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REVIEW

POLYPHENOL OXIDASES IN PLANTS

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Key Word Index—Higher plants; polyphenol oxidases; catechol oxidase; laccase; properties; distribution; physiological function.

Abstract—Recent progress in the study of plant polyphenol oxidases is critically reviewed. Two main groups are recognized: the catecholoxidases and the laccases. Their purification, subcellular location and protein properties are described. Attention is also given to their activation and induction, their function and evolution.

INTRODUCTION

We selected the title polyphenol oxidases in plants for our review as being the most encompassing. The commission on enzymes originally referred to two enzymes in this category—laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2) and catechol oxidase or *o*-diphenol oxygen oxidoreductase (EC 1.10.3.1). The commission has now revised the nomenclature, lumping them together as EC 1.14.18.1 monophenol monooxygenase. This revised nomenclature is in many ways unfortunate. Catechol oxidase which is also frequently referred to as phenolase, polyphenol oxidase, tyrosinase, catecholase or cresolase, is quite distinct from laccase. Laccase oxidizes a very wide range of substrates including monophenols, triphenols and ascorbic acid as well as *o*- and *p*-diphenols. The name *p*-diphenol oxidase is therefore somewhat misleading [1]. However, its ability to oxidize *p*-diphenols is diagnostic and we will refer to it only as laccase. In contrast, the inability of *o*-diphenol oxygen oxidoreductase under any circumstances to oxidize *p*-diphenol is a typical property of the enzyme. It does, however, oxidize in some cases monophenols and this property is of great significance. We will use the term catechol oxidase for this enzyme despite the fact that it is not really descriptive. The terms catecholase and cresolase refer to its oxidation of diphenols and monophenols respectively. We will use these terms only in special cases, for the sake of clarity.

The general topic comprising both these enzymes in plants. has been rarely reviewed completely. Much of the early work was discussed by Mason [2] and by Bonner [3]. Laccase was reviewed by Levine [4] in a fairly comprehensive fashion, and by Malmström [5] and catechol oxidase by Brooks and Dawson [6]. Since then the topic has appeared sporadically in the literature, generally with reference to the property of the enzymes as typical oxygenases. Most recent reviews have concentrated on the mechanism of action of the enzymes and particularly on the function of copper in them [7–9].

The enzymes to be discussed are very widespread in distribution. Although there is much similarity between catechol oxidases from plants and animals, we will confine ourseves to plant tissues, but will include fungi and bacteria in the discussion.

ASSAY OF THE ENZYMES

Both catechol oxidase and laccase oxidize phenolic substrates, utilizing molecular oxygen. Since oxidized phenolic substances undergo many secondary reactions, both with each other and with protein, it is difficult to measure product formation in routine assays. Since some of the products react with reagents for phenolic substances, it is also difficult to routinely assay for residual substrate. The most convenient, but in some ways inaccurate, method is to follow the initial rate of formation of the quinone spectrophotometrically. It is, however, much more preferable to measure O_2 uptake directly again taking into consideration only initial rates, since the enzyme may undergo rapid inactivation during catalytic performance [6, 10–12]. It is important to note that the ratio oxygen consumed/substrate oxidized changes with time of the reaction and depends on substrate type and concentration and on the pH of the reaction mixture, the buffer used, etc. [13–16]. An additional problem concerning the assay is the low affinity of phenol oxidases to oxygen. Most assays, and particularly the spectrophotometric ones, are therefore carried out at oxygen concentrations far below the K_mO_2 of the enzyme. It is evident that true V_{max} values are seldom determined. Some of these problems have been considered by Kean [17]. Mayer *et al.* [15] and Yamaguchi [18].

An elegant way of following the hydroxylation reaction has been described by Pomerantz [19], who measured the release of tritiated water during the hydroxylation of a [3, 5^3-H] substrate (in his case tyrosine). This method has not been used as extensively as might have been expected from its sensitivity. Recently methods have been proposed to follow catechol oxidase or laccase spectrophotometrically, using coupling reactions with the quinone formed. In one case quinone formation is followed by a trapping reaction and in the other disappearance of the coupling reagent is determined

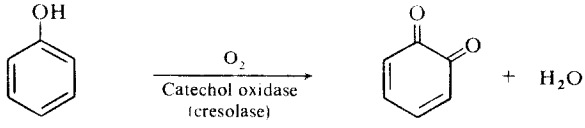
[20, 21]. Good linearity with time and enzyme concentration was observed in both cases, but the exact stochiometry of the reactions was not determined. Direct observation of the disappearance of substrate still appears to be preferable.

It is extremely important to differentiate between catechol oxidase and laccase on the one hand and peroxidase on the other. Peroxidative oxidation of phenols is frequently mistaken for catechol oxidase or laccase. Peroxidase can be easily distinguished from the polyphenol oxidases. Addition of catalase and alcohol to the reaction mixture results in destruction of peroxides. Under these conditions no oxidation of phenols occurs [22]. Ways to distinguish between the enzymes in gels after electrophoretic separation have also been described [23]. Catechol oxidase and laccase can be distinguished by a number of criteria: (1) oxidation of *p*-diphenols or *p*-diamines: (2) response to CO [22, 24]; (3) response to phenylhydrazine [25]. These aspects will be amplified in later sections. Much of the earlier work and even recent publications fail to take adequate precautions in this respect and confusion often exists.

CATECHOL OXIDASE AND ITS DISTRIBUTION

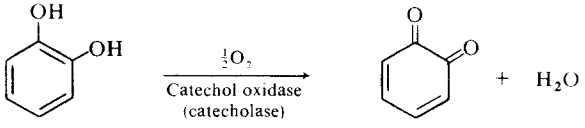
Catechol oxidase is a copper enzyme which catalyses two distinct reactions:

(a) The insertion of oxygen in a position *ortho* to an existing hydroxyl group, usually followed by oxidation of the diphenol to the corresponding quinone, often referred to as cresolase activity:



and

(b) The oxidation, with hydrogen abstraction, of *o*-diphenol



often referred to as catecholase activity. Molecular oxygen participates in both reactions.

Catechol oxidase is widely distributed in the plant kingdom. Being easily detectable, it was among the first enzymes to be studied [26–28]. It has since been reported to occur in several bacteria [e.g. 29–38], numerous fungi, algae [39–42], bryophytes [43–45], pteridophytes [46], gymnosperms [47] and practically every order (and possibly family) of angiosperms where it has been looked for.

However, the enzyme is by no means ubiquitous. Although it has been reported from several algae (e.g. *Nitella* [40–42] and *Monostroma* [39], it has not been detected in unicellular algae, except for a questionable report on *Dunaliella* [48]. Cambie [47] has found catechol oxidase activity in 20 of 44 species of Gymnospermae examined. Since it is only one of several phenol oxidizing enzymes known to occur in plant tissues, cases of mistaken identity, especially when crude preparations have been used, cannot be ruled out. Latency of catechol oxidase and the occurrence of endogenous inhibitors may, on the other hand, account for failure to detect the enzyme in some tissues.

In contrast to the laccases, catechol oxidases do not appear to be restricted to any particular part of the plant and their presence has been reported from a variety of plant organs and tissues. Some particular cases which should be mentioned are pollen grains [e.g. 49], the latex of some plants [50, 51], crown-gall tissue [52] and a relatively high level in guard cells [53]. Reports on differences in the properties of the enzyme from various parts of the same plant or from different organelles in same cells are widespread in the literature [e.g. 39, 54–58].

The level of catechol oxidase often changes markedly during the development of the plant [e.g. 59–66] and may be considerably affected by growth conditions [44, 67–70]. Such effects have also been observed in tissue cultures of various plants [71–75].

Mutations affecting the level of the enzyme [76–79], its properties [76, 77, 80–84] or its control [80–82, 84–86] have been reported from various fungi and from a liverwort [45]. Marked differences in both the level of catechol oxidase activity and in the content of its substrates have been observed between different cultivars of fruits and vegetables [e.g. 61, 87–90]. Such differences are important in commercially exploited plants in which browning is a problem.

SUBCELLULAR LOCATION

Excluding a few cases in fungi where tyrosinase has been reported to be excreted into the medium [91, 92], catechol oxidase is an intracellular enzyme. In contrast, many reports on laccase describe its excretion by mycelia at one stage or another of the life cycle of fungi. The only cases where catechol oxidase might be considered as 'extracellular' in plants are those locating the enzyme in the cell-wall fraction [40, 93, 94].

Fungal catechol oxidase appears to be a 'soluble' enzyme, not bound to membranes. The same is true for most of the cases which have been studied in bacteria, except for the *Mycobacterium leprae* enzyme, which is tightly bound to a 'particulate' fraction [33].

Plant catechol oxidase, including the enzymes from *Marchantia* [43], *Monostroma* [39] and *Nitella* [40], has been located in a variety of cell fractions, both in organelles [e.g. 55, 57, 68, 95–98], where it may be tightly bound to membranes and in the soluble fraction of the cell [57, 98–101]. Tanning reactions, taking place after the disruption of tissues rich in phenols may cause binding of soluble catechol oxidase to a 'particulate' fraction. Craft [100] concluded from a comparison of the properties of catechol oxidase in various cell fractions of potato tubers, that the enzyme was originally soluble and became non-specifically attached to 'particles' during grinding and fractionation [102]. Sanderson [99] showed that the enzyme from tea leaves remains soluble if isolation is carried out the presence of polyamide, which adsorbs endogenous phenols. He concluded that earlier reports on the particulate nature of the enzyme from this tissue [103] were a result of artefacts. However, Kato *et al.* [97] reported recently that part of the activity, in this tissue can be shown to be located in well defined particulate fractions even when phenols are removed with Polyclar AT.

In spite of possible artefacts, the weight of evidence indicates the wide occurrence of membrane-bound catechol oxidases, particularly in chloroplasts. Although many reports localizing the enzyme in organelles are based merely on differential centrifugation, some more careful studies involving density gradient centrifugation and correlation with chlorophyll content and activities of 'marker' enzymes [96, 97, 104, 105] reached the same conclusion. Furthermore, histochemical work employing dopa as a substrate and observations with the electron microscope showed the enzyme to be bound within chloroplast lamellae and grana [106–108, and Ben Shalom, Harel and Mayer, unpublished data] and in mitochondria [109 and Ben Shalon, Harel and Mayer, unpublished].

Apart from its localization in chloroplasts [e.g. 55, 57, 58, 95, 96, 103, 107, 108, 110–112], catechol oxidase has been reported to be localized in mitochondria [55, 95, 98, 113, 114] and in peroxisomes and 'microsomes' [97, 104]. These latter reports must be considered as exceptional at present.

