# DIOXYGENASES IN HIGHER PLANTS—A REVIEW

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## ABSTRACT

*Several pathways have been delineated for the degradation of aromatic compounds in microorganisms and higher animals. However very few reports have appeared in the literature on the elucidation of such pathways in higher plants. Studies from our laboratory and elsewhere have revealed the occurrence of several ring cleaving systems in plants. Dioxygenases, such as, indole oxidase, homogentisate dioxygenase, protocatechuate* 3, 4-*dioxygenase and pyrocatechuate* 2, 3-*dioxygenase have been partially purified from plants. The properties of these enzymes have been compared with the microbial enzymes. Our current knowledge on these dioxygenases and other dioxygenases in plants have been reviewed.*

Keywords: Review, plant dioxygenases.

## INTRODUCTION

Metabolism of aromatic compounds in plants has been the subject for extensive research during the past two to three decades and yet rapid strides have been made in very few areas of phenolics metabolism. Investigations, primarily of an analytical nature in the early part of this century had revealed the diversity and enormity of plant phenolics, ranging from simple molecules like hydroxylated benzenes to the more complex polymeric structural materials like lignins and tannins. Such a comparative study in several plant species evoked a keen interest, particularly in taxonomists and attention was soon directed towards the assessment of the contribution of these results to plant systematics. Elucidation of a few key pathways, substantiated by the advances towards the understanding of biogenetic relationships between phenolic substances and plants, has kindled attempts at determination of their precise functions in these systems.

A cursory glance at the literature outlining the functional significance of phenolics in plants illustrates that umpteen roles, a few exotic, have been

attributed to these compounds [1–4]. They act as substrates for polyphenol oxidases and other enzyme systems which build the structural and colouring materials such as malanins, tannins and lignins. They are involved in the oxidative browning of fruit products, and in tea fermentation reactions [5–8]. They are also implicated as disease resistance factors in a number of plants against the pathogens [9–10]. Some of them have been shown to be growth regulators for several plants [11–13]. And they serve as the source of carbon and energy for several microorganisms [14–16].

Despite their recognized importance, the metabolism of polyphenols has not been studied in detail, especially in plants. Recently the subject has been reviewed with a view to understand the biogenetic relationship among these phenols [17, 18]. With the operation of the most prevalent "shikimate pathway", some of the key aromatic compounds are synthesized. The simple aromatic compounds thus formed are utilized to build the more complex structures like alkaloids, flavonoids, lignins, etc. During this process, a variety of biochemical reactions are employed and chemically diversified aromatic compounds are biosynthesized. Oxygenases form a class of important enzymes involved in such biotransformations.

Molecular oxygen, in addition to serving as the ultimate acceptor of electrons in the respiratory chain, is also utilized as a biosynthetic and biodegradative device for a variety of essential biochemicals in the living cell. Enzymes catalyzing such reactions are termed 'oxygenases'. Ever since Mason *et al.* [19] and Hayaishi *et al.* [20] simultaneously and independently demonstrated the fixation of molecular oxygen into the aromatic compounds with the use of $^{18}O_2$, there have been increasing reports on the occurrence of such enzyme systems in microbes, higher plants and animals. Such studies necessitated the classification of the enzymes catalysing the oxygen fixation reactions into two main categories. Mono-oxygenases [21], which are also called as mixed function oxidases [22], are enzymes catalyzing the incorporation of one atom of molecular oxygen into the substrate and reducing the other atom to water utilizing appropriate electron donors, while dioxygenases [21] are enzymes catalyzing the fixation of both the atoms of molecular oxygen into the substrate.

Dioxygenases are a particularly interesting class of enzymes because they convert the inert aromatic ring into aliphatic products which can be appropriately metabolized to useful biochemicals. They can cleave 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. 1). With the substituted catechols, multiple modes of cleavage are possible. These are illustrated in Fig. 1.

All the three modes of cleavages are known in a few cases of substituted catechols. For example, protocatechuic acid and homoprotocatechuic acid undergo three different modes of cleavage yielding aliphatic products [14, 23]. These dioxygenases have been extensively studied from microbial sources as evidenced by the large number of review articles that have appeared in the literature time and again [14–16, 24–29].

Although pathways for the degradation of aromatic compounds by microorganisms have been delineated in detail, very little attention has been devoted to the study of such biotransformations in higher plants. Studies

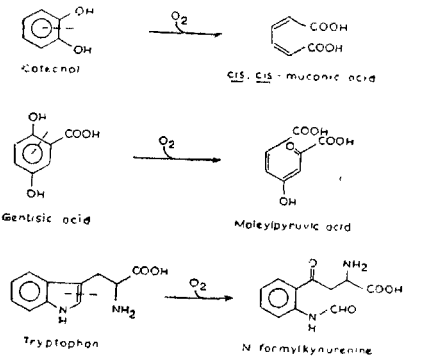



FIG 1a THREE TYPES OF DIOXYGENASES

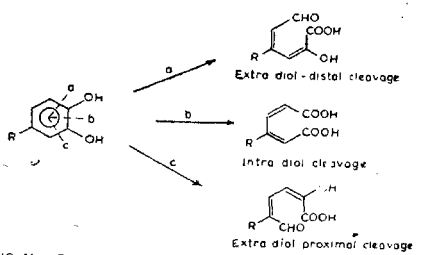



FIG 1b THREE MODES OF CLEAVAGE OF A SUBSTITUTED CATECHOL

on enzyme systems from plant sources have been greatly hampered by the problems encountered in (*a*) eliminating the interfering microbial metabolism, (*b*) low levels of metabolites and enzyme activities and (*c*) the occurrence of enzymes such as polyphenol oxidase which utilize rapidly the added phenolic compounds to build up the polymeric materials.

