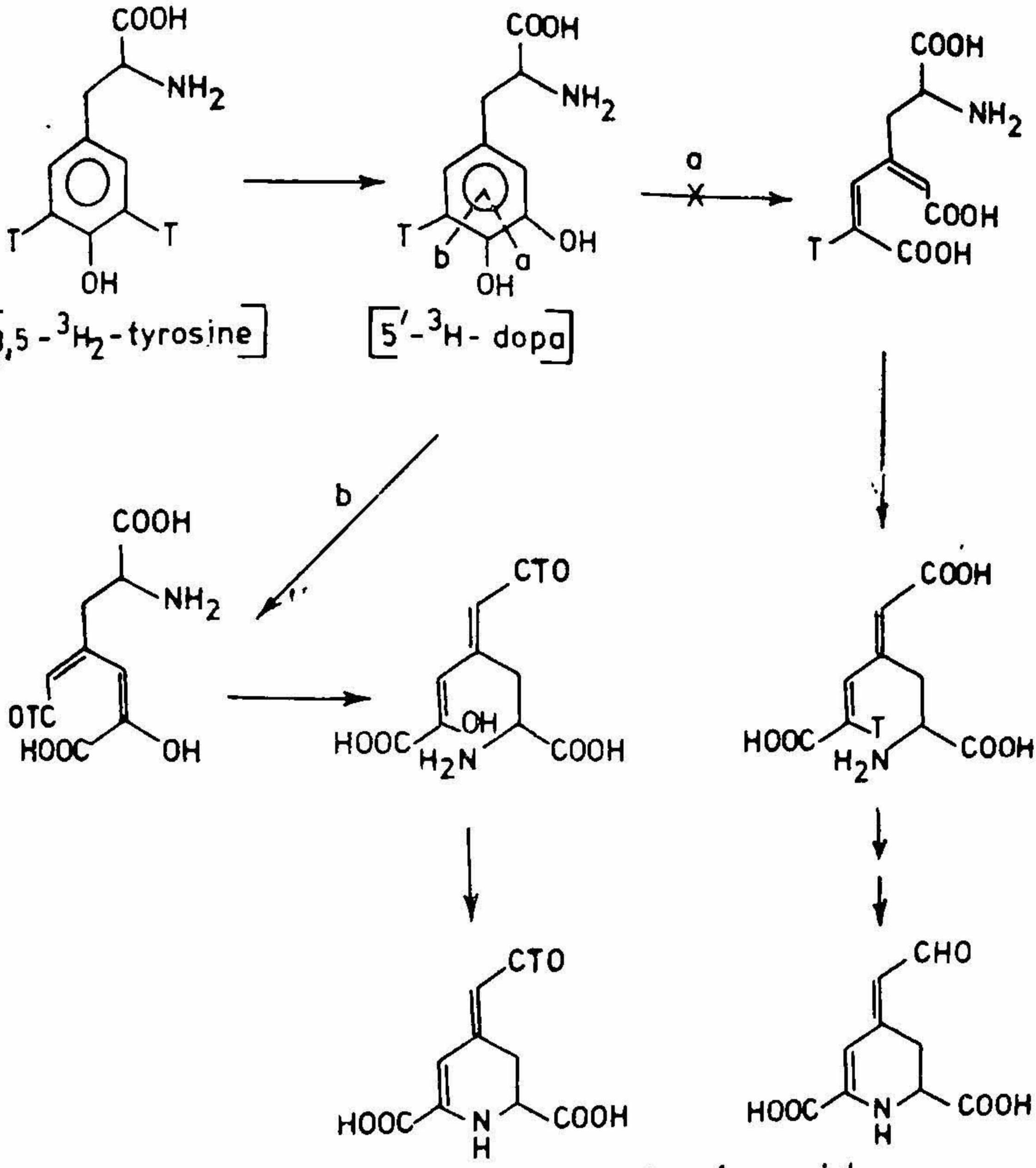


Fig. 18. Mechanism of betalamic acid formation from 3′, 5′-ditritiated tyrosine.

nate at extradiol proximal position to produce 2, 6-dioxo nona-4-en-1, 9-dioic acid (Fig. 20).

(*h*) *Homoprotocatechuic acid*

Homoprotocatechuate-2, 3-dioxygenase was first demonstrated in the cell-free extracts of *Pseudomonas ovalis*[196]. Subsequently, this enzyme was purified to homogeneity and crystallized[346, 347]. The crystalline enzyme has a molecular weight of 140,000 and contains 4–5 gm atoms of iron per mole of enzyme. Besides homoprotocatechuate, a