The strength of binding of catechol oxidase to membranes appears to vary depending on the tissue and the stage of development of the plant. Thus, in tobacco, washing with buffer suffices to release the enzyme from chloroplast lamellae [115]. In most cases, more drastic conditions are required for the solubilization of membrane-bound catechol oxidase, such as the use of detergents, e.g. digitonin [58, 102, 116], Triton X-100 [57, 58, 111, 117], SDS [18, 118], Manoxol OT [119], butanol [67, 120] or limited digestion with proteolytic enzymes [106, 121]. These treatments evidently cause changes in the enzyme's structure and/or conformation and are frequently accompanied by activation [119, 121, 122] as well as changes in the substrate specificity, pH optimum and other properties of the enzyme [58, 118, 119, 121, 122]. Apparently, conversion of particulate forms of the enzyme to 'soluble' ones occurs in tissue cultures of apple fruit following exposure to 'stress' conditions such as low humidity and deficiency in nutrients or growth substances [74, 75]. Solubilization occurs also under more 'natural' conditions, e.g. ripening of fruits or aging. Thus, apple [61] and grape [66] catechol oxidases become increasingly soluble during fruit ripening. The proportion of the soluble enzyme in tissue cultures of apple fruit increases with the age of the culture [74]. This is the case also with catechol oxidase in sugar beet leaves [60]. The enzyme in green olives is tightly bound to membranes and requires drastic treatments for its solubilization. However, when the olive ripens and turns black the enzyme is completely soluble [105].

The compartmentation of the phenolic substrates of the enzyme, both in special cells [123–126] and within the cell [127] have been reported. This results in the separation between the enzyme and the bulk of its phenolic substrates *in situ*.

PURIFICATION

Although catechol oxidase has been partially purified from many plant tissues, reports on purification to homogeneity and data on amino acid composition, copper content, etc., are relatively few. Most of the available information comes from fungi, particularly mushrooms and *Neurospora*, with only few reports from higher plants, and even fewer in bacteria. Catechol oxidase is a relatively difficult enzyme to purify. Tanning reactions, taking place during the isolation of the enzyme, result in changes in its properties as well as in apparent multiplicity [128]. Such reactions can be partially prevented by isolation under N_2 [129–130], or in the presence of reducing agents or phenol adsorbing agents such as polyethyelene glycol, polyamide or polyvinyl pyrrolidone [99, 131–136]. However, some of these agents inhibit the enzyme irreversibly [57, 66, 137].

The binding of catechol oxidase to membranes in many tissues further complicates its isolation. Solubilization, usually achieved after preparation of acetone powder or extraction with detergents and other agents, undoubtedly result in modification of enzyme structure and properties. A widely observed phenomenon is the marked decrease or complete loss of the 'cresolase' activity upon solubilization of a membrane bound enzyme [57, 58, 111, 118]. An additional obstacle to purification is the extensive multiplicity of the enzyme and the interconversions between forms which continually occur during purification steps and during storage of purified preparations.

Procedures which have been employed in the purification of catechol oxidases include removal of inactive protein by precipitation with protamine sulphate [128, 138] or Ca acetate [139, 140], fractionation with ammonium sulphate, ion exchange chromatography—DEAE-cellulose and hydroxyl apatite being the most widely used—and gel filtration. It appears that Celite offers some advantages for the purification of catechol oxidase, being a relatively specific binder of copper proteins [141, 142]. Preparative isoelectric focusing has also been employed in some cases [66, 143].

Attempts to purify catechol oxidases by affinity chromatography have had only limited success [144–146 and Lerner, Mayer and Harel, unpublished data]. This is apparently due to changed affinity of the phenolic ligands to the enzyme upon their binding to the support, to the oxidation of ligands during the operation of the column [145 and Lerner, Mayer and Harel, unpublished data], and to the relatively low affinity of the enzyme for its phenolic substrates.

PROPERTIES OF CATECHOL OXIDASE

Molecular weight and quaternary structure

Crude extracts of mushroom catechol oxidase show multiplicty, part of which is due to polymeric forms [130, 147–149]. The MW of the predominant form has been often reported to be between 116 000 and 128 000 daltons [129, 130, 149–154]. The minimal MW observed by sedimentation was 26–32 000 [155, 156]. The enzyme was supposed to consist of four identical subunits (MW *ca* 30 000) each containing one copper atom [130, 155]. This assumption was strengthened by observation of association–dissociation equilibria among the various forms, which were concentration dependent. Jolley *et al.* [149] reported that the equilibrium could be shifted toward association by increasing protein concentration and by the presence of Ca^{2+}. Dissociation was facilitated by elevated temperatures (50°), increased ionic strength, acid pH (4.4) and by the presence of SDS or EDTA. Urea (8 M) caused complete dissociation of the 'tetramer' [129, 157].

Association–dissociation phenomena were observed also in *Neurospora* [141] with a MW range of 35 000 to

120000. A monomer weight of 33000 having one copper atom per molecule was proposed [141, 158]. The MW of catechol oxidase purified from *Streptomyces glaucescens* was 29000 [35] while that from *S. nigrifaciens* was reported to be 18000 [159], each with one copper atom per molecule.

Following the observation that copper atoms in mushroom catechol oxidase occur in pairs [160–164] Strothkamp *et al.* [165] reinvestigated recently the quaternary structure of the mushroom enzyme. SDS-acrylamide gel electrophoresis showed that the enzyme contains two types of polypeptide chains, a 'heavy' (H, MW 43000) and a 'light' (L, MW 13400) chain, differing in their amino acid composition. It was concluded that the previously designated 'monomer' of 26–34500 MW was a dimer of the L form, that the predominant form found in extracts (MW *ca* 120000) was a tetramer of the quaternary structure L_2H_2 and the active enzyme (apparent MW 69000) may then by L_2H, having two copper atoms. The distribution of the (apparently two) copper atoms among the L and H subunits is yet to be determined.

Observations by other groups might turn out to be in agreement with this recently revised quaternary structure of *Agaricus bispora* catechol oxidase. Thus Lerch [166], in contrast to Guttridge and Robb [158], reported that the *Neurospora* enzyme contains two copper atoms per functional unit of 42000 MW. An earlier observation of a low MW (8–10000) isozyme in *Agaricus hortensis* [167] and a 42000 MW 'monomer' having a copper content greater than one atom per molecule in *Podospora anserina* [138] may also be consistent with the recent observation of the Portland group.

However, several earlier observations are not readily explained by the revised model. Strothkamp *et al.* [165] suggested that the widely reported 30000 'sub-unit' was a dimer of their 'light' subunit. The fact that this 'dimer' exhibited enzymic activity has to be reconciled with the new concept of the functional unit of catechol oxidase and with the distribution and function of copper atoms in this unit. It is also not clear how the reported association–dissociation phenomena, resulting in transitions between the 120000, 60000 and 30000 MWs arose. In addition, if the 42000 MW functional unit of the *Neurospora* enzyme [166] has two copper atoms, how did the previously reported 30000 MW species originate?

The problem of the MW and quaternary structure of higher plants catechol oxidase is even more complex. Most reports on the MW of the enzyme are based on estimates (e.g. by employing gel filtration or acrylamide gel electrophoresis) using partially purified preparations: the values reported cover a wide range. In many cases, crude or partially purified preparations show a multiplicity of forms which may have resulted from association–dissociation. Thus, Harel *et al.* [116] observed 3 forms of catechol oxidase from apple fruit, having MWs of 30–40, 60–70 and 120–130 K. These were shown to undergo interconversions. Demenyuk [114] observed 24, 67 and 13 K forms in apple, the high MW one being transformed into the lower MW forms during ripening and storage of the fruit. Samorodova-Bianki *et al.* [168] reported values of 32–36 and 60 K from the same tissue The MW of *Quercus pedunculata* catechol oxidase was estimated to be 67 K [135].

Multiplicity of MWs has also been observed in catechol oxidases from tea leaves (30, 60, 120 K [169]), avocado (14, 28, 56, 112 K [170]), potato tubers (aggregates of 2, 4, 8 and 16 subunits of 36 K MW [171, 172], sugar cane (32 K and 130 K [101]), banana and apple leaves (42–45 K, 85–100 K and 150–180 K [173]). Purified catechol oxidase from cotton leaves supposedly has a MW of 5300 with *ca* one copper atom per molecule in addition to a form with 11700 MW [174]. This is the only report of such low MW of a catechol oxidase and it may well be the result of proteolytic degradation during isolation. Thus, even enzyme degraded by trypsin to a MW of less than 10000 retained activity [121]. Other catechol oxidases which have been purified to homogeneity have MWs around 36–40 K [171, 175, 176] with *ca* one g atom copper per mol and this might represent the basic subunit of plant catechol oxidase. It is not clear how these values are related to the reports on the fungal enzymes and whether they are reminiscent for example of the 42 K subunit of *Agaricus* and *Neurospora*. Does plant catechol oxidase also have a 'heavy' and a 'light' subunit? The 11700 MW form of the cotton enzyme and the 14 K form from avocado might represent such a 'light' subunit while values of 23500 [177], 25 K [178] and 28 K [170] might be its dimers and the frequently observed forms of 60–70 K MW—the L_1H and L_2H combinations. However, this is pure speculation at the moment, while the contribution of artifacts in the reported observations might be much more real. Gregory and Bendall [179] concluded that native tea catechol oxidase has a MW of 144 ± 16 K, the multiplicity observed being the result of partial degradation and tanning reactions. Harel *et al.* [180] reported that the predominant form of grape catechol oxidase (55–59 K MW) underwent 'dissociation' upon storage, dilution or exposure to acid pH or urea, forming a 31–33 and 20–21 K 'subunits'. However, such 'dissociation' could be imitated by a short exposure of the enzyme preparation to the action of pepsin or trysin.

Amino acid composition and primary structure

The amino acid composition of several bacterial and fungal catechol oxidases has been reported over the years [35, 141, 155, 157, 181]. These reports show a considerable similarity in amino acid composition among the mushroom, *Neurospora* and *Streptomyces* enzymes. Unfortunately, all reports were calculated and presented for the 30000 MW subunit and will now have to be reconciled with the recent report by Strothkamp *et al.* [165] showing marked differences in the amino acid composition of the heavy and light chains of *Agaricus* catechol oxidase and with the report that the subunit of the *Neurospora* enzyme has MW 42000 [166]. Potato tuber [171], spinach beet [176] and grape [175] catechol oxidases also show considerable similarity in amino acid composition, both among themselves and compared to the fungal enzymes and to silkworm [182] and mouse melanoma tyrosinases [183]. The similarity is particularly striking in the basic amino acids and in the content of threonine, serine, glycine, alanine and isoleucine. A relatively low content of sulphur amino acids has been reported in catechol oxidases from various sources. Thus Robb *et al.* [119] found neither methionine nor cysteine in broad bean catechol oxidase while in potato tubers [171] and grapes [175] the enzyme did not contain methionine.

There are few reports on non-amino acid constituents in the structure of catechol oxidase. Balasingam and Ferdinand [171] reported that RNA accounts for half

of the weight of purified potato tuber catechol oxidase. The RNA could not be digested by RNase but its removal by other methods resulted in complete loss of enzyme activity: this result requires confirmation. Yunusov *et al.* [184] claimed that 26% of the enzyme from cotton (out of a MW of 5600!) consist of an arabinose, xylose, galactose and glucose-containing polysaccharide. They also reported [185] that the carbohydrate moiety is attached through the hydroxyl of a *C*-terminal serine. The *N*-terminal amino acid in the cotton enzyme is histidine [184]. These findings, seen only in abstract by the reviewers, should be treated with considerable reservation. In *Agaricus*, isoleucine is the *N*-terminal while valine is the *C*-terminal amino acid. An *N*-terminal pentapeptide isolated from *Neurospora* catechol oxidase has the sequence N-Ac-Ser-Thr-Asp-Ile-Lys [186].