This review attempts to give a detailed description of the metabolism of aromatic compounds *via* the ring fission reactions in higher plants.

*Indole Oxidase*

A powerful indole oxidase system which converts indole to anthranil, evidently by a 2, 3-dioxygenase type of reaction, was isolated from our laboratory in 1964 [30]. The partially purified enzyme from fresh, mature leaves of *Tecoma stans*, oxidized indole with the consumption of three atoms of molecular oxygen. By trapping experiments, the immediate ring cleaved product was identified as N-formyl-2-aminobenzaldehyde. Based on chemical and enzymological studies, the reaction sequence shown in Fig. 2 was established to be the one which is involved in the conversion of indole to anthranil.

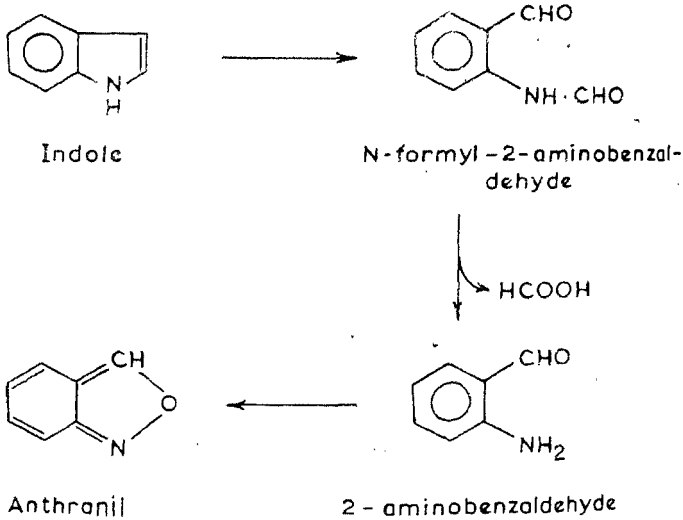



FIG. 2. Metabolism of indole in *Tecoma stans*.

The enzyme system showed a pH optimum of 5 and was found to be remarkably stable at acidic pH. It was highly susceptible to pH changes in neutral and alkaline conditions. There was a sudden drop in activity in the pH range 5·8–6·2; almost 60% of the activity being lost over the range of 0·4 pH unit.

Among various metal ions tested only $Hg^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ were inhibitory to the reaction. Contrary to the 2, 3-dihydroxyindole-2,3-dioxygenase, an indole oxidase purified from a soilborne gram positive coccus [31] indole oxidase of *Tecoma stans* was not inhibited by 1, 10-phenanthroline. Nevertheless, other metal chelating agents such as 8-hydroxyquinoline, diethyl dithiocarbamate and salicylaldoxime inhibited the *Tecoma* enzyme to varying degrees. The inhibition caused by the latter two reagents could be reversed only by $Cu^{2+}$ and not by any other metal ions. These studies taken together with the reconstitution of enzyme activity in the dialyzed preparations by $Cu^{2+}$ ion, showed that indole oxidase is a cuproprotein. Unlike tryptophan and indolamine 2, 3-dioxygenases [29] both *Tecoma* enzyme and bacterial enzyme did not possess heme cofactor.

In addition to $Cu^{2+}$, the indole oxidase system also required FAD. Atebrin inhibited the reaction drastically and the inhibition could be reversed by the addition of FAD. The dialysed enzyme, which is inactive, could be reactivated by the addition of both $Cu^{2+}$ and FAD, thereby showing the flavin requirement for the oxygenation reaction. Though it is possible to explain the FAD requirement for the second oxidation step, in view of the finding that pyridine dioxygenase requires flavin to show full activity [32], it can be speculated that flavins are involved in the indole dioxygenase reaction as well. However, further purification and fractionation of the individual enzymes are necessary to answer this problem.

Studies on the effect of sulfhydryl reagents and sulfhydryl compounds revealed that a sulfhydryl-cupric-ion complex at the active site is essential for the reaction [30].

*Pyrocatechuic Acid Metabolism*

Pyrocatechuic acid is an important phenolic acid precursor for the biosynthesis of iron-sequestering agent enterochelin [33]. We detected this acid in the *o*-dihydroxy-phenolic acid fraction of *Tecoma stans* [34] and isolated an enzyme system, which cleaves the aromatic ring of 2, 3-dihydroxybenzoic acid, from the fresh tender leaves of *Tecoma stans* [35]. The enzyme was partially purified by calcium acetate treatment, pH treatment,

protamine sulfate treatment, negative adsorption on DEAE cellulose, ammonium sulfate precipitation and tricalcium phosphate gel treatment. The partially purified enzyme oxidized pyrocatechuic acid rapidly. From the reaction mixture a compound was isolated and identified to be a γ-dilactone carboxylic acid by spectral studies. Based on this identity, it was concluded that an intradiol cleavage of pyrocatechuic acid had occurred and the reaction scheme shown in Fig. 3 was proposed.