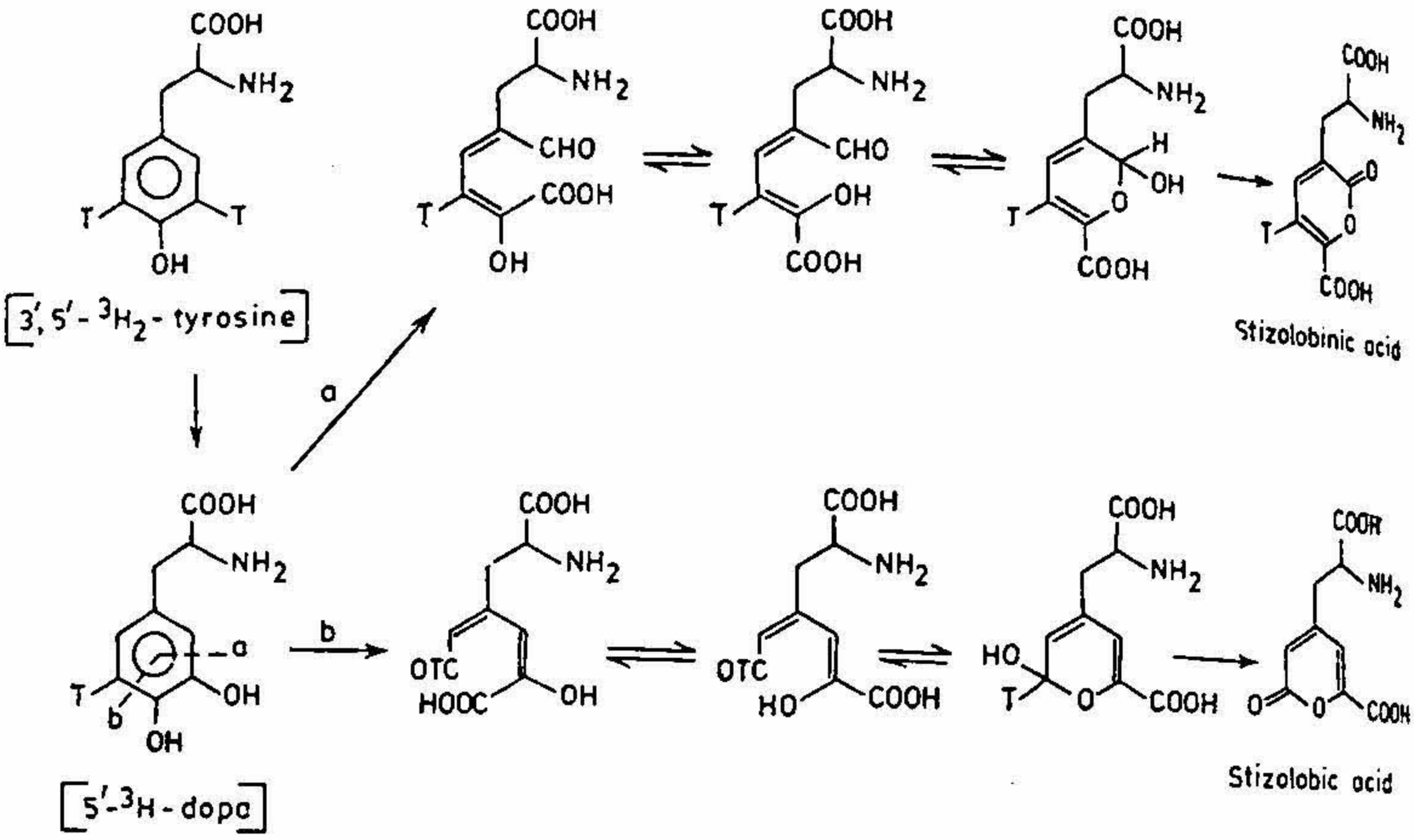


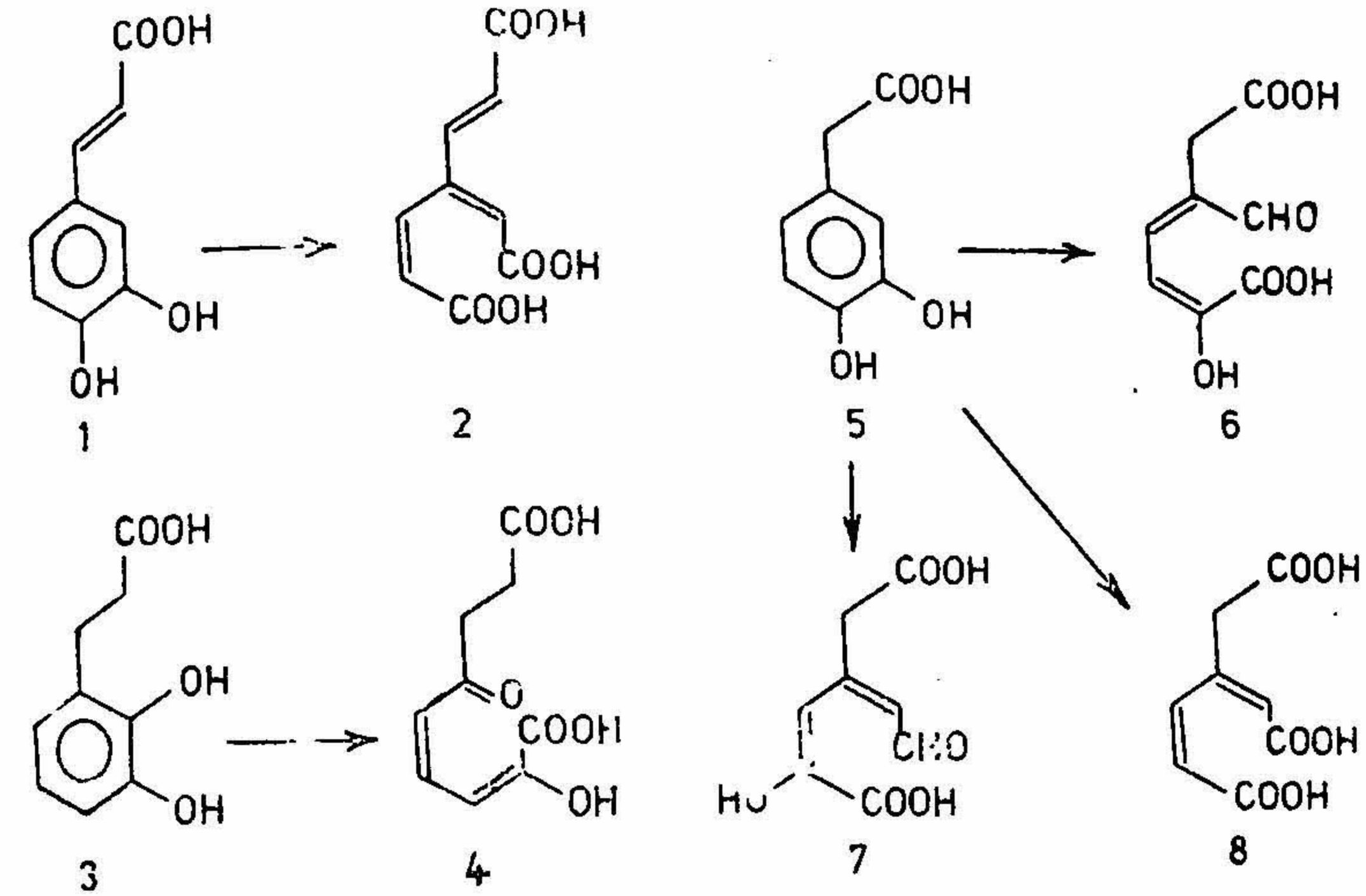
FIG. 19. Biosynthesis of stizolobinic acid and stizolobic acid from 3′, 5′-ditritiated L-Tyrosine.