Copper

Numerous reports on enzymes from various sources suggest a copper content of one atom per polypeptide chain or subunit in catechol oxidase [35, 119, 129, 138, 139, 141, 158, 159, 174–176, 187]. However, recent observations with *Agaricus* and *Neurospora* catechol oxidases [162, 164, 188] indicate that the functional unit of the enzyme contains a pair of Cu ions and raise the question as to whether such a pair is attached to a single polypeptide chain or to two identical or different subunits [165]. Some earlier reports in the literature might suggest the presence of more than one copper atom per polypeptide chain [177, 179]. Kertesz *et al.* [189] suggested that two non-equivalent copper atoms are present per each catalytic site of mushroom catechol oxidase.

Determination of the copper content of catechol oxidase is further complicated by the apparent loss of copper from the enzyme during its purification [129, 149, 155, 175]. Dawson and Tarpley [10] described the loss of Cu from the enzyme below pH 5.0. The exchange of enzymic copper with [$^{64}Cu^{2+}$] [190] and its loss during catalytic activity [6] are also well documented. The reconstitution of active enzyme from apoenzyme and copper, originally demonstrated by Kubowitz [191] and later extended to catechol oxidases from other sources [129, 136] was shown by Kertesz [192] to require the addition of copper prior to that of substrate.

The ideas about the state of copper in proteins and its involvement in the catalytic action of catechol oxidase and other copper enzymes have been extensively reviewed [7, 193, 194] (see also section on reaction mechanism). The structure of the active site of catechol oxidase and the ligands present in it have not yet been characterized. It appears that cysteine could be eliminated as the ligand for copper, at least in some cases, either because of its absence [119] or where it has been shown to be linked in an intrachain disulfide bridge [181].

Substrate specificity

While catechol oxidases from animal tissues are relatively specific for tyrosine and dopa [2], the fungal and higher plants enzyme act on a wide range of mono- and *o*-diphenols. In addition, the specificity for optical isomers, which is clear cut in the mammalian enzyme [195, 196] is less evident in catechol oxidases from fungi [197] or higher plants [93].

The rate of oxidation of *o*-diphenols by lettuce catechol oxidase increases with increasing electron withdrawing power of substituents in the *para* position [198]. The rate of oxidation corresponds to the 'substituent constant' values in the Hammett equation. Similar observations were reported also for apple [57] and cherry [199] catechol oxidases. As the electron withdrawing ability of the substituent increased, K_m and k_{cat} decrease in the order H > SCN > $COCH_3$ > CHO > CN > NO_2, using a purified enzyme from mushrooms [155]. Except for the k_{cat} values for the two most slowly oxidized substrates, both K_m and k_{cat} conformed to Hammett relationships when the substituent parameter σ was used.

o-Diphenol substitution at one of the positions adjacent to the —OH groups (e.g. by —CH_3 or an additional —OH) usually are not oxidized. It was suggested that these positions should remain free for oxidation to take place [198]. The *o*-diphenol might be undergoing ketonization in the process of its oxidation by the enzyme. Additional support for this suggestion may come from the observation that 2,3-naphthalenediol is not a substrate of catechol oxidase, but inhibits the enzyme competitively [22]. This compound cannot undergo keto–enol tautomerism and does not form a quinone due to fixation of the double bonds near the hydroxyl groups [200].

There is a continuing argument on the physical relationships between the cresolase and catecholase functions. While some workers suggested that both functions are catalysed by a single site [191, 201], others implied the participation of two sites, either on the same enzyme molecule [10, 190, 202–205] or on different ones [46, 206]. Contrary to the enzyme from animal tissues, different fractions or isozymes of which show a constant ratio of cresolase to catecholase activity [e.g. 207], there are reports from fungi and higher plants showing different ratios among isozymes [6, 58, 147]. However, Long *et al.* [208] did not find such differences in isozymes of mushroom catechol oxidase and Jolley *et al.* [181] could not relate the differences they observed to differences in the primary structure of the isozymes.

Many preparations of catechol oxidase from plants are devoid of cresolase activity [93, 136, 199, 209–211], although it is difficult to assess whether these represent genuine 'native' forms of the enzyme. The phenomenon of the lability of cresolase activity is well known [10, 154, 201, 212] and it has been suggested that the loss of cresolase activity results from changes in the structure of the protein during its purification. Thus, Matheis and Belitz [213] observed that only high MW forms of the potato tuber enzyme are capable of oxidizing monophenols. Loss of cresolase activity on solubilization of membrane-bound catechol oxidases has been reported by Harel *et al.* [57, 58, 180] while Vaughan [212] observed a sharp decrease in cresolase activity upon aging. Activity could be restored by the addition of bovine serum albumin. On the other hand, cresolase activity might be induced or increased after treatment with hormones [214], trypsin [215] or urea [119] or following infection [216].

More interesting in a way are cases in which certain isozymes show apparently only cresolase activity. According to Taneja [64, 217] the time of appearance of cresolase isozymes differs from that of catecholase isozymes during the development of wheat grains. The cresolase isozymes were restricted to the endosperm while the catecholase ones were found also in other parts of the seed. Separation of isozymes having only cresolase activity was reported also from *Sorghum* [218] and carrot [216] tissue.

The suggestions of different sites for the cresolase and catecholase functions were based in some cases on differential effects of inhibitors or other treatments on the two activities [17, 219–221], including a specific protein inhibitor of cresolase [222]. However, the interpretation of such observations is complicated by the lag in cresolase activity and by the effect of mono- and diphenols, and of oxidizing and reducing agents on it.

Although the rate of oxidation of *para* substituted *o*-diphenols corresponds to the Hammett relationship, it appears that catechol oxidases from various sources show a preference for certain phenolic substrates. Yasunobu [153] concluded from a comparison of the substrate specificity of various catechol oxidases that although the enzyme could oxidize a wide range of phenolics, the individual enzymes tend to 'prefer' a particular substrate or a certain type of phenolic compound. In some cases, the preferred substrate is also the most abundant phenolic in the particular tissue [e.g. 93, 223–226]. Several authors reported the isolation of enzyme fractions or isozymes which differ in substrate specificity [58, 102, 172, 173, 178, 210, 227, 228].

Although most catechol oxidases do not oxidize quinol, resorcinol, pyrogallol or phloroglucinol, the enzyme from several tissues does oxidize some of these compounds. Thus the enzyme from tea leaves oxidizes quinol and *p*-phenylenediamine [179] and pyrogallol [229]. Abukharma and Woolhouse [230] reported that potato tuber catechol oxidase oxidizes quinol while the enzymes from *Monostroma* [39] and sugar beet [60] are able to oxidize pyrogallol. The possibility of artefacts due to coupled oxidation must, however, always be borne in mind. Another curious property of the enzyme from tea leaves has been reported by Coggon *et al.* [231]. A purified preparation of catechol oxidase, free from peroxidase and flavanol-gallate esterase activities was able to catalyse epimerization of tea flavanols at position C-2, independent of whether oxidation of the flavanols did or did not take place.

The affinity of plant catechol oxidases for the phenolic substrates is relatively low. The K_m is high, usually around 1 mM [57, 58, 93, 96, 180, 211, 225, 227, 232]. This value is higher than the values reported for fungi [e.g. 151, 155, 159] and bacteria [37]—*ca* 0.1 mM. However, several authors reported higher affinities for the phenolic substrates in catechol oxidases from some sources—0.01–0.1 mM in potato tubers [102, 140, 226, 230], cotton [174] and banana [93], with an exceptionally high affinity [K_m *ca* 1 μM] in *Papaver somniferum* [50].

The affinity of catechol oxidases to oxygen is also relatively low, similar to other copper containing oxidases [2, 233, 234]. The values reported are in the range 0.1–0.5 mM [39, 102, 151, 211, 230, 235]. The affinity for oxygen depends on the phenolic substrate being oxidized [57, 155] and could vary also among different forms of the enzyme isolated from the same tissue [58]. The reaction of catechol oxidase with H_2O_2 and the formation of 'oxytyrosinase' is discussed in the section on reaction mechanism.

Inhibitors

There are two main types of catechol oxidase inhibitors—reagents which interact with the copper in the enzyme and compounds which affect the site for the phenolic substrate. With some inhibitors of the first type it is possible to show competitive inhibition with oxygen, inhibition being non-competitive towards the phenolic substrate. The reverse is true for some inhibitors of the second type. Thus Duckworth and Coleman [155] showed, using mushroom catechol oxidase, that cyanide was competitive to oxygen while benzoic acid competed with the phenolic substrate. Competitive inhibition for both substrates by 2,3-naphthalenediol and diacetyl has been reported [236]. In addition to cyanide, inhibitors which act on the copper in the enzyme include diethyldithiocarbamate salicylaldoxime, K ethylxanthate and thiurea derivatives such as phenylthiourea. Other metal ion chelators, less specific for copper, also inhibit the enzyme in some cases—Na azide [210, 230] and EDTA [227]. 3,4-Dichlorophenylserine was reported to be a relatively effective inhibitor of catechol oxidase, being a specific reagent for copper [230, 237]. Catechol oxidase copper is sometimes relatively inaccessible to inhibitors. Enzyme activated by urea or detergents was markedly more sensitive to inhibition by copper reagents [238]. The inhibition of catechol oxidase by CO, contrary to the inhibition of heme-containing enzymes, is not reversed by light [191, 239, 240].

The inhibition of catechol oxidase by —SH compounds and other reducing agents is well documented. Apart from the effect of compounds such as bisulfite, thiosulfate, GSH and cysteine, there are reports on effective inhibition by relatively low concentrations of certain thio-compounds, e.g. complete inhibition by Na mercaptobenzothiazole at 5 μM which is, however, slowly overcome due to the apparent oxidation of the inhibitor [241]. It is generally assumed that these compounds too react with the enzyme's copper [242]. It should be remembered, however, that reducing agents can affect the enzyme reactions in several ways and that the observed effect would be dependent on the assay used.

As competitive inhibitors of the phenolic substrate can serve compounds which are slowly oxidized substrates, e.g. 4-nitrocatechol [35], *p*-nitrophenol [39, 210, 243], 4-chlorophenol [243] or compounds which are not oxidizable but resemble the substrate's structure (e.g. benzoic acid [155], OH-benzoic acid and other aromatic carboxylic acids [211, 244] and 2,3-naphthalenediol [22, 58, 111]). The interpretation of the mode of inhibition of catechol oxidase by various compounds is further complicated in relation to the cresolase and catecholase functions. Differential inhibition of the two activities by some compounds has been interpreted to indicate separate sites for mono- and diphenols [e.g. 219, 220]. However, as has been pointed out [7], the interpretation of such observations should take into account the multi-substrate nature of the catalysis and the consequent complexity of the observed kinetics (see also section on substrate specificity).

The kinetics of inhibition of catechol oxidase by a series of substituted cinnamic acids indicated that the cinnamic acid derivatives do not act on the site for the phenolic substrate but on a specific 'inhibitor site' [245]. A similar conclusion was reached earlier by Lerner *et al.* [221] regarding the inhibition by phenylhydrazine. The action of this inhibitor is dependent on the presence of oxygen and is relatively specific for catechol oxidase from several sources [221, 246]. SH-reagents may act as inhibitors [230, 247], but this is apparently not a general phenomenon [82] probably because catechol oxidases usually have a low content of cysteine.