This was the first report on the occurrence of aromatic ring fission by an isolated enzyme system from a higher plant. It was also the first report on a direct intradiol cleavage of 2, 3-dihydroxybenzoic acid in any system. In bacteria, however, it undergoes an extradiol cleavage [36, 37] and in fungi, a decarboxylation to yield catechol which undergoes intradiol cleavage [38].

Detailed studies on the enzymes of pyrocatechuic acid metabolism from *Tecoma stans* revealed the operation of 3-oxoadipic acid pathway for the utilization of this aromatic compound. The formation of 2-carboxy-*cis*, *cis*-muconic acid, muconolactone(s) and 3-oxoadipic acid and evolution of carbon dioxide during the oxidation of pyrocatechuic acid could be demonstrated with the cell-free extracts of *Tecoma* leaves [39].

Spectral studies demonstrated the formation of 2-carboxy-*cis*, *cis*-muconic acid from pyrocatechuic acid. The enzymatic product showed an absorption maximum at 268 nm. On heating the product, a bathochromic shift from 268 to 274 nm occurred with an increasing absorption coefficient. The absorption at 268 nm completely disappeared under acidic conditions.

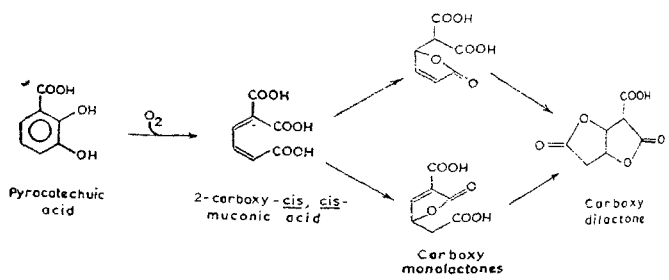



FIG. 3. Proposed pathway for the formation of dilactone from pyrocatechuic acid in *Tecoma stans*.

Such transitions have been attributed to the isomerization of *cis, cis*-muconic acid to *cis, trans*-muconic acid [40–42]. Hence the proposed intradiol cleavage could be established for the degradation of pyrocatechuic acid. While the final purified enzyme oxidized pyrocatechuic acid to 2-carboxy-*cis, cis*-muconic acid with the consumption of one mole of molecular oxygen, a partially purified enzyme [39] oxidized the same with the evolution of carbon dioxide to a compound which answered the Rothera's test (3-oxoadipic acid) [43]. In the reaction mixture, both muconolactone and dilactone could be detected. These studies led to the proposal of a new pathway for the degradation of pyrocatechuic acid in *Tecoma stans* (Fig. 4).

Pyrocatechuic acid 2,3-dioxygenase was further purified from the leaves of *Tecoma stans* about 63 fold with a recovery of 46% [39]. The enzyme was extremely labile and had a half-life of 3–4 hr. Addition of glycerol, 10% acetone, low concentration of substrate and its analogues or various metal ions failed to stabilize the enzyme.

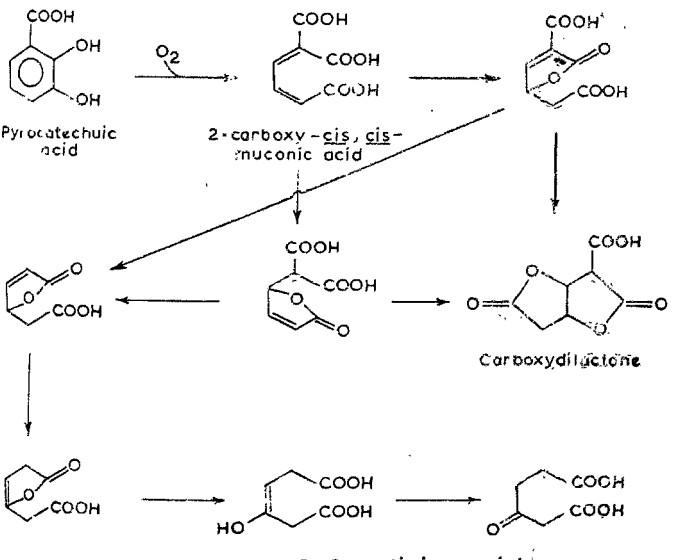



FIG. 4. Proposed pathway for the degradation of pyrocatechuic acid in *Tecoma stans*.

The enzyme oxidized one molecule of pyrocatechuic acid stoichiometrically to one molecule of 2-carboxy *cis*, *cis*-muconic acid with the consumption of one molecule of oxygen. Apart from 2, 3-dihydroxybenzoic acid, it also oxidized 2,3-dihydroxy-*p*-toluic acid and 2,3-dihydroxy-*p*-cumic acid, albeit at a slower rate. However, none of the other dihydroxyphenolic acids were utilized. The enzyme seems to require a 2, 3-dihydroxybenzoic acid moiety in the substrate to exemplify the activity.