number of 4-substituted catechols are attacked, but to a lesser extent, by the enzyme[348]. The holoenzyme consists of 4 identical subunits of molecular weight 35,000[349]. Though the iron atoms in the native enzyme are not detectable by ESR, a signal at $g = 4{\cdot}3$, characteristic of ferric ion, develops when excess substrate is added under aerobic conditions. When the oxygen is used up, the signal disappears ; when catechol, a slowly reacting substrate, is added to the enzyme under aerobic conditions, a sharp signal at $g = 4{\cdot}3$ and a deep violet colour appear. These changes are attributed to the formation of a ternary complex of the enzyme iron, substrate and oxygen[350]. This enzyme has also been purified from *Bacillus stearothermophilus*[351] and *Arthrobacter*[352].

A different mode of *meta* cleavage is observed in *Pseudomonas* sp[353]. The partially purified enzyme from this organism requires Fe^{2+} for activity and cleaves homoprotocatechuate between C–4 and C–5. Subba Rao *et al.*[354] have partially purified a dioxygenase from the fungus *Tilletiopsis washingtonensis* which cleaves homoprotocatechuate by an intradiol fashion (Fig. 20). This enzyme exhibits a broad substrate specificity and attacks protocatechuate, homoprotocatechuate, 3, 4-dihydroxymandelate, caffeate, 3, 4-dihydroxyphenylpropionate and dopa in the decreasing order.

(*i*) *Methyl catechols*

Pseudomonas desmolyticum oxidizes 4-methyl catechol by an extradiol proximal as well as an intradiol cleavage mechanism[355]. *Pseudomonas* B13 grown on 3-chlorobenzoate while cometabolizing 3-methylbenzoate develops a less specific dioxygenase which cleaves both 3- and 4-methyl catechols by an intradiol cleavage mechanism[356]. In fact, the