Polyvinylpyrrolidone (PVP) adsorbs phenols and has

been widely used to protect plant enzymes from inactivation by phenolics and their oxidation products [131, 132]. It also acts as an inhibitor of catechol oxidase. Hulme *et al.* [248] suggested that PVP inhibits the enzyme by combining with a catechol oxidase-substrate complex, probably by attachment to the phenolic substrate moiety of such a complex. Harel *et al.* [57] observed that PVP and its monomer, *N*-vinyl-2-pyrrolidone, inhibited the enzyme irreversibly and were able to act in the absence of added substrate. Compounds structurally related to the monomer (e.g. *N*-vinylsuccinimide and *N*-vinylphthaleimide), also acted as inhibitors [Harel and Mayer, unpublished results]. Fusaric acid, a toxin excreted by *Fusarium lycopersicum*, is an effective inhibitor of catechol oxidase from various sources [249 and Harel and Mayer, unpublished results]. α-Picolinic acid and 5-ethylpicolinic acid which resemble fusaric acid in their structure, are less effective inhibitors. Fusaric acid is a chelator of copper cobalt and iron [250]. *N*-vinyl-2-pyrrolidone, fusaric acid and related compounds which inhibit catechol oxidase contain groups similar in structure to a peptide bond. This in turn might be related to the inhibition of copper enzymes by short chain polypeptides, which is discussed later in this section.

Inhibition by halides has been demonstrated for catechol oxidase from many sources [106, 118, 227, 243, 251]. Inhibition is pH dependent [118, 251] and may be caused by the formation of a complex between the halide ion and copper in the enzyme, which is stabilized at low pH values [118]. Other catechol oxidase inhibitors which have been described are borate [252] and quatenary NH_4^+ compounds [253]. The inhibition by chloride and by PVP are interesting from a commercial point of view for food processing [254] since most other inhibitors of catechol oxidase are toxic to man. Salicylhydroxamic acid has been shown to inhibit catechol oxidase at quite low concentrations [255].

There are several reports in the literature on 'natural' inhibitors of catechol oxidase which have been detected or isolated from various tissues. A low MW compound with an absorption peak at 280–290 nm isolated from cultures of *Penincillium expansum* inhibited apple and tobacco catechol oxidases but did not affect fungal laccase [256]. The inhibitor was excreted by the fungus into the host and disappeared towards sporulation. Harel *et al.* [257] reported that an inhibitor of galactose oxidase [258] isolated from cultures of *Dactylium dendroides* also inhibited mushroom and apple catechol oxidases. The inhibtor was a polypeptide of 13 amino acids, supposedly reacting with the copper in both enzymes. A short polypeptide isolated from *Agaricus hortensis* competitively inhibited the enzyme from the same tissue [259, 260]. It had a MW 1000–1200 and consisted of equivalent amounts of phenylalanine, aspartate and glutamate. The natural occurrence of protein inhibitors of catechol oxidase was reported by Karahanis and Frieden [261] and by Bull [151]. Nilova *et al.* [222] isolated a protein (34000 MW) from potato tubers which strongly inhibited cresolase activity of the enzyme from the same source.

pH optima

The pH optimum of most of the catechol oxidases studied is between pH 5.0 and 7.0. However, even a scanty survey of the literature reveals conflicting reports on the pH optimum of the enzyme from the same source—e.g. in potato tubers [140, 262] and in apples [57, 58, 232, 263]. A feature which recurs in many reports is the presence of two peaks, a peak and a prominent shoulder [57, 58, 95, 111, 114, 140, 230, 243, 264], or a wide optimum in the pH curve of enzyme activity [46, 102, 105, 210, 263]. There are also many reports on differences in the pH optimum, depending on the phenolic substrate being oxidized [102, 210, 230, 243].

Changes in the form of the pH curve during development, or as a result of changes in growth conditions or treatment of the isolated enzyme with various agents have often been reported. These include changes in the pH optimum upon aging of tissue cultures [265 and Volk, Harel and Mayer, unpublished], exposure of the tissue to stress conditions [Volk *et al.*, unpublished] and treatment of the enzyme with denaturing agents such as detergents [58, 118, 238], urea [266], elevated temperatures [122, 267] or a short exposure to acid pH [66, 266]. Such changes are sometimes accompanied by activation of the enzyme in the neutral to alkaline region of its pH curve. They might be explained by partial denaturation of the enzyme and/or conformational changes which result in shifts in its pH optimum. It is not unlikely that similar changes in the pH curve take place also *in vivo* during aging of the tissue, following release of a membrane bound enzyme to the soluble fraction of the cell during fruit ripening [105] or upon damage incurred by stress conditions.

Multiple forms

Reports on multiple forms of catechol oxidase started to flood the literature as soon as methods of protein fractionation such as ion exchange chromatography, gel filtration, gel electrophoresis and isoelectric focussing were introduced to study the enzyme. Most reports on catechol oxidase mention multiple forms or 'isozymes.' Vanneste and Zuberbühler [7] properly called the section dealing with multiplicity in their review: "heterogeneity—true or artifact", reflecting the main problem concerning the multiplicity of catechol oxidase and probably of many other enzymes. The heterogeneity which results from various degrees of aggregation of subunits has been dealt with in an earlier section. In addition, there is good evidence for differences in the primary structure between isozymes in mushroom catechol oxidase [181] including the characterization of different subunits [165], and in *Neurospora*, where the genetic basis for such differences has been studied [81]. True isozymes differing in primary structure occur also in higher plants [e.g. 119, 214], although reports based on a thorough examination are relatively few.

The discussion of multiplicity obviously includes arguments as to whether it is a 'native', genuine phenomenon or results from the release of membrane bound forms, partial denaturation, fragmentation, proteolysis, activation of latent forms, tanning reactions, etc. [119, 128, 147, 154, 179, 180, 181]. Interconversion between forms, apparently due to association–dissociation phenomena, have been reported for the enzyme from several sources [116, 148, 149]. Such interconversions, which occur spontaneously can be accelerated by factors such as ionic strength, concentration or dilution, the presence of certain ions, urea, etc., and by presence of the phenolic substrate [268–270]. The content of five hydrophobic residues (leucine, isoleucine, proline, valine and phenyl-

alanine) in mushroom catechol oxidase is 30% [181]. According to Van Holde [271], a hydrophobic residue content of 30% is the approximate point of overlap between the single and multichain structure in globular proteins. Jolley *et al.* [181] suggested that such an overlap is responsible for the dissociation–association phenomena, a view supported by calculations based on the relative side-chain hydrophobicities [272, 273]. These suggestions have implications with regard to catechol oxidases in general, considering the close resemblance in amino acid composition in enzymes from various sources.

A great deal of attention has been paid to differences in the properties of multiple forms of the enzyme and to the possible 'physiological' significance of such differences. These include differences in affinity and specificity to phenolic substrates and to oxygen [58, 172, 178, 213, 227, 274], sensitivity to inhibitors [58, 178, 227, 228], pH optimum [227, 229] and inactivation by heat [82, 83, 118, 141, 228]. Differences in 'isozyme pattern' have been reported also in connection with subcellular organelles [58], the stage of development of the tissue [63–65] and as a result of attack by pathogens [275, 276] or of treatment with plant hormones [277, 278]. Although such differences in 'isozyme patterns' might have a physiological significance, the establishment of such significance requires further evidence.

Latency, activation and induction

Latency and activation of plant catechol oxidases were first studied by Kenten [122, 267] in *Vicia faba*. Activation was achieved by short exposure to acid (pH 3.0–3.5) or alkali (pH 11.5) or by incubation with ammonium sulphate at pH 5.0. The activation was ascribed to the removal of an inhibitory protein which was assumed to be attached to the enzyme. Activation by anionic detergents was interpreted to act by combining with cationic groups of the enzyme which result in dissociation of the 'enzyme–inhibtor complex' [122]. Latency due to combination of the enzyme with a diffusable inhibitor was suggested in spinach chloroplasts [279]. Mayer and Friend [280] and Mayer [67] observed that various detergents caused activation of membrane bound catechol oxidase in sugar beet chloroplasts without causing solubilization of the enzyme. Solubilization and activation were brought about by water saturated with butanol and by limited proteolysis with trypsin or carboxypeptidase [106, 121]. A 30-fold activation due to treatment with trypsin was observed by Tolbert [96] in isolated chloroplasts. He concluded that the activation was not due to proteolytic action since it took place even with denatured trypsin preparations. Activation by detergents has been observed also in mushroom catechol oxidase [281, 282]. It should be noted, however, that what might be considered as 'induced' latency is frequently observed after preparing acetone powders [118, 282]. Such latency might well be a result of the acetone-treatment rather than a native property of the enzyme.

The activation of broad bean catechol oxidase [122, 267] was further studied by Swain *et al.* [238]. They interpreted the effects of detergents and short exposure to acid, alkali or urea as involving a limited conformational change rather than dissociation or aggregation. Activation was reversed upon removal of the denaturing agent and did not involve a change in the sedimentation behaviour of the enzyme. Conformational changes as the cause of activation were also suggested by Lerner *et al.* [266]. They demonstrated that a short exposure to acid pH or urea caused an up to ten-fold activation within 1–3 minutes. The activation was due primarily to an increase in the V_{max} while affinity for the phenolic substrate decreased and that for oxygen increased. The process was reversible and could be repeated several times with the same preparation. Lerner and Mayer [283] further showed that activation was accompanied by a change in the Stoke's radius of the enzyme indicating the involvement of a conformational change. Conformational changes caused by a long exposure to pH 2–3 were observed also in purified mushroom catechol oxidase [155]. However, these changes were not reversed by dialysis against pH 7.0 buffer. Irreversible activation which apparently involves conformational changes were observed in grape catechol oxidase [266, 283] following long exposures to acid pH or urea. The activation of *Podospora anserina* catechol oxidase by heat was accompanied by changes in the electrophoretic mobility of the enzyme with no change in its sedimentation behaviour [138].

Induction of catechol oxidase due to *de novo* synthesis of the enzyme has been extensively studied in *Neurospora* by Horowitz and coworkers. The enzyme, which normally appears in the reproductive phase of the life cycle, could be induced when vegetative growth was inhibited by starvation, metabolic inhibitors, or inhibitors of nucleic acid and protein syntheses including amino acids analogues [80, 84, 85, 284, 285]. *De novo* synthesis of the enzyme [81, 82, 85, 285] was inhibited by cyclohexamide, actinomycin D and puromycin [86]. The effects of various inducers, inhibitors of nucleic acid and protein syntheses and the behaviour of mutants were interpreted to indicate derepression of catechol oxidase synthesis through interference with the formation of a rapidly turning-over protein repressor [84–86]. Feldman and Thayer [286] demonstrated that cyclic-AMP and inhibitors of cAMP-diesterase induced catechol oxidase synthesis in *Neurospora*. They suggested that induction by cyclic-AMP is related to the induction phenomena observed by Horowitz and coworkers.