The substrate protected the enzyme against heat inactivation at 55° C for 15 min. Both sulfhydryl compounds and sulfhydryl reagents inhibited the enzyme activity. While preincubation with substrate reversed the inhibition caused by sulfhydryl reagents, it failed to do so with the sulfhydryl compounds.

Interestingly the dioxygenase was found to be a cuproprotein. Cyanide, sulfide and azide were potent inhibitors of enzyme activity even at very low concentrations. The inhibition caused by salicylaldoxime, neocuproine and diethyldithiocarbamate could be specifically reversed by the addition of cupric ions. Dialysis irreversibly denatured the enzyme. The apoenzyme could be prepared, however, by ammonium sulfate treatment followed by chromatography on Sephadex G–25. The holoenzyme was reconstituted by the addition of cupric ions alone and not by any other metal ions. Incidentally other copper containing dioxygenases are indole oxidase of *Tecoma stans* [30], quercetinase of *Aspergillus niger* [44], tryptophan 2, 3-dioxygenase of *Pseudomonas* [45] and indoleamine 2, 3-dioxygenase of rabbit intestine [46].

In addition to its occurrence in the soluble fraction, the enzyme was also found to be present in the chloroplast fraction [47] of *Tecoma* leaves. The chloroplast enzyme was more stable than the soluble enzyme. Several attempts to solubilize the enzyme from chloroplast membranes under a variety of mild conditions yielded only inactive enzyme. In contrast to the soluble enzyme which exhibited a pH optimum of 5·2, the chloroplast enzyme had a pH optimum of 5·6. While the former showed simple Michaelis-Menten kinetics towards the substrate, the latter exhibited an initial lag at low concentration of substrate and a sudden increase at high substrate concentration (Fig. 5) [48].

Interestingly the inhibition of the chloroplast enzyme activity caused by sulfhydryl reagents could be reversed by the addition of sulfhydryl compounds [47] unlike the soluble enzyme where the inhibition could not be reversed [39]. The bound and soluble enzyme differed markedly in their

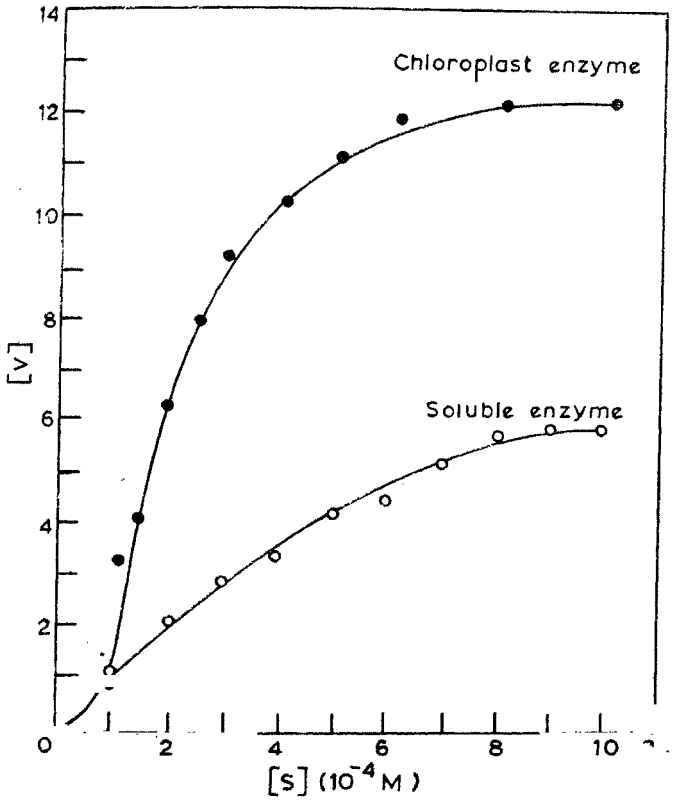



FIG. 5. Effect of substrate concentration on the velocity of pyrocatechuic acid-2, 3-dioxygenase from *Tecoma stans*.

response towards various metal ions. The chloroplast enzyme was also found to be a cuproprotein like the soluble enzyme. However, unlike the soluble enzyme, the chloroplast enzyme was stable to dialysis.

The *Tecoma* pyrocatechuic acid 2, 3-dioxygenase exhibited dramatic seasonal variation and photoperiodicity. Further studies are necessary to elucidate the mechanisms operative in triggering this ring cleaving enzyme.

*Protocatechuic Acid Metabolism*

The experiments of Tateoka [49, 50], with slices of hypocotyls of mung-bean seedlings suggested that protocatechuic acid might be degraded in this plant through the 3-oxoadipic acid pathway. Using cell suspension cultures, Berlin *et al* [51] demonstrated the catabolism of ring labelled protocatechuic acid to labelled carbon dioxide in mung bean (*Phaseolus aureus* Roxb.) as well as soybean (*Glycine max*). However, no attempts were made to elucidate the pathway for the degradation of protocatechuic acid by these workers.