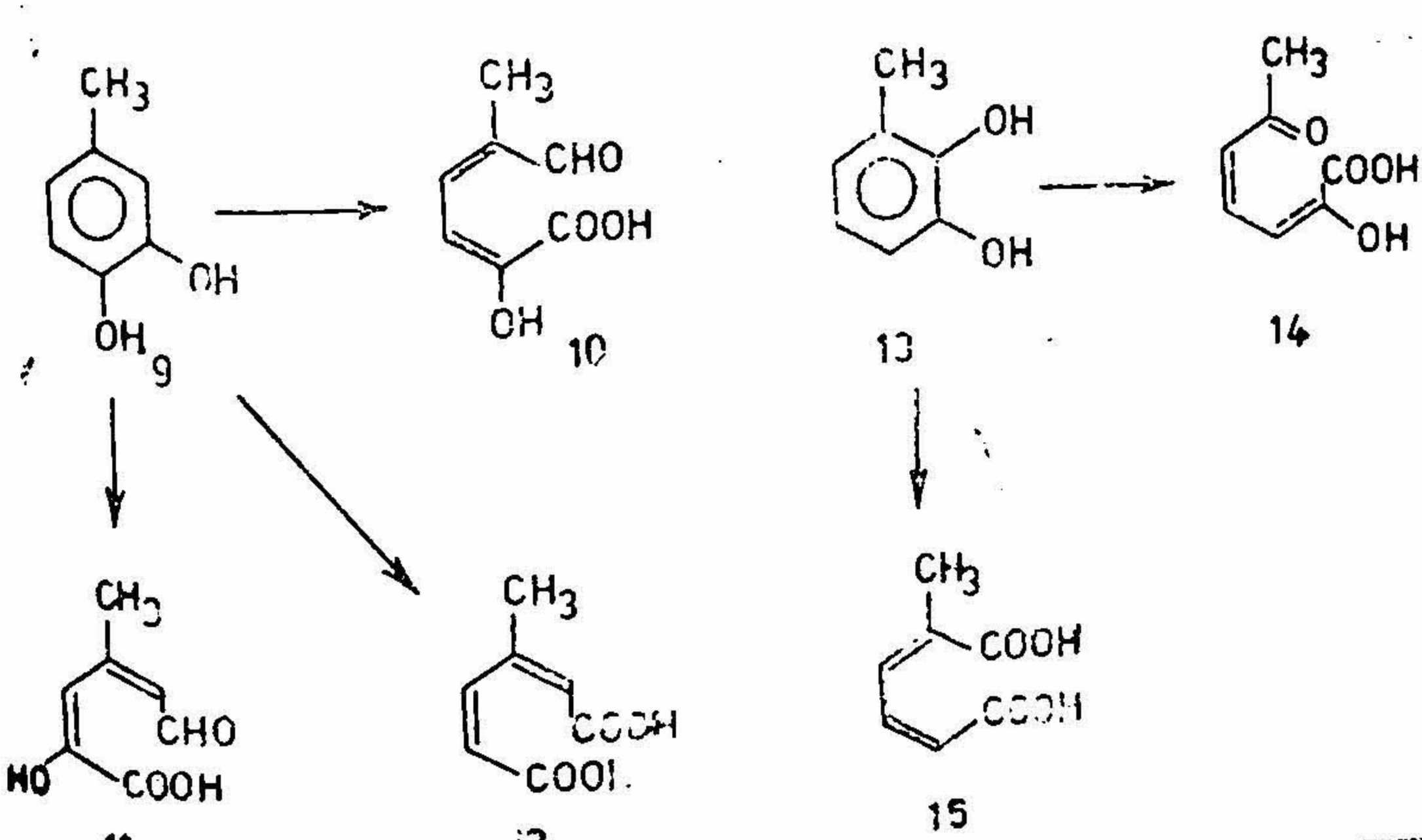


FIG. 20. OXIDATION OF CAFFEIC, 2, 3-DIHYDROXYPHENYLPROPIONIC AND HOMOPROTOCATECHUIC ACIDS AND METHYLCATECHOLS. 1. Caffeic acid. 2. 2-(β-carboxyethylenyl)-*cis*, *cis*-muconic acid. 3. 2, 3-Dihydroxyphenylpropionic acid. 4. 2,6-Dioxonona 4-en-1, 9-dioic acid (enol form). 5. Homoprotocatechuic acid. 6. 2-Hydroxy-5-carboxymethyl muconic semialdehyde. 7. 2-Hydroxy-4-carboxymethyl muconic semialdehyde. 8. 3-Carboxymethyl-*cis*,*cis*-muconic acid. 9. 4-Methylcatechol. 10. 2-Hydroxy-5-methyl muconic semialdehyde. 11. 2-Hydroxy-4-methyl muconic semialdehyde. 12. 3-Methyl-*cis*, *cis*-muconic acid. 13. 3-Methylcatechol. 14. 2-Hydroxy-6-oxo-2, 4-heptadienoic acid. 15. 2-Methyl-*cis*, *cis*-muconic acid.

purified catechol-1, 2-dioxygenase from *Brevibacterium fuscum* catalyzes the conversion of 3- and 4-methyl catechols to the corresponding muconic acid derivatives[357].

However, many organisms seem to oxidize methyl catechols by an extradiol proximal cleavage. Formation of an yellow coloured compound during the oxidation of 3-methyl catechol was attributed to this type of reaction[61, 69, 358]. The product was identified to be 2-hydroxy-6-oxo-2, 4-hepta dienoic acid by Catelani *et al*[359]. A *Pseudomonas* strain[62, 63], *Pseudomonas arvilla*[360], *Pseudomonas putida*[64] and *Bacillus stearothermophilus*[44] oxidize 3-methyl as well as 4-methyl catechols in this way. The crystalline catechol-2, 3-dioxygenase from *Pseudomonas arvilla* catalyzes the extradiol proximal cleavage of a number of 3- and 4-methyl catechols[361]. It is interesting to note the oxidation of 3-methyl catechol occurring at the same site, on catechol-1, 2-dioxygenase through an intradiol as well as an extradiol proximal cleavage mechanisms[362].

On the contrary, Hashimoto[65, 70] has observed the extradiol distal cleavage of 4-methyl catechol by yeast strains (Fig. 20).