The increase in activity or appearance of new isozymes in higher plants has also been ascribed to *de novo* synthesis. The rise in enzyme activity following wounding of Jerusalem artichoke tubers was inhibited by inhibitors of nucleic acid and protein syntheses [287]. Hyodo and Uritani [288] reported that the increase in catechol oxidase activity during the incubation of sliced sweet potato tissue was inhibited by actinomycin D, puromycin and blasticidin S. They concluded that the rise in enzyme activity resulted from *de novo* synthesis. 2-Thiouracil inhibited the rise in catechol oxidase activity associated with acquired resistance in virus infected soybean [289]. Synthesis of the enzyme has also been suggested to be involved in the 'induction' of catechol oxidase in germinating wheat seeds [278], which was affected by actinomycin D or cycloheximide. However, claims on the involvement of *de novo* synthesis should obviously be based on evidence more substantial than the effect of inhibitors of nucleic acid and protein syntheses. Appearance of, or marked rises in catechol oxidase activity following infection [290, 291] or wounding [292, 293] could be caused by activation rather than through re-synthesis. Infection by *Botrytis* was said to cause activation of the latent catechol oxidase in leaves

of *Vicia faba* [290]. The effect of *Fusarium* in increasing enzyme activity in tomato plants could be imitated by the use of detergents [291]. Activation was probably involved also in the increase in catechol oxidase activity observed within 10–20 hours of the exposure of apple tissue culture to low relative humidity [74].

A curious phenomenon of inactivation of catechol oxidase was observed by Volk *et al.* [75] in suspension cultures of apple fruit. The addition of 0.5–1.0 mM ethionine, which induces enzyme synthesis in *Neurospora*, caused a rapid disappearance of catechol oxidase activity in apple cell suspension cultures. This disappearance took place even if ethionine was removed after 4 hours and the activity could not be restored by the addition of methionine [Volk, Harel and Mayer, unpublished results]. Similar, though less rapid decreases in catechol oxidase activity were observed in tissue cultures following the addition of an antiauxin or ethylene, as Ethrel [75]. Such inactivation of catechol oxidase might have practical applications.

REACTION MECHANISM

The mechanism by which catechol oxidase reacts with its substrates is complicated by two problems. First it is clear that the cresolase reaction involving apparently a hydroxylation reaction differs from the oxidation of the *o*-diphenols, the catecholase reaction. Secondly, as already stated, the copper in catechol oxidase does not give an EPR signal. Consequently, it is much more difficult to follow the reaction of copper. The enzyme is also devoid of an absorption peak in the visible spectrum, so that the spectrophotometric analysis is also more complicated.

For long it was maintained by some authors that the hydroxylation reaction was non-enzymatic and only the dehydrogenation reaction was enzymatic. This view has been proposed by Onslow and Robinson [294] and by Keilin and Mann [24] and vigorously advocated by Kertesz and Zito [192, 295]. However, the evidence is overwhelmingly against this view. The salient arguments have been summarized [7, 203] and need not be repeated here. The state of copper in the enzyme is still not satisfactorily described. The fact that catechol oxidase is inhibited by CO and reagents for monovalent copper would indicate that copper is present as Cu^+ [12, 192] but the work of Jolley *et al.* [296] raises some doubt on this point. These workers showed that the resting enzyme reacts with hydrogen peroxide to eventually form enzyme containing reduced, i.e. cuprous, copper and oxygen. Nevertheless, Cu^+ is obviously involved at some stage of catalysis [162]. Most of the the copper is present as a pair of antiferromagnetically coupled Cu^{2+}; it was possible to obtain an EPR signal for the copper following acid denaturation under anaerobic conditions [164]. In marked contrast, *Neurospora* catechol oxidase of 33000 MW and one copper per molecule can oxidize phenolic substrates [158]. These findings show that in this case there is no concerted attack of several metal ions during oxygen reduction, and that a single copper ion is sufficient. No aggregation of subunits is needed for activity. Lerch [166] reinvestigated the same enzyme and claims that the MW determined by Guttridge and Robb [158] is incorrect, being based on wrong extinction coefficients of the protein. He assigns a MW of 42000 with 2 Cu/mol. This leads to the view that a pair of copper ions is required for oxidation of substrate. On the basis of EPR studies a copper pair was also shown in *Neurospora* enzyme by Deinum *et al.* [188]. That the monomer of the enzyme is active also emerges from other work [58, 116, 141, 171, 192]. If all monomers contain a copper pair, all problems can be resolved. However, it is also possible that monomers undergo aggregation in the presence of phenolic substrate [268–270], leading to an 'effective copper pair". even if the monomer only contains a single copper.

Makino *et al.* [164] suggested that at the active site a Cu-disulphide pair complex might be present, but no experimental evidence is available for this view. The recent revision by Mason's group of the quaternary structure of mushroom catechol oxidase will lead to a reassessment of many of the proposed mechanisms of copper binding [165]. The disulphide bridge seems no longer tenable and whether copper is located on the light or heavy subunit or both is still unknown. Some evidence for the necessity of histidine for catechol oxidase activity, based on photochemical oxidation of the enzyme, was recently provided [297]. Thus although the presence of copper in catechol oxidase is not disputed, and its participation in the oxidation reaction is well established, just what happens with the copper is not adequately described. The copper in the resting enzyme can apparently be either oxidized or reduced or bound to peroxide [7]. The enzyme can catalyse a pseudo-peroxidase reaction [298]. It has been suggested that the copper in catechol oxidase also plays a role in determining the stereospecificity of catechol oxidase toward D and L *o*-diphenols such as dopa, adrenaline and noradrenaline [197]. A control site binding to the side chains by chelation of copper was supposed to function as a reaction rate modulator. It should be recalled that practically all the studies on the changes in the state of oxidation of copper are based on studies using mushrooms (*Agaricus*) catechol oxidase. No comparable studies on higher plant catechol oxidase have been carried out, apart from the demonstration that the spinach beet enzyme reacts with H_2O_2 to form an oxygenated enzyme absorbing at 340 nm [299].

The mechanism of the hydroxylation reaction has been discussed by many workers and several schemes have been put forward [1, 7, 190, 203, 220, 300]. The question of an intermediary *o*-diphenol formed during the cresolase reaction has been discussed repeatedly. The problem, which relates to the reaction kinetics, is whether the diphenol is an intermediary step which is released from the enzyme or whether the reaction goes directly to completion to give an *o*-quinone. It has been argued that the diphenol, if formed, does not normally appear in the free form [190, 301]. However, this question is extremely difficult to resolve and other workers argue in favour of the release of *o*-diphenol from the enzyme. Thus, McIntyre *et al.* [300] demonstrated the conversion of *p*-coumaric to caffeic acid by spinach beet catechol oxidase and proposed a kinetic mechanism from this. This question is closely related to a further unresolved problem: does the enzyme have separate binding sites for mono- and diphenols? Dressler and Dawson [190] on the basis of [$^{64}Cu^{2+}$] exchange studies in the presence of either of the substrates argue in favour of separate binding sites. Inhibition studies on potato tuber [206] and grape [221] catechol oxidases also favoured the view that two distinct binding sites exist. *Streptomyces* catechol oxidase also behaved as if two binding sites were present

[35] while Guttridge and Robb [158] hold the view that there is a single binding site in the *Neurospora* enzyme. It is also well known and frequently observed that the catecholase/cresolase activity ratio can vary depending on the source of the enzyme, its mode of preparation and on whether monomer, dimer or tetramer structure is involved. It is difficult to reach any satisfactory conclusion on this point. In a recent review it has been argued that most of the evidence is consistent with a single binding site [7], but too few data on various catechol oxidases are available to justify this conclusion.

The onset of cresolase activity is often preceded by a lag period and electron donors, including *o*-diphenols, shorten or abolish this lag. It is not clear whether this lag is inherent in cresolase activity or an artefact due to changes induced in the enzyme during preparation and isolation.

Only in isolated cases have detailed kinetic analyses of catechol oxidase been carried out and only in very few species. The first detailed study was with the French prune enzyme [302]. It was concluded in this early study that O_2 must bind first, although the K_m for O_2 depended on the phenolic substrate. Tea catechol oxidase was one of the first to be studied in detail [179]. This enzyme is anomalous as it slowly oxidizes quinol and the purified form is blue. The enzyme might be intermediary between 'normal' catechol oxidase and laccase. Its kinetics indicate that the oxidized and reduced states of the enzyme form binary complexes with phenol or O_2, respectively, followed by formation of a ternary complex by addition of the second substrate. From this ternary complex the product is released. Cleland kinetics were applied to *Streptomyces* catechol oxidase [35]. This enzyme again differs somewhat from fungal catechol oxidase. Kinetic analysis indicated that k_{cat} and K_m were dependent on the oxygen concentration when *o*-diphenol was the substrate, but not when a monophenol was oxidized. No detailed reaction mechanism was suggested. Mushroom catechol oxidase was studied by Duckworth and Coleman [155]. Again during oxidation of *o*-diphenol it showed differences in K_m for O_2 depending on the *o*-diphenol substrate. Two binding sites were proposed. It is pointed out that oxidation of monophenols is difficult to analyse kinetically. These authors propose a quaternary complex of enzyme, oxygen and two molecules of catechol. This seems a rather unlikely event. However, the authors present the first detailed kinetic study of the mushroom enzyme. The kinetic analysis of *Neurospora* catechol oxidase showed random binding of phenol and O_2 [158]. Another enzyme whose kinetics have been studied is catechol oxidase from spinach beet [300]. In this case kinetics of the hydroxylation of *p*-coumaric acid were analysed. The suggested sequence was binding of O_2 to reduced enzyme, binding of *p*-coumaric acid, followed by release of caffeic acid and oxidation of the enzyme. The caffeic acid is then supposed to be bound again and the quinone formed and released, binding being at cofactor site. The cofactors, probably acting as electron donors, are suggested to produce catalytic amounts of caffeic acid. The need to involve a cofactor in this reaction complicates the reaction and as stated by the authors the kinetics may be more complex. Catechol oxidase of grape chloroplasts has also been studied kinetically according to Cleland kinetics. The proposed sequence is: first, random binding of an oxygen and an *o*-diphenol molecule followed by release of a product molecule prior to binding of the second *o*-diphenol molecule. The second product molecule is then released [235]. The reaction rates are consistent with a bireactant mechanism, since the first sequence is rate limiting. Oxidation of monophenol could not be analysed kinetically, but the results indicated that the mechanism differed from that for *o*-diphenol. In potato catechol oxidase O_2 is supposedly the first substrate to be bound to the enzyme [303].

During the oxidation of diphenols, the ease of abstraction of electrons from the substrate determines the rate of their oxidation. A study of the rate of oxidation of various substrates shows that this observes Hammett's relationship reasonably well [57, 155, 198, 199] (see also above). Vanneste and Zuberbühler [7] pointed out that such relationships oppose those expected if the phenol were to bind coordinately with copper, but would properly fit formation of a charge transfer complex with an electron donating amino acid residue of the protein.

It is somewhat surprising that despite the many detailed studies on catechol oxidase, its reaction mechansim is still so unclear. It must be remembered that it is not always certain what form of the enzyme is being studied. As discussed in the previous section, catechol oxidase can undergo monomer–dimer–tetramer transitions. It is not always certain which of the forms is being investigated from the point of view of mechanism. Possibly, although this is unlikely, the mechanism or binding of the substrate changes during during transition. Furthermore, it is important to remember that catechol oxidase can undergo conformational changes induced by the substrate O_2 and by pH [266, 283]. The changes in conformation were accompanied by changes in the K_m of the enzyme for both its substrates, oxygen and diphenol. Thus, if we postulate that binding of a substrate induces a conformational change in the enzyme, which in turn changes the binding of the second substrate, many of the discrepancies found in the literature on the mechanisms, number of binding sites and substrate dependent K_m can probably be accounted for. It would undoubtedly be very important to establish that the behaviour shown to exist for the grape enzyme is much more general in nature.