Recently, we undertook studies on the metabolism of this phenolic compound in *Tecoma stans* and found that it is converted to 3-oxoadipic acid (unpublished results). The immediate product of protocatechuic acid oxidation was identified to be 3-carboxy *cis*, *cis*-muconic acid in this plant. Further studies revealed the presence of an enzyme system which decarboxylated 3-carboxy *cis*, *cis*-muconic acid to *cis*, *cis*-muconic acid in the acetone fraction. We have also obtained evidence for the presence of a lactonizing enzyme which utilizes both 3-carboxy *cis*, *cis*-muconic acid and *cis*, *cis*-muconic acid. However, whether the same enzyme or two different enzymes catalyze these two reactions remains to be elucidated. Based on these results we have proposed the pathway shown in Fig. 6 for the catabolism of protocatechuic acid in *Tecoma stans*.

We have isolated protocatechuate 3, 4-dioxygenase from the fresh tender leaves of *Tecoma stans*. The enzyme was specifically involved in protocatechuic acid metabolism and was different from polyphenoloxidase of *Tecoma stans*. By employing heat treatment, manganese sulfate treatment, ammonium sulfate precipitation and DEAE cellulose column chromatography the dioxygenase was purified to near homogeneity with a recovery of 33%.

Protocatechuate 3, 4-dioxygenase from *Tecoma stans* exhibited a strict, substrate specificity and did not oxidize any of the substrate analogues like the bacterial enzymes [52, 53]. Contrary to the bacterial enzymes [52, 53], the *Tecoma* enzyme was not inhibited by various substituted catechols other than protocatechualdehyde which showed only 10% inhibition. Even the $K_m$ value for protocatechuic acid was significantly different for the bacterial and the plant enzymes (Table I).

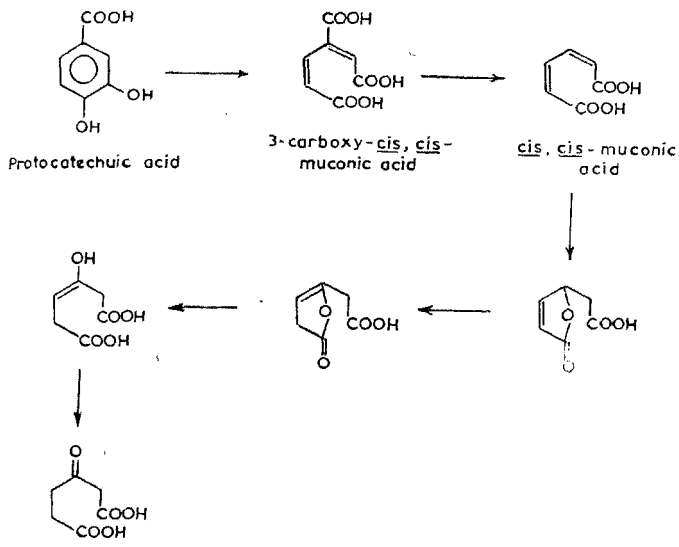



FIG. 6. Proposed metabolism of protocatechuic acid in *Tecoma stans*.

While the molecular weight of plant enzyme as determined by gel filtration technique was 155,000, that of bacterial enzymes were 677,000 and 700,000. Interestingly the plant enzyme showed an acidic pH optimum, while bacterial enzymes had an alkaline pH optimum (Table I). The other properties, like temperature optimum, stability and absorption spectrum, were significantly different for the plant and microbial enzymes (Table I).

A comparison of the effect of various metal ions on protocatechuate 3, 4-dioxygenase from these two sources revealed interesting differences (Table II). While $Fe^{3+}$, and $M_0^{2+}$ completely inhibited the *Tecoma* enzyme, they did not have any effect on the *Acinetobacter* enzyme [53]. While ferrous ion was essential for the plant enzyme to exhibit its activity, it was inhibitory to the bacterial enzyme. Sulfhydryl reagents especially 4-chloromercuribenzoate inhibited both *Acinetobacter* and *Tecoma* enzymes, but *Pseudomonas* dioxygenase was not affected at all [52, 53]. Interestingly DTNB and

TABLE I

*Comparison of properties of bacterial and plant protocatechuate-3, 4 dioxy-genases*

| Property | *Tecoma* enzyme | *Acinetobacter* enzyme [53] | *Pseudomonas* enzyme [52, 53] |
|---|---|---|---|
| Molecular weight | 155,000 | 677,000 | 700,000 |
| pH optimum | 5·2 | 8·5–9·0 | 8·0 |
| Temperature optimum | 40° C | 35° C | 35° C |
| Stability at 4° C | 7 days | 6 months | 6 months |
| Absorption maximum at visible region | No absorption | 410 and 450 nm | 410 and 450 nm |
| Iron content | N.D. | 7·27 g atom/mole | 7·0 g atom/mole |
| $K_m$ value for protocatechuic acid | $3·3 \times 10^{-4}$ M | $0·714 \times 10^{-4}$ M | $0·3 \times 10^{-4}$ M |
| $K_m$ value for oxygen | N.D. | $5·88 \times 10^{-5}$ M | $4·3 \times 10^{-5}$ M |
| $K_m$ value for iron | $4·0 \times 10^{-5}$ M | .. | .. |
| Energy of activation | N.D. | 0·93 Kcals | 1·33 Kcals |

N.D. = Not determined.

iodoacetamide inhibited the plant dioxygenase and not the *Acinetobacter* enzyme [53].