(*j*) *Pyrocatechuic acid*

Ribbons first reported the oxidation of pyrocatechuic acid to pyruvate, acetaldehyde, formate and carbon dioxide by a battery of inducible enzyme systems in *Pseudomonas fluorescens*[363]. Subsequently, the dioxygenase which requires Fe^{2+} was isolated and the products of the reaction were identified to be 2-hydroxymuconic semialdehyde and carbon dioxide[364]. In order to elucidate the site of ring cleavage, Ribbons and Senior used 2, 3-dihydroxy-*p*-toluic acid, a non-inducing substrate analog for the dioxygenase and showed the ring fission at 3, 4-position on the basis of 2, 6-dioxo heptanoic acid formation[365].

Simultaneously, Madhyastha *et al.*[366] showed the oxidation of 2, 3-dihydroxy-*p*-cumic acid and pyrocatechuic acid by a soil psuedomonad grown on *p*-cymene by a meta-pyrocatechase type of reaction. These authors postulated a ring fission between C-1 and C-2 of pyrocatechuate based on the formation of β-isopropyl pyruvate and acetaldehyde from 2, 3-dihydroxy-*p*-cumate[366]. However, *Pseudomonas putida* PL strain, grown on *p*-cymene, oxidized 2, 3-dihydroxy-*p*-cumic acid to 2, 6-dioxo-7-methyl oct-4-enoic acid[38]. Formation of this product could be accounted for only by ring fission at C-3 and C-4 of pyrocatechuic acid moiety.

Recently, a dioxygenase from *Tecoma stans* has been isolated in this laboratory[367, 368] which cleaves pyrocatechuic acid to give 2-carboxy-*cis*, *cis*-muconic acid. Interestingly, this enzyme seems to require copper and not iron for activity[368]. Oxidative degradations of pyrocatechuate and its derivatives are illustrated in Fig. 21.

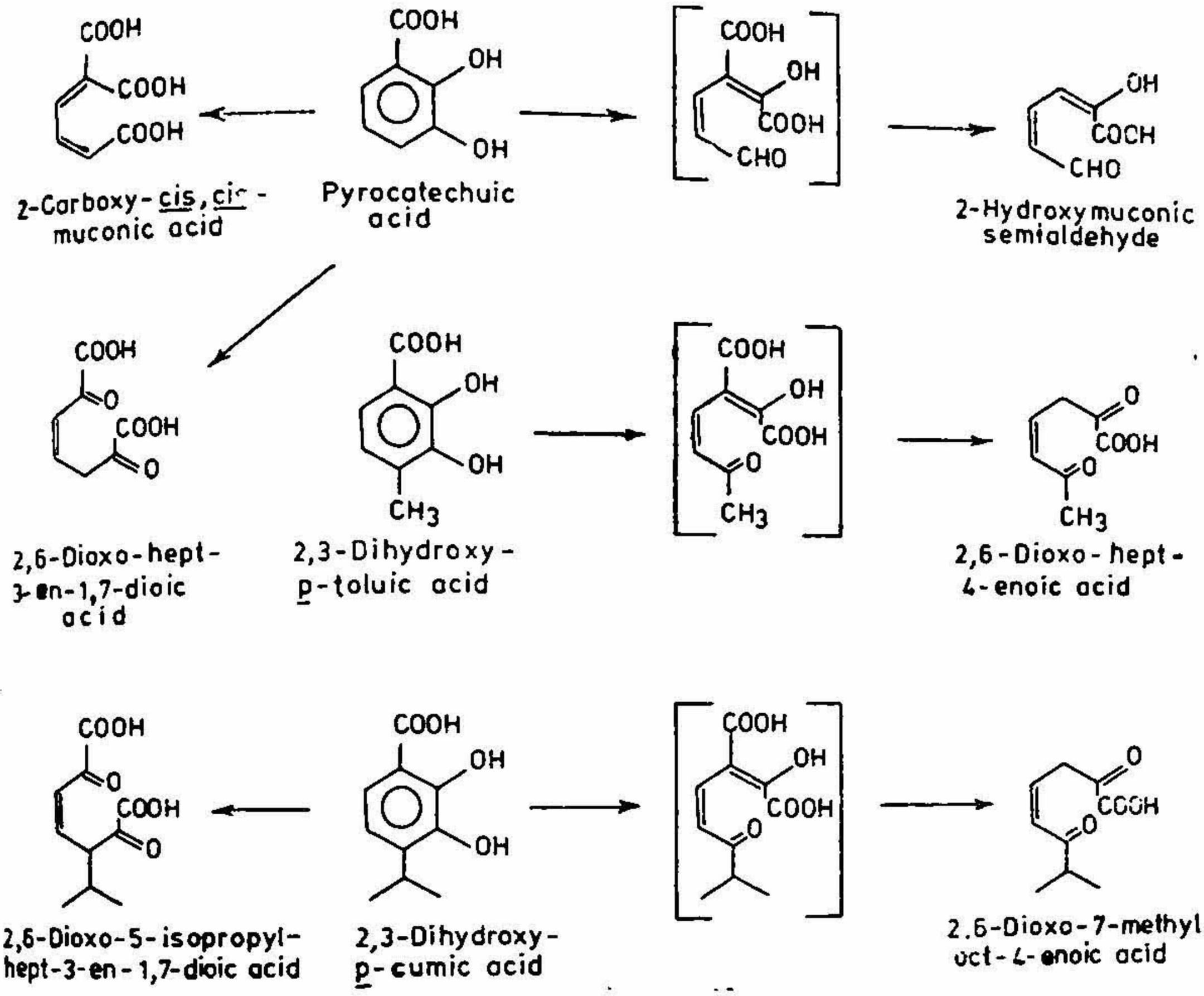


FIG. 21. Oxidation of pyrocatechuic acid and its derivatives.