In considering the reaction mechanism of catechol oxidase, it must also be remembered that the enzyme undergoes inactivation during the oxidation of diphenols. This inactivation depends very much on the substrate oxidized. It is very rapid when catechol is being oxidized but the enzyme is quite stable during the oxidation of, for example, 4,5-dimethylcatechol. It has been shown by Wood and Ingraham [304] that the quinone formed during the oxidation reaction undergoes a Michael reaction with an amino group of the enzyme, leading to irreversible binding. This binding becomes impossible if the 4 and 5 positions of the substrate are blocked. This view has been confirmed by Brooks and Dawson [6] who conclude that Cu is lost from the enzyme at the same time. Inactivation is less marked or altogether absent in the cresolase reaction.

Lastly, in considering the mechanism of reaction of catechol oxidase the question of latency must be recalled. The changes in the enzyme, which transform it from latent to active, have been considered in the previous section. They are obviously an important feature of this enzyme. The changes occurring are such that binding

properties change radically. Such changes are obviously relevant to the question of mechanism, but have not yet been studied adequately from this point of view.

PHYSIOLOGICAL FUNCTION

The physiological function of catechol oxidase in plants and fungi can be considered in several ways—in the fungus, in the plant and in fungal infection of plants. As will be seen below, there is a disturbing lack of clarity in all three areas. This lack of clarity is particularly disturbing because catechol oxidase is one of the oldest enzymes known. Its general properties are well known and its distribution is virtually universal. It may be recalled that this enzyme is known throughout all living organisms although we are only considering here its presence in plants.

Fungi and bacteria

As shown above, catechol oxidase is present in every or almost every fungus examined as well as in bacteria. The level of the enzyme in fungi seems to change during development and frequently its formation correlates with formation of fruiting bodies, e.g. in *Hypomyces* [305], in *Schizophyllum* [78, 260, 306], in *Agaricus* [307], and in *Aspergillus* [308]. Wilson [305] reported that the addition of mushroom tyrosinase to the growth medium of *Hypomyces* markedly increased the number of perithecial primordia formed by the fungus. Mutants of *Penicillium*, which did not form cleistothecia when grown on a minimal medium, formed fruiting bodies when tyrosine and $CuSO_4$ were added to the medium [79]. The recovery of 'fertility' was accompanined by the appearance of two phenol oxidases which were present in the wild type but were absent from the 'infertile' mutants. However, in *Aspergillus* and probably in most fungi the presence of catechol oxidase is not confined to one specific phase of development. Furthermore, although spore formation is often accompanied by melanin formation, which is mediated by catechol oxidase, melanin formation is not essential for fructification. This has been shown, for example, in sclerotia formation [309]. In *Sclerotium* the number of isozymes of catechol oxidase increased during formation of fruiting bodies [310]. The correlation often observed between enzyme activity and melanin formation has prompted various authors to assign the function of melanin formation to catechol oxidase. It has been suggested that the importance of melanin is to increase resistance to lytic enzymes particularly those originating in bacteria [309, 311, 312]. The cell walls of a melanin-less mutant of *Aspergillus* were much more susceptible to attack by bacterial glucanase and chitinase [311]. Although such a view seems reasonable one would like to see much more convincing evidence, referring to many more organisms, before accepting it. An entirely different view has been ascribed to *Aspergillus* catechol oxidase by Bull and Carter [151]. These authors claim that the enzyme is important in oxidizing extra-mitochondrial dinucleotides under aerobic conditions. We will see that similar suggestions have been made for plant catechol oxidases but the evidence is not really very convincing. Apart from the suggested role of catechol oxidase in dopa and melanin synthesis, its involvement in the biosynthesis of specific metabolites has also been implied, e.g. in the synthesis of the actinomycin chromophore [313].

Inadequate attention has been given to the very elaborate control of catechol oxidase in fungi. A detailed model for the control of enzyme formation has been presented [86] which suggests that an unstable repressor protein is operating. The *Neurospora* model is further complicated by the apparent involvement of cyclic AMP, acting as an inducer of synthesis [286]. In addition to the apparent genetic control, and the control by inducers such as phenolic substrates [92], the presence of specific endogenous inhibitors of polypeptide nature has also been reported [151, 259–261]. The delicate and elaborate control of both the level and activity of catechol oxidase in fungi would lead one to suppose that it will be very difficult to assess their role merely by assaying enzyme activity in extracts. Such activity will not take into account control mechanisms involving inhibitors. Moreover, activity in extracts may be modified by factors previously absent or in contact with the enzyme. Inhibitors or activators may be modified *in vitro*, changing the entire expression of enzyme activity. Such factors have in our opinion been inadequately considered when ascribing a physiological role to catechol oxidase in fungi.

Plants

Any attempt to ascribe a physiological function to catechol oxidase in green plants, from the algae to higher plants, must take into account a number of properties of the enzyme. These properties are: (1) its subcellular location—the enzyme may be particulate or soluble and it often appears in several subcellular fractions; (2) the phenomenon of latency; (3) the activation of the enzyme under certain conditions due to conformational changes; (4) the presence of native inhibitors; (5) the enormous variation in enzyme level at different periods of growth and development; (6) the separation of the enzyme from most of its substrate due to compartmentation, cellular or subcellular. A possible interpretation of these very diverse properties is a formidable task.

One of the oldest suggestions of a physiological role is that of synthesis of *o*-diphenols. This suggestion is based on the undoubted ability of many catechol oxidase preparations to oxidize monophenols to the corresponding *o*-quinone. In this regard there are two crucial questions to be answered. First, why is the ratio between the so-called cresolase to catecholase activities so very variable? Often cresolase activity is low or absent and generally it is much lower than catecholase activity. Secondly, is the *o*-diphenol ever released as a distinct product during oxidation of monophenols? This aspect has already been discussed with regard to the reaction mechanism. Under strongly reducing conditions, e.g. in the presence of excess ascorbic acid, the formation of *o*-diphenol can easily be demonstrated. The question then is whether such conditions could prevail under normal physiological conditions. This seems to be very doubtful, although not impossible. An alternative possibility could be that the monophenol is oxidized to the corresponding *o*-quinone which is then reduced by a specific quinone reductase. No such enzymes have been shown to exist in higher plants although they do exist in certain fungi [314]. Thus, although plant catechol oxidases have been shown to hydroxylate naturally occurring phenolics, e.g. flavonoids in spinach beet [315], the physiological significance of these observations was not established. In *Vicia faba*, it was shown that catechol oxidase is not involved in dopa synthesis [70] nor was it active in the synthesis of

flavone glycosides in cell suspension cultures of parsley [316]. It has been claimed that catechol oxidase activity correlates with alkaloid content [317], but attempts to show that catechol oxidase is required for, or involved in, synthesis of morphine were unsuccessful [51, 318]. Elstner *et al.* [319] suggested that catechol oxidase and dopamine participate in ethylene formation by sugar beet leaves, following wounding. The enzyme was claimed to be involved in the formation of the superoxide radical required for the formation of ethylene from methional. This claim requires further verification. A biosynthetic role of catechol oxidases seems therefore doubtful.

A second long suggested role of catechol oxidases is in electron transport. Since quinones are powerful oxidizing agents it is easy to imagine a reaction in which a diphenol is oxidized by catechol oxidase and the quinone then reduces some cell constituent, such as a nucleotide, non-enzymatically. Long ago Kubowitz [191] claimed to have demonstrated such a reaction and since then catechol oxidase has been suggested to be involved one way or another in aerobic respiration [e.g. 3, 168, 320–322]. There is really nothing to suggest that the non-specific, non-enzymatic reduction of quinone is a functioning, normal physiological process active in electron transport. It has been often claimed that the cyanide-insensitive respiration of plant tissues might be mediated by catechol oxidase. Against this suggestion is the relatively high sensitivity of catechol oxidase to cyanide, although admittedly it is much less sensitive than cytochrome oxidase. However, a very interesting finding by Rich and Bonner [255] demonstrates that substituted hydroxamic acids are powerful inhibitors of catechol oxidase from mushrooms. Apple catechol oxidase is similarly affected [Mayer, unpublished]. The substituted hydroxamic acids are known inhibitors of the cyanide insensitive, or alternate electron transfer pathways. These results suggest for the first time that catechol oxidase may after all have some function in this pathway, at least in certain cell fractions such as the mitochondria.

The presence of catechol oxidase in chloroplasts, which has been known for a long time, is particularly puzzling. The enzyme is more or less tightly bound to the thylakoid membranes and is released from them by various treatments, such as detergents or tryptic digestion. The release is closely connected in some cases with the phenomenon of latency and generally the enzyme is activated following its release from the membrane (see section on latency). There is at present no evidence to show that release from membranes, activation of the enzyme and its solubilization are phenomena which occur during normal plant growth and development, particularly in the chloroplasts. It should be recalled that enzyme activation may involve pH-induced conformational changes in the enzyme structure [221, 266] and that O_2 can also induce such changes. Oxygen concentration and pH are factors which change within chloroplasts during their normal function. In contrast the well known activations induced by detergents [119, 238, 280] are not normal metabolic events. Whether in a functional chloroplast the enzyme may be released or activated by proteolytic enzymes is doubtful. Therefore, the tryptic activation of the enzyme, which also may involve conformational changes, may be an experimental artifact [96, 106, 112, 121]. Nevertheless, the catechol oxidase is present in a well defined membrane fraction of the thyalkoid having a rather high Chl a/b ratio [112] which is not, however, identical with the fraction having maximal PS I activity. Thus although the enzyme is present in chloroplasts, and although conformational changes may occur in them, there is at present no proof or even any indication that catechol oxidase is involved in any way in photosynthesis. The enzyme has a low affinity for oxygen. One might then suggest that it acts only when the oxygen concentration in the chloroplast is high, but even then it is unclear what it might be doing. Possibly it may bind oxygen transiently, thus reducing its concentration, or it might be involved in the outward release of oxygen, when activated. This is at present no more than speculation. Possibly a photosynthetic metabolite might partially control latency. A low MW compound has been described which reacts with catechol oxidase giving an inactive complex [279]. Such a substance might arise during metabolism. In addition it has been reported that 2,3-dihydroxybenzaldehyde induced association of the monomer present in chloroplasts to a dimer with change in activity [270]. Again, a similar process might occur *in situ*. Nevertheless, whether activity is controlled by conformational change, by activation or inhibition or by changes in the membrane, the purpose of such events remains to be uncovered.

The involvement of catechol oxidase in affecting the regulation of plant growth has been implied in various ways. Gordon and Paleg [323] demonstrated that quinones can interact with tryptophan to form, ultimately, IAA. They suggested, however, that the reaction occurs only in wounded tissues. Various plant phenolics have been shown to affect IAA oxidation [e.g. 324] and the destruction of auxin catalysed by catechol oxidase was demonstrated in plant extracts [325–327]. However, it appears that the involvement of catechol oxidase in determining auxin concentration *in vivo* is rather limited if not doubtful [e.g. 328, 329]. Catechol oxidase appears in carrot tissue cultures prior to root formation. Its appearance, possibly cAMP induced, might be related to differentiation [330, 331].