Although these enzymes catalyze the same reaction, they differ significantly in their properties. These results may reflect the fundamental differences in their primary and quaternary structures.

*Tyrosine Metabolism and Homogentisate Dioxygenase*

As early as 1961, Ibrahim *et al* [54] presented evidence for the cleavage of the aromatic ring of tyrosine. These workers administered uniformly labelled tyrosine to the leaf discs of angiosperms and observed the incorporation of more than 33% of radioactivity in non-aromatic compounds. They attributed such high conversions to the aromatic ring cleavage system.

TABLE II

*Effect of metal ions on the activity of bacterial and plant protocatechuate-3, 4-dioxygenases*

| Salt | Percentage inhibition at $2 \times 10^{-3}$ M concentation | |
|---|---|---|
| | *Tecoma* enzyme | *Acinetobacter* enzyme [53] |
| Ferrous ammonium sulfate .. | 0 | 30 |
| Ferric ammonium sulfate | 100 | 0 |
| Nickel chloride | 91 | 43 |
| Nickelous chloride .. | 100 | 36 |
| Molybdenum dichloride .. | 91 | 0 |
| Cuprous chloride | 89 | 68 |
| Cupric sulfate .. | 100 | 40 |
| Cobalt sulfate .. | 45 | 50 |
| Cobaltous chloride .. | 100 | 68 |

Hills and Isoi [55] also observed high rate of conversion of tyrosine to aliphatic products in the *Eucalyptus* plant.

During his studies on the biotransformations of hydroxyphenylacetic acids in plant, Kindl [56] observed the utilization of homogentisic acid in the detached leaves of *Sinapis alba* and *Astilbe chinensis*. To demonstrate unequivocally the potentiality of plant tissue cultures to split the aromatic ring, Ellis [57] undertook a careful study and observed the degradation of ring labelled tyrosine and homogentisic acid in ten different plants using cell suspension culture technique.

Though from these studies it was clear that exogenously supplied tyrosine and homogentisic acid were extensively metabolized by tissues of higher plants, operation of the homogentisate ring cleavage pathway was not established with certainty until Durand and Zenk [58, 59] reported its occur-

rence in cell suspension cultures of higher plants. Using crude homogenates of cell suspension cultures of *Drosophyllum lusitanicum*, these investigators unequivocally demonstrated the presence of tyrosine aminotransferase, 4-hydroxyphenylpyruvate dioxygenase, homogentisate dioxygenase and fumarylacetoacetate hydrolase in the above plant [59].

Employing the same steps used for the isolation of the bacterial enzyme [6], Durand and Zenk [59] purified *Drosophyllum* homogentisate dioxygenase to about 190 fold with a yield of 39%. Like the microbial enzyme, the plant enzyme was also inhibited by iron-chelating agents notably α, α'-bipyridyl. To establish the widespread occurrence of homogentisate dioxygenase these authors purified and demonstrated the presence of this enzyme from ten species of plants belonging to seven different families. Thus the

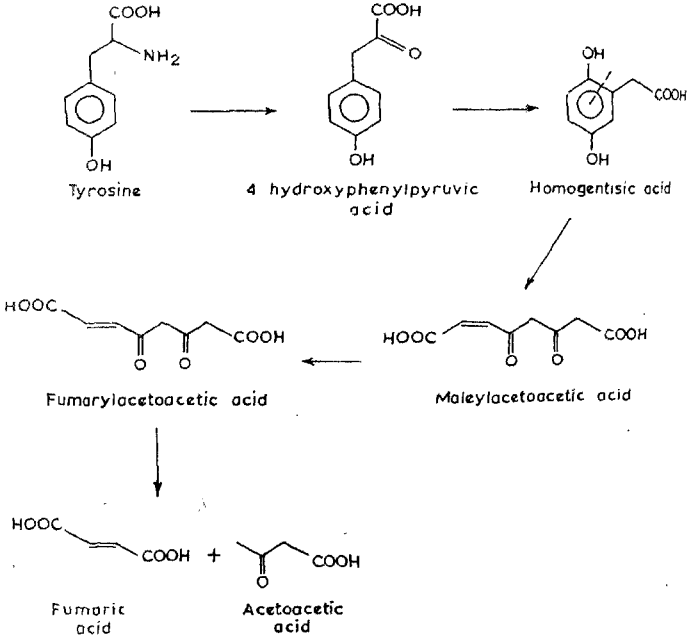



FIG. 7. Metabolism of tyrosine in *Drosophyllum lusitanicum*.

mechanism of conversion of L-tyrosine to acetate and fumarate *via* homogentisate seems to be same in all living organisms and shows a remarkable phylogenetic constancy (Fig. 7).

*Biosynthesis of Betalain Pigments*

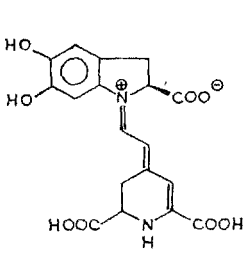
In 1957, crystalline samples of the pigment betanin were obtained [61, 62] and subsequently the chemical nature of betanin and related pigments were determined [63, 64]. It was postulated that betanidin arises from the assembly of two molecules of L-dopa [62, 65, 66].