(k) *Protocatechuic acid*

Protocatechuate-3, 4-dioxygenase which catalyzes the conversion of protocatechuic acid into 3-carboxy-*cis*, *cis*-muconic acid, has been studied from a number of microorganism, including soil bacteria[369], *Pseudomonas*[370–372], *Acinetobacter calcoaceticus*[373], *Nocardia*[374] *Neurospora*[172, 375] and *Tilletiopsis washingtonensis*[354]. *Pseudomonas*[372] and *Acinetobacter*[373] enzymes have been purified to homogeneity ; the dioxygenase has been crystallized from the former source. While the enzyme from these sources has a molecular weight of 700,000, *P. putida* enzyme has a molecular weight of 420,000 only[371]. The dioxygenase contains 8 gm atoms of iron per mole and appears to consist of eight subunits, each subunit having one substrate binding site[376]. These subunits in turn seem to consist of four smaller subunits of two non-identical types $(\alpha_2\beta_2)$[377]. The iron is tightly bound, but can be removed by extensive dialysis under anaerobic conditions in the presence of o-phenanthroline and dithionite. The holoenzyme can be reconstituted by incubating the apoenzyme with Fe^{2+} and dithionite[378]. Steady state kinetic analysis revealed the operation of an ordered Bi Uni mechanism with the formation of a tertiary complex by the addition of substrate first followed by oxygen[379]. The above mechanism and the ternary complex formation have also been established by stopped flow studies[380].

The enzyme is red in colour and shows broad absorption from 400 to 650 nm. Reduction bleaches the colour and on reoxidation, the colour reappears[376]. It has a sharp ESR signal at $g = 4{\cdot}31$, which diminishes on addition of substrate under anaerobic conditions. These results are attributed to the presence of ferric iron whose ligand field is affected by the addition of substrate[376].

The dioxygenase, in which cobalt substitutes for iron, has been obtained by growing the organism on a medium containing cobalt in the place of iron. The cobalt containing enzyme has catalytic properties similar to that of iron containing enzyme, but differs from the latter in its spectral and ESR properties[381].

Protocatechuate-4, 5-dioxygenase was the first *meta* cleaving dioxygenase to be characterized[66]. It was purified from *Pseudomonas testosteroni* and shown to attack gallate and 3-0-methylgallate as well[382]. Hayaishi's group[383] have purified this enzyme to near homogeneity. It has one gm atom of iron per mole of enzyme (molecular weight 150,000). Although the enzyme is highly unstable, it can be stabilized by 10% ethanol. Rapid inactivation during catalysis[382, 383] is prevented by L-cysteine[382]. The inactivation could be due to the removal of iron, as the external addition of ferrous iron, fully reactivates the enzyme[383].

Zabinski *et al.*[384] purified the enzyme from *P. testosteroni* and showed the presence of 4 gm atoms of iron per mole of enzyme (molecular weight 140,000). The iron in the native enzyme is not detected by ESR, but the addition of substrate or its analog under anaerobic conditions results in the appearance of a signal at $g = 4{\cdot}3$ which is typical of a high spin ferric iron. Mossbauer studies on $^{57}Fe^{2+}$-reconstituted enzyme indicate that either the iron atoms are in a low spin ferrous state or the enzyme has two active sites, each containing two anti-ferromagnetically coupled high spin ferric ions[384].

A new dioxygenase, which oxidizes protocatechuate by extradiol proximal cleavage has been obtained from *Bacillus circulans* recently, and is shown to have a narrow substrate specificity[385]. All the three modes of ring cleavage of protocatechuic acid are depicted in Fig. 22.

(l) Catechol

Catechol-1, 2-dioxygenase (Fig. 22), also called pyrocatechase, is the first enzyme shown by $^{18}O_2$ studies to be dioxygenase[386]. It has been purified from *P. arivilla*[387], *P. fluorescens*[388], *Acinetobacter calcoaceticus*[389] and *Brevibacterium fuscum*[357]. *Pseudomonas* and *Acinetobacter* enzymes have molecular weights of 90,000 and two gm atoms of iron per mole of enzyme, while *Brevibacterium* enzyme has a molecular weight of 64,000 and one gm atom of iron. Even the substrate specificities of *Pseudomonas* and *Brevibacterium* enzymes are different. The former has a narrower specificity than the latter[362]. *Pseudomonas* enzyme catalyzes not only the intradiol cleavage, but also an extradiol cleavage, when 3-methylcatechol is used as a substrate[362]. However, *Brevibacterium* enzyme cleaves this substrate only by the intradiol cleavage[362].