It is a special characteristic of many if not all plant tissues that phenolic substances, which act as substrates for catechol oxidases, are either sequestered in special cells, or in the vacuole, away from the enzyme. It is a common observation that enzyme activity and the amount of possible substrates do not change together. Often one increases, while the other decreases or the changes in them are not in phase [61, 62, 105]. Thus it is obvious without further consideration that catechol oxidase is not involved in the oxidation of, or reaction with, the bulk of the phenolic compounds present in intact tissues. It is possible that in intact tissues the normal substrate is only a small fraction of the total which may well be located in the tissue so as to be available to the enzyme. Present analytical procedures, which analyse only total phenolics or the total of the different phenolics present, without considering subcellular location, cannot give any clue to the correctness of the above suggestion.

A new unexpected role of catechol oxidase may be in reactions which render seed coats impermeable to water. In seeds in which the enzyme is absent the coats are permeable, while its inhibition prevents darkening and loss of water permeability [332–334].

Following injury of plant tissue, especially mechanical injury, the tissues rapidly brown or blacken. This reaction is catalysed by catechol oxidase. In it at least two or

three distinct reactions may be involved. The oxygen tension at the active site of the enzyme may increase, the sequestered phenols become accessible to the substrate, and latent enzymes may become activated, possibly due to limited proteolysis, aggregation reactions or conformational changes. The critical question remains what benefit does the darkening reaction confer on the tissue. Possibly it might promote wound healing, temporarily prevent or reduce infection or decrease the rate of infection. Again it is difficult to understand why such a very delicate and complex mechanism is operating to achieve what appears to be a rather limited aim. These questions have prompted many workers to study the possible function of catechol oxidases in relation to the plant–fungal interaction which we will next consider.

Interaction between higher plants and fungi

The presence of phenolic compounds in plants, their oxidation following injury, either mechanical or due to infection and the relatively high toxicity of the oxidation products have long drawn attention. The possible relationship of these properties to plant resistance to disease has prompted many research workers to ascribe a role to catechol oxidase in disease resistance. The subject has been reviewed a number of times [335–338]. Kosuge [339] concluded that in most cases there was inadequate evidence to show that catechol oxidase plays a significant role in disease resistance. From the review of Kosuge and the articles discussed by him the following general conclusions can be drawn. Catechol oxidase does indeed increase in activity following infection by virus, bacteria fungi or mechanical injury. The increase may be due to activation of host latent enzyme, due to solubilization of host catechol oxidase which is normally particulate or even due to *de novo* synthesis. Even nematode infection may induce increased catechol oxidase activity [340]. The increase in catechol oxidase activity can be induced by treatment of potato tubers with purified endopectate transeliminase, but freezing and thawing of the tubers had the same result [341]. Release of latent catechol oxidase in tomato plants infested by *Fusarium* [291] and in *Vicia* by infection with *Botrytis* [290] has also been demonstrated. An increase in non-particulate enzyme has been shown following *Phytophthora* infection of potatoes [342]. However, both phenolic content and peroxidase and catechol oxidase activities were higher in tomato cultivars susceptible to *Verticillium* attack, compared to resistant ones. Moreover, increases in phenolic content and phenol oxidizing enzymes correlated with the amount of viable fungus observed in infected plants [343]. In potato tubers, no correlation was found between the level of catechol oxidase and resistance to either blackspot [344] or rot caused by *Pectobacterium* [345]. All these support the general conclusions reached by Kosuge [339]. Kosuge also concluded that generally speaking quinones are quite toxic to extracellular enzymes produced by pathogens, due to their great reactivity. Thus catechol oxidase may function by producing such quinones. However, the oxidase itself is rapidly inactivated by its product, a factor often ignored by research workers. In favor of the view that the oxidation reaction is important are the observations that reduction of the quinones increases infectivity and decreases resistance [346] and that inhibition of catechol oxidase decreases resistance [347]. Host resistance in *Ribes* to the pathogen *Sphaeotheca* is said to depend on the levels and interaction of catechol oxidase, chlorogenic acid and a reducing agent such as ascorbic acid [348]. It must, however, be remembered that the quinone is not always the toxic compound. Thus in the case of pear trees infected by *Erwinia*, it is the quinol produced from arbutin which is toxic while *p*-quinone rapidly polymerizes with loss of toxicity [349]. This would imply that the pathogen could itself produce an enzyme to detoxicate the protective compounds produced by the host. This type of defensive mechanism may be quite common. Catechol oxidase or laccase produced by the pathogen may in certain cases have such a role [350]. Thus, phenylthiourea and related inhibitors of copper enzymes have been shown to act as systemic fungicides [351]. The role of catechol oxidase in hypersensitivity has also been frequently discussed. It seems to become increasingly clear that the necrotic reaction is a secondary one and not the prime cause of hypersensitivity [352, 353]. Catechol oxidase apparently has no immediate role in the reaction. The mycorrhizal endophytic infection of orchid corms has been suggested as a model for studying host–pathogen interactions [354]. In this case the spread of the endophyte is limited by the host reaction, which among other things includes a very marked rise in catechol oxidase activity.

Perhaps the most crucial question which must be asked is: do resistant and susceptible lines of the same species differ in their reaction to infection and does the catechol oxidase react in the way expected from its supposed role in resistance? The answer in most cases seems to be ambiguous. Hyodo and Uritani [276] showed that in sweet potato slices washing alone could produce increases in catechol oxidase, apparently by *de novo* synthesis. Nematode infection caused equal increases in the enzyme in both susceptible and resistant lines of tomato [340]. However, in millet leaves catechol oxidase was found to be more active in the resistant lines. The oxidized phenols were shown to inhibit germination, growth and pectolysis by the pathogen *Helminthosporium* [355] as well as in *Verticillium* [356]. In injured sugar beet leaves damaged by *Lygus* (Hemiptera), catechol oxidase activity rose 1–3 days after injury. This would seem to be a rather delayed reaction which cannot afford resistance [293]. Again in apples *Erwinia* infection induces a delayed increase in catechol oxidase, both in virulent and avirulent infection [357]. In *Setaria italica*, the susceptible lines had a higher catechol oxidase content than the resistant ones [358], but supposedly tomato roots resistant to *Fusarium* showed an increase in catechol oxidase activity following infection which was absent in susceptible ones [359]. The enzyme pattern was the same in both varieties. Increased lignification of potato tubers following infection by *Phytophthora* did not involve catechol oxidase activity [360]. Increased activity of catechol activity in infected musk melons could be shown to be due entirely to enzymes originating in the infecting fungus *Fusarium* [361]. It has been claimed that the normal browning reaction of apples is suppressed by the infecting fungus *Penicillium* [362].

As we survey the literature, the ambiguity about the function of catechol oxidase in disease resistance is brought home forcibly. The present picture certainly does not preclude a role in disease resistance, but it also fails to prove such a role. One is tempted to speculate that the tests made so far are of the wrong kind. Generally, quite long term effects in the enzyme have been studied.

Perhaps the very rapid release or change in activity modifies other metabolic pathways in the host cell. It might be these which are significant. To go further than this at present would be wild speculation, not based on any known data.

LACCASE AND ITS DISTRIBUTION

Since its first detection by Yoshida [363] and Bertrand [26] in the genus *Rhus*, laccase from the lacquer trees has attracted much attention. The enzyme is easily extracted, stable and readily purified. Laccase was later detected in a number of fungi including *Lactarius*, *Polyporus*, *Aspergillus*, *Pleurotus*, *Polystictus*, *Psalliota*, *Glomerella*, *Podospora*, *Botrytis*, *Neurospora* and *Russula* (see also Franke [364]). Laborde [365] was probably the first to describe its presence in fungi. Although the early workers were well aware of the distinction between laccase and catechol oxidase, insufficient attention was paid to this question in subsequent reports on the presence of laccase in tissues of higher plants. The use of impure preparations containing endogenous phenols would cause a coupled oxidation of *p*-diphenols by catechol oxidase leading to the erroneous conclusion that a laccase was present. The classical, elegant work of Keilin and Mann [24, 366] provided the tools for distinguishing between the two activities. Catechol oxidase is inhibited by CO while laccase is not. It is fairly clear today that laccase is present in very many fungi, but insufficient data are available to draw conclusions about any possible taxonomic significance about their occurrence. They are present in Ascomycetes, Basidiomycetes and Fungi Imperfecti. In contrast, authentic reports on laccase in higher plants are much more limited. The genus *Rhus* has been investigated in considerable detail [367]. Joel *et al.* [368] recently reported that laccase occurs in the cavity of the secretory ducts of all the members of the Anacardiaceae, but at least in mango fruit it is absent from the rest of the tissue. The Burmese lacquer tree, *Melanorrhea*, which also contains a laccase [366], also belongs to this family. The other authentic proof of laccase in higher plants is the report of its presence in peaches [22, 369, 370], and in *Aesculus* [371]. A *p*-diphenol oxidase, which may be a laccase, has been reported to be present in many gymnosperms especially in species of the Podocarpaceae. Although the enzyme was prepared from *Cryptomeria*, it cannot unequivocally be regarded as a laccase [47]. Other reports of the alleged presence of laccase in higher plants should be treated with some reservation, including the listing of Franke [364]. A list of fungi of the Agaricales containing laccase is given by Harkin *et al.* [372].

SUBCELLULAR LOCATION

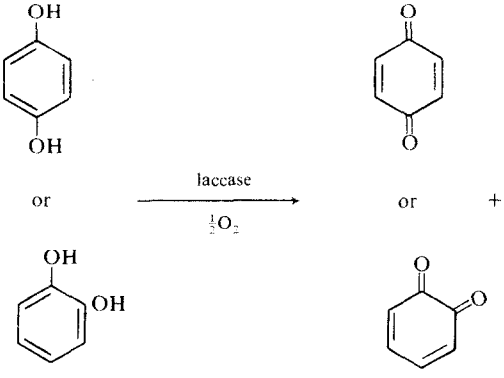
Levine [4] referred to fungal laccase as being exclusively extracellular, i.e. an enzyme excreted by fungi into the medium. However, Froehner and Eriksson [373] showed that in *Neurospora* there exists both an extracellular and an intracellular laccase, as is the case for *Glomerella* [253]. Both are soluble. It appears to be true that generally fungal laccase is readily excreted by the hyphae, e.g. in *Botrytis* [374] and *Polyporus* [375], but in *Podospora* the enzyme is clearly intracellular [77]. Its excretion by lignin digesting fungi may be of functional importance, while its intracellular location in the Ascomycetes may be related to colour formation during fruiting. In any case fungal laccase has never been reported to be other than a cytoplasmic enzyme.

The higher plant laccases also are, as far as is known, cytoplasmic, soluble. No reports are known of laccase occurring in a well defined subcellular particle [367–369].

PROPERTIES OF LACCASE

Substrate specificity, pH optimum and inhibitors

All laccases described to date catalyse the reaction:



This reaction is characteristic of laccase and indeed is the main criterion according to which the enzyme is classified. In addition, all known laccases oxidize *o*-diphenols of the catechol type as well as ascorbic acid. Generally the affinity for ascorbic acid is much higher than that for the phenolic substrates. In addition most laccases oxidize various other *p*-diphenols, *m*-diphenols and *p*-phenylenediamine. Laccases do not oxidize tyrosine. However, their ability to oxidize *p*-cresol is less clear. *Rhus* laccase was unable to oxidize *p*-cresol [24, 376] but Benfield *et al.* [350] claimed that the enzyme does oxidize this substrate. The report by Benfield *et al.* [350] should be treated with reserve. The enzyme, of unspecified purification, was incubated with substrate for nine (*sic*!) days. If the reaction was indeed due to the *Rhus* laccase it must have been very slow indeed. Neither peach laccase [369, 370] nor the laccase present in the secretory ducts of *Mangifera indica* oxidize *p*-cresol [368]. In addition, syringaldazine [377] which may be regarded as a cresol derivative is apparently not oxidized by the *Mangifera* enzyme [368]. In contrast to higher plant laccases, there is no doubt about the ability of most of the fungal laccases to oxidize *p*-cresol [77, 281, 373, 374, 378]. The resulting oxidation product is a white precipitate, of ketonic structure [379]. However, laccase from *Aspergillus*, *Glomerella* and *Lactarius* were reported not to oxidize *p*-cresol [253, 380, 381]. These claims merit further verification. In this respect there may therefore exist a significant difference between fungal and higher plant laccases. Differences in substrate specificity of laccases II and III from *Podospora*, indicating structural differences, have been reported [382].

The pH optimum of the different laccases shows quite considerable variation, being 6.4 or 7.5 for *Rhus* depending on the species and 4.5 for the Burmese lacquer tree *Melanorrhea usitata* [376]. The pH optimum of *Neurospora* and *Podospora* laccase towards quinol is around pH 6.0, that of *Ganoderma* around 5.4; *Botrytis* laccase shows optimal activity at pH 4.7 and *Polyporus* around 4.0 [77, 373, 374, 378]. The pH optimum differs also depending on the substrate oxidized. Stability of laccases from various sources is reported to be pH

dependent and changed with the buffer and other factors in the medium [383]. Most laccases from fungi are unstable at alkaline pH, the most extreme case being *Botrytis* laccase which is rapidly inactivated above pH 7.0 [374]. *Rhus* laccases seem to be more stable to changes in pH than fungal ones [384]. Laccase is usually more sensitive to inactivation by heat, compared to catechol oxidases [77, 385]. Fungal laccases have a fairly high affinity for the their phenolic substrate, around 10^{-4} M or somewhat less for quinol [374, 382, 386]. However. the *Rhus* and peach laccases have a much higher K_m for quinol around 10^{-2} M [370, 376]. The K_m for O_2 of the enzyme also shows variability—10–20% O_2 (equilibration with this amount of oxygen in the atmosphere) for peach laccase, and 25% for *Botrytis* [Mayer and Marbach, unpublished results]. Surprisingly few accurate determinations of the K_m have been made.

All laccases so far investigated are glycoproteins. The reported sugar content is between 32–45% for *Rhus* [387, 388], 11% for *Neurospora* [373], 14% for *Polyporus* II [389] and 23% for *Podospora* [383]. Both *Botrytis* and peach laccases are glycoproteins but the carbohydrate content has not yet been determined [369, 390]. Variability in the carbohydrate content of purified preparations of *Podospora* laccase has been reported [391].

The activity of laccases is not inhibited by CO [22, 366] nor by phenylhydrazine [25, 368] or 2,3-naphthalenediol [22, 370] all of which inhibit catechol oxidase. The enzyme is usually inhibited by the copper chelator diethyldithiocarbamate [368. 369] as well as by cyanide, azide and EDTA [374]. The response to inhibitors permits therefore an easy differentiation between laccase and catechol oxidase even in impure preparations. This method has not always been used sufficiently.

Molecular weight, structure, amino acid composition and copper content

The MW of various laccases differs considerably. That of *Rhus* laccase has been reported to vary between 101 000 and 140 000 depending on the species and even variety, e.g. Chinese and Japanese *Rhus vernicifera* [376, 387, 388]. The MW of the fungal laccase varies between *ca* 56 000 for *Botrytis* [390], 67 000 for *Lactarius* [381], 63–65 000 for *Russula* [392], 60–65 000 for the main *Polyporus* laccase [389], 390 000 for *Podospora* laccase I and 71 and 78 000 for laccase II and III [393], 64 800 for *Neurospora* laccase [373] and 107–117 000 for *Aspergillus* [380]. Peach laccase has a MW of 73 500 [369]. *Polyporus* and *Podospora* laccases exist in more than one form [394, 395] of varying MW.

These data suggest that laccases are a heterogenous group of glycoproteins. having a basic structural unit of between 50–70 000 MW which can undergo aggregation to give larger units. Presumably, the laccase I of *Podospora* contains 4 such subunits [393]. The differences in the precise MW reported for a given laccase may be ascribed to species differences and such differences as the length of the attached carbohydrate chain. Some of the detailed properties (spectral, analytical, etc.) of the three laccases studied in great detail (*Rhus*, *Polyporus* and *Podospora*) are listed by Fee [8], by Molitoris and Esser [383] and by Coleman [12].

As already mentioned, laccases are glycoproteins with varying carbohydrate content. The basic subunit seems to be a polypeptide chain of 50–70 000 containing 15–41% carbohydrate. The carbohydrate includes hexosamine, glucose, mannose, galactose in *Podospora* and *Polyporus* [383, 389] while the *Rhus* laccase contains hexosamines, frucose, mannose, galactose, glucose and arabinose [388]. No detailed analysis appears to be available for other laccases. The amino acid composition of laccase has been reported [367, 369, 373, 387, 388, 390, 391]. The amino acid composition shows very considerable variation. All except the peach laccase contain appreciable amounts of sulphur amino acids including cysteine. The absence of this in the peach laccase may be the result of the method of its preparation. *Botrytis* laccase has an exceptionally low content of basic amino acid and very high serine and threonine content. In all the other laccases aspartic acid is the amino acid present in largest amount while glutamic acid is present in much smaller amounts. The differences in amino acid composition as well as many of the other properties already mentioned emphasize again that we are dealing with a 'family' of enzymes and not with a single enzyme which underwent minor modifications during evolution.

All laccases contain copper. In those cases where the enzyme has been purified and isolated under carefully controlled conditions. avoiding loss of copper, the single polypeptide chain contains 4 atoms of copper. Some of the copper is bound to or near one cysteine sulphur and three N atoms [396]. According to Briving and Deinum [397] SH groups, although present in *Rhus* and fungal laccase do not coordinate type I or II copper. The ligand for both Cu I and Cu II appears to be an imidazole group [398], presumably in histidine.

The purified laccases are characteristically blue in colour, i.e. they contaln a blue copper centre. Copper may be present in protein in a form detectable by electron paramagnetic resonance and intensely blue (Type I), EPR detectable but not blue (Type II) and not detectable by EPR, Type III. It is generally accepted today that in laccase all three types of copper are present, one atom each of types I and II and two atoms of type III [8, 9, 12, 399]. Since this subject has been discussed in great detail in the reviews mentioned, the earlier literature will not be cited here. A significant difference exists between *Rhus* and fungal laccases. *Rhus* laccase can be reconstituted from copper and apoprotein while fungal laccases cannot be reconstituted readily. However, in *Lactarius* laccase apparently such reconstitution was possible [381]. The copper can be removed quite readily from all laccases, with loss of activity. The state of copper in laccases has been investigated by a series of modern techniques including optical absorption, EPR, circular dichroism and optical rotary dispersion and Raman spectroscopy. It is possible to remove the type II copper selectively with loss of enzyme activity, while blue colour is retained [400]. Cu II can be restored, the reconstituted enzyme being active [401]. A certain amount of monovalent copper seems to be present even in oxidized laccase preparations. This Cu^+ does not participate in the redox system. It may, however, be involved in substrate binding [402]. The protein structure of *Rhus* laccase seems to be organized as β structure and random coil [403], but no further details seem to have been published since then.

REACTION MECHANISM OF LACCASE

The first basic question to be answered about the

reaction mechanism was whether a free radical was involved. Nakamura [404, 405], showed conclusively that the first step in the oxidation of quinol by laccase was the formation of the semiquinone, with transfer of an electron from substrate to the copper in the enzyme. The second step was a non-enzymatic disproportionation reaction between two semiquinone molecules to give one molecule of quinone and one of quinol. The binding sequence of O_2 and quinol does not appear to have been studied. However, it has been suggested that a 'ping-pong' mechanism is operating and that no binary or ternary enzyme–substrate complexes are formed [9]. It would be most interesting to determine this experimentally and also to find out whether any definite sequence of substrate binding exists, as seems to be the case for the catechol oxidase reaction (see earlier section).

The most extensively investigated part of the reaction mechanism is the function of copper and this has been reviewed at length and in great detail a number of times in recent years [8, 9, 12, 194, 367]. This aspect will therefore not be discussed in detail here. The techniques employed for studying this question have been: reaction of the copper with inorganic ions such as ferrocyanide, fluoride or H_2O_2, EPR, temperature jumps methods and spectrophotometric methods of various kinds. It is still not entirely clear what the precise sequence of events is in the reduction of copper. Malmström and coworkers seem to favour successive one electron transfers to type II Cu but some revision of their views has been published recently [406, 407]. Others tend to favour the view of reduction of oxygen by the almost simultaneous transfer of four electrons to molecular oxygen [8]. From various studies it is clear that H_2O_2 if formed at all, is reduced to H_2O on the enzyme and is not released from it. Fee [8] concluded that the present available data are insufficient to allow the formation of a general catalytic reaction scheme for *Rhus* laccase. He also suggested that *Rhus* and fungal laccases probably use the same catalytic mechanisms. However, Holwerda *et al.* [9] point to the considerable differences in rate of oxidation of substrate by *Rhus* and fungal laccase. They suggest that in *Rhus* type I Cu is deeply buried in the polypeptide structure, making it much less reactive. They also suggest that in fungal laccase perhaps type I copper is first reduced while *Rhus* type II copper is reduced with subsequent reduction of the type II copper pair. However, in *Rhus* a direct electron transfer between type III copper and oxygen has been suggested [408]. Moreover, the type III copper pair may act not as a cooperative two electron acceptor. Instead, strong reductants may induce efficient one electron transfers to the type III Cu site [409]. On the basis of EPR studies using ^{17}O it has been suggested that type II copper is a component of the oxygen reducing site in both *Rhus* and fungal laccase [410]. This would be in accord with the general model proposed by Andreasson and Reinhammar [407]. But the recent results of Pecht and his group [409] may necessitate a revision of this model. Moreover, it still appears that fungal and *Rhus* laccase show very considerable differences, if only because their redox potential is so very different. Thus in addition to the two basic types of laccase differing in basic structure and properties they may also differ in the precise sequence of events leading to reduction of copper and transfer of electrons between the different copper atoms.

PHYSIOLOGICAL FUNCTION OF LACCASE

Much work has been concerned with a study of factors inducing increased laccase secretion into the culture medium. The reports are extremely confusing, not to say contradictory. However, it does appear to be true that addition of Cu^{2+} at the right stage causes an increase in laccase in the medium [373, 411, 412]. In some cases, e.g. *Neurospora*, cycloheximide, actinomycin D and puromycin induce production and secretion of the enzyme into the medium [412]. Fahraeus *et al.* [413] used 2,5-xylidine to induce laccase production in *Polyporus*. Some, but not all, phenolic substances promote laccase formation [414–416]. On the other hand, presence of an adequate carbon source in the medium depressed laccase production or its secretion [415]. In *Botrytis*, gallic and tannic acid appeared to promote secretion while 4-methylcatechol was ineffective [Mayer