Piattelli and his group investigated the pigments of *Centrospermae* systematically and isolated forty-four kinds of the new pigments from seven families of the order *Centrospermae* [67–72]. The most widespread plant pigments, *viz.*, anthocyanins appear to be replaced by these new pigments in flowering plants belonging to the above order. These pigments include a distinctive group of red-violet (betacyanins) and yellow (betaxanthins) pigments which possess a dihydropyridine moiety (Fig. 8). It was suggested that the dihydropyridine ring system might originate from L-dopa by an oxidative ring fission followed by ring closure [62, 65, 66, 73, 74].

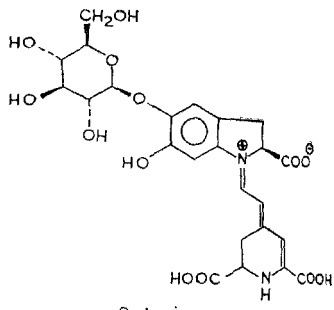
Subsequently various groups of workers showed that $^{14}C$ labelled dopa and tyrosine were readily incorporated into these pigments [71, 75–79]. Utilizing $^{15}N$ labelled tyrosine, it was also demonstrated that even the amino nitrogen was incorporated into the pigments [74]. Though these studies showed that dopa is the precursor of these pigments, they did not give much information on the mode of conversion of dopa to these pigments.

It is generally accepted that betalamic acid (Fig. 8) is the precursor of these pigments. Purely on the basis of structural chemistry, one can rule out the extradiol proximal cleavage of dopa for the biogenesis of betalamic acid. This leaves the option between extradiol distal cleavage and intradiol cleavage, though the former seems to be more likely to occur.

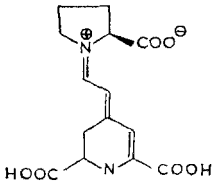
To decide between these two possibilities, Dreiding's group carried out an experiment in cactus fruits (*Opuntia decumbens*) using 3', 5'-dititiated tyrosine [80]. As tyrosine to dopa conversion does not involve any NIH shift, 3', 5'-dititiated tyrosine would be converted to 5'-tritiated dopa *in vivo*. If an intradiol cleavage of this 5'-tritiated dopa occurs followed by ring closure, then the newly formed betalamic acid would have lost the remaining tritium atom during the process. On the contrary, an extradiol distal cleavage would give rise to tritiated betalamic acid. Since in the



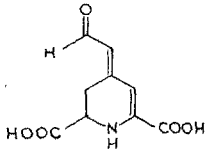

Betanidin




Betanin




Indicaxanthin




Betalamic acid

FIG. 8. Structures of some betalain pigments and betalamic acid.

*in vivo* experiments tritiated betalamic acid derivative was formed, it was concluded that an extradiol distal cleavage of dopa had occurred (Fig. 9).

Piattelli's group also established the occurrence of extradiol distal cleavage of dopa, simultaneously and independently by demonstrating the presence of one atom tritium in indicaxanthin synthesized *in vivo* from 3', 5'-ditritiated ($^{14}$C—COOH) tyrosine [81].

Whether or not betalamic acid lies on the pathway to betalains biosynthesis is still an open question. Because it is still uncertain whether cyclization of the ring cleaved product to give the dihydropyridine ring system takes place before or after the condensation with the other part of the pigment, and whether it is enzymatic or spontaneous. However, these studies would

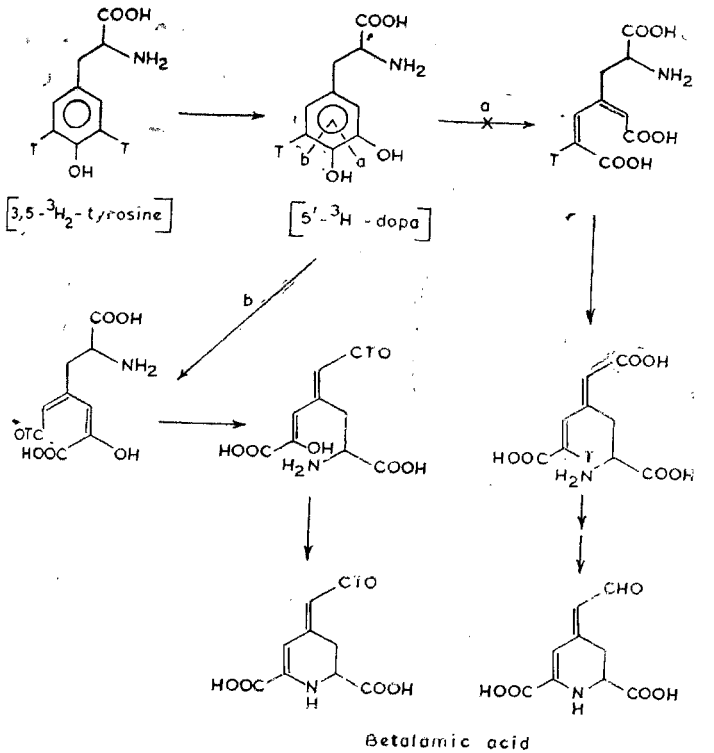



FIG. 9. Mechanism of betalamic acid formation from 3', 5'-ditritiated tyrosine.

not alter the established mode of ring fission of dopa. The enzymology of the biosynthesis of these pigments is a promising field for future studies.