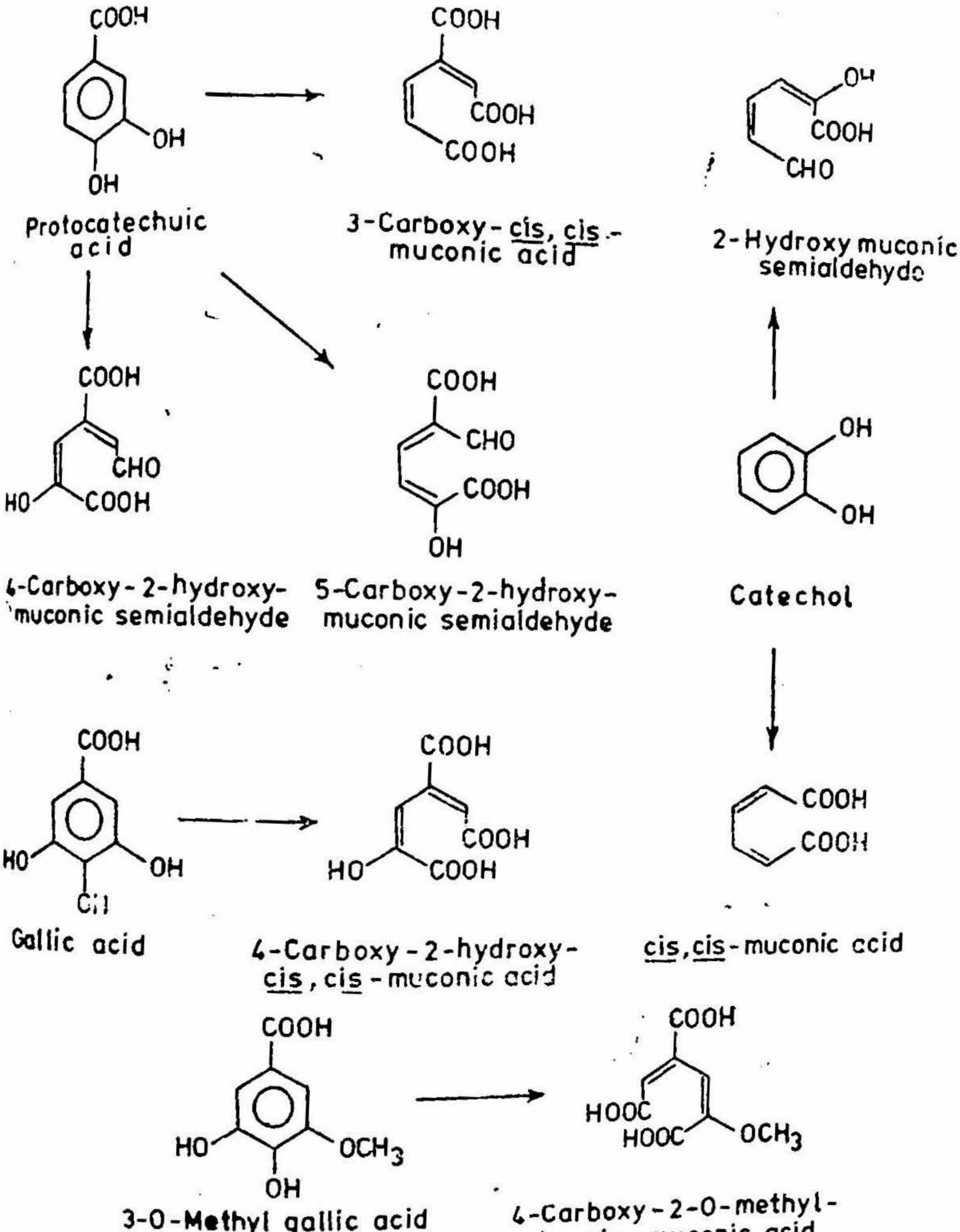


FIG. 22. Oxidation of protocatechuic, gallic and 3-O-methyl gallic acids and catechol.

Pyrocatechase is bright red coloured with a broad absorption band between 390 and 650 nm[387]. Both the apoenzyme and the dithionite reduced enzyme are colourless. On reconstitution of the apoenzyme with Fe^{2+} and reoxidation of the reduced enzyme, the red colour as well as the enzyme activity are restored. The enzyme shows a sharp ESR

signal at $g = 4{\cdot}28$, characteristic of Fe^{3+}. Addition of substrate under anaerobic conditions results in the disappearance of this signal which may be due to the formation of ferrous ion. The signal reappears when oxygen is let in[350, 390].

Catechol-2, 3-dioxygenase (Fig. 22), also known as metapyrocatechase, was the first dioxygenase to be obtained in pure crystalline form[391]. Low concentrations of organic solvents such as ethanol and acetone stabilize the enzyme. It has a molecular weight of 140,000 and seems to be made up of 3–4 subunits, each having one gm atom of iron[392, 262]. It exhibits rather a broad specificity and attacks a variety of catechols as