*Biosynthesis of Stizolobic Acid and Stizolobinic Acid*

Hattori and Komamine [82] for the first time isolated strizolobic acid and stizolobinic acid—new types of heterocyclic non-protein amino acids—from the sap of the etiolated epicotyl tips of the plant *Strizolobium hassjoo*.

Subsequently, these amino acids were shown to be γ-pyrone-6-carboxylic acid derivatives with an alanyl side chain, at the 3 position for stizolobinic acid and at the 4 position for stizolobic acid [83, 84]. They postulated dopa to be the precursor of these amino-acids and proposed an oxidative ring cleavage followed by cyclization and dehydrogenation reactions for the biogenisis of these compounds [84–86].

In addition to *Stizolobium,* these amino-acids were also detected in *Mucuna* and *Amanita* species [87, 88]. Recently, the Japanese group examined the incorporation of labelled phenylalanine, tyrosine and dopa into these amino-acids and found that dopa and to a lesser extent tyrosine were readily incorporated into these heterocyclic compounds by the etiolated seedlings of *Stizolobium hassjoo* [89]. Ellis confirmed these results using the plant, *Mucuna deeringiana* [88].

The site of cleavage of the aromatic ring of dopa was determined by using doubly labelled tyrosine [90]. After feeding equal amounts of (U) $^{14}$C-tyrosine and L-(3, 5-$^{3}H_2$) tyrosine to the etiolated epicotyls of *S.hassjoo*, labelled stizolobinic acid and stizolobic acid were isolated and the $^{3}H/^{14}C$ ratio determined. If these compounds are biosynthesised by extra diol cleavages, then stizolobinic acid would be free from tritium. This was found to be true thereby proving the proposed biogenetic pathway shown in Fig 10.

*Miscellaneous Compounds*

It would be interesting and intriguing to examine the available evidences on the ring cleavage of other compounds. Despite the difficulties encountered in the study of plant phenolic metabolism, a number of reports have appeared describing the ability of plants to split the aromatic ring.

Zaprometov [91] who studied the oxidation of $^{14}$C labelled catechins by tea plants accounted for 73–82% of the absorbed activity as $^{14}CO_2$ after 30 hr. This result definitely suggests that catechins in tea plants serve as important respiratory substrates. The ability of higher plants to split the aromatic ring of labelled chalcone [92], toluene [93], benzene [93, 94] and phenol [95] has been well authenticated.

Kindl [56] observed the conversion of labelled 2, 3- and 3, 4-dihydroxy phenylacetic acids to D-glucose in the leaf cuttings of *S. alba* and *A. chinensis* and attributed such conversions to the ring cleavage reactions. Ellis and Towers [96] undertook a careful study of the degradation of phenylalanine

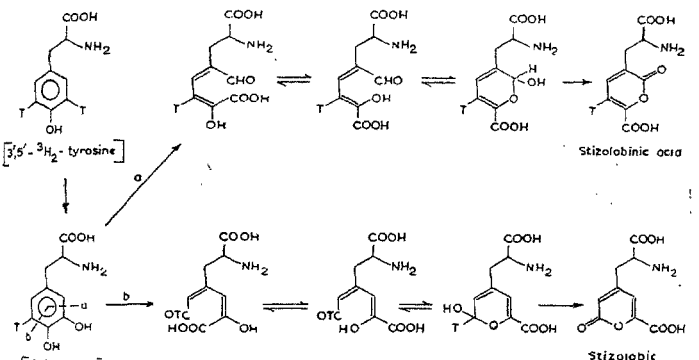



FIG. 10. Biosynthesis of stizolobinic acid and stizolobic acid from 3', 5'-Ditritiated L-Tyrosine.

cinnamic acid and tryptophan employing aseptic tissue cultures *Ruta* and *Melilotus* and surmised that these plants degraded both phenyl propanoid and indole compounds. Towers group [97] also demonstrated the oxidation of labelled DL-phenylalanine to labelled carbon dioxide in nine species of marine algae.

Ellis [57] reported the oxidation of labelled dopa and catechol to carbon dioxide by ten different plant species employing cell suspension cultures. Labelled caffeic acid and catechol were degraded to carbon dioxide in cell suspension cultures of mung bean and soybean [51]. Unpublished observations from our laboratory show that both 3-hydroxyanthranilic acid and catechol undergo ring fission in the leaves of *Tecoma stans*, although such a catabolic route has not yet been unequivocally established.

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*Note:* After this paper has been sent to the press, we came across an article [K. Saito and A. Komamine, *Eur. J. Biochem.*, **68**, 237–243 (1976)] in which the authors have reported the partial purification of stizolobic and stizolobinic acid synthesising enzyme system from the cell free extracts of the etiolated seedlings of *Stizolobium hassjoo*. This enzyme system which showed maximum activity at pH 7·5 and required the presence of $NADP^+$ or $NAD^+$, converted L-dopa into Stizolobic and Stizolobinic acid under aerobic conditions.