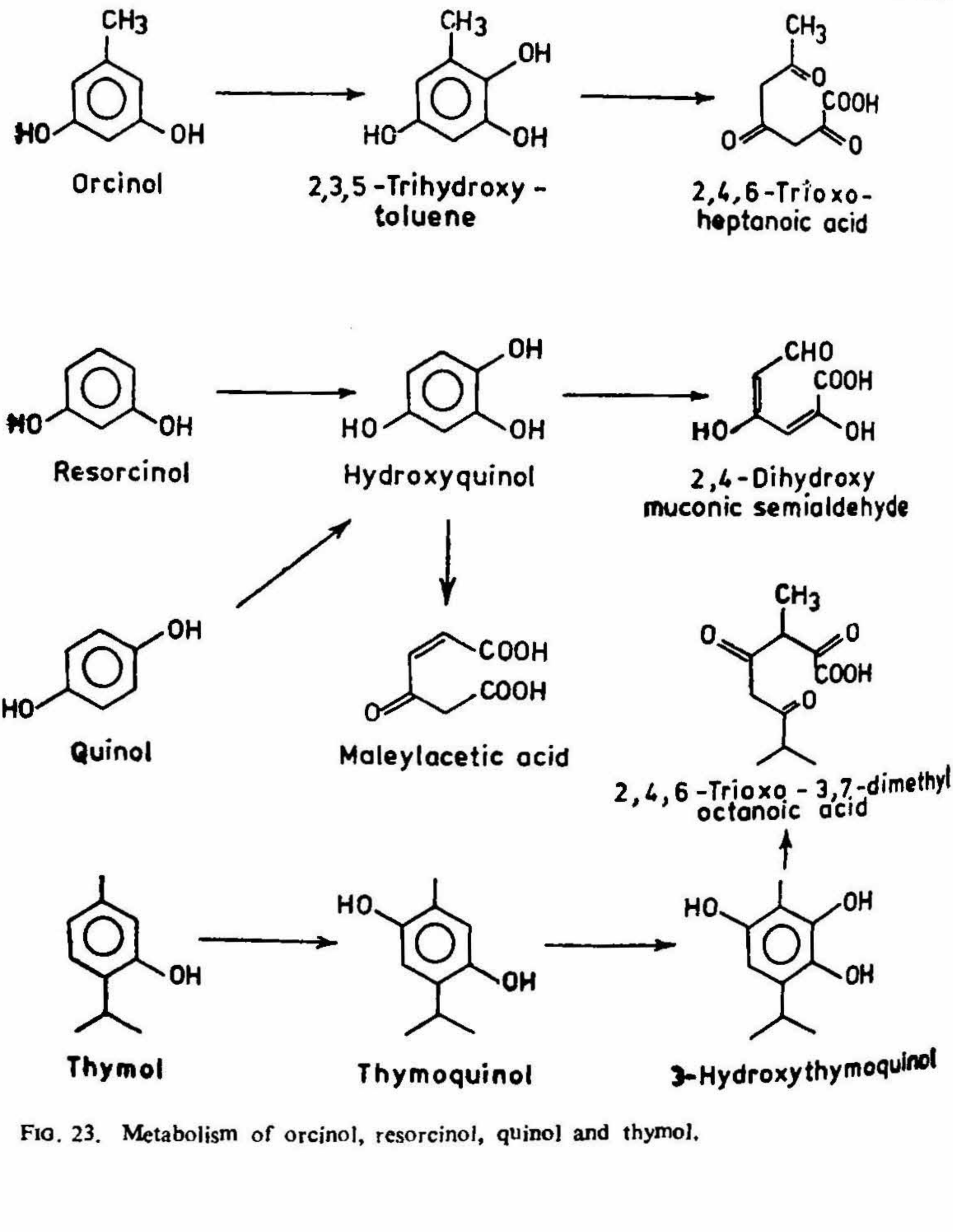


FIG. 23. Metabolism of orcinol, resorcinol, quinol and thymol.

Steady state kinetic analysis reveals the operation of an ordered Bi Uni mechanism in which the organic substrate combines first with the enzyme followed by oxygen[379]. It is colourless and shows neither significant absorption in the visible range nor any ESR signal at around $g = 4 \cdot 3$. However, H_2O_2 treatment results in an inactive enzyme which shows a broad ESR signal at $g = 4 \cdot 2$, characteristic of ferric iron. These results are consistent with the fact that the iron in the native enzyme is present in the form of ferrous ion[262].

(m) *Miscellaneous compounds*

Studies on the metabolism of syringic and gallic acids in *P. putida* reveal the presence of two gallate dioxygenases, one specific for gallate and the other attacking both gallate and 3-0-methylgallate[393]. The former is present in gallate grown cells while the latter is encountered in syringate grown cells. Both open up the aromatic ring of gallic acid at 3, 4-position to give 2-hydroxy-4-carboxy-*cis*, *cis*-muconic acid (Fig. 22). Interestingly, the protocatechuate-4, 5-dioxygenase also attacks gallate at the same position[394].

Although two hydroxyl groups are sufficient to labilize the aromatic ring, some compounds seem to undergo hydroxylation reaction to form trihydroxyphenols, which are then cleaved. Thus, *P. putida* hydroxylates orcinol as well as resorcinol to the corresponding hydroxylated products, and then oxidizes them[395]. While hydroxyquinol undergoes both *ortho* and *meta* cleavage reactions depending upon growth conditions, trihydroxytoluene undergoes only *meta* cleavage[395]. Similarly, during the metabolism of thymol, hydroxylation of thymoquinol occurs[396]. The product, hydroxythymoquinol is the substrate for ring cleaving enzyme[396]. Hydroxylation of quinol followed by intradiol cleavage of hydroxyquinol has also been reported[397]. The metabolic transformations of orcinol, resorcinol, quinol and thymol are given in Fig. 23.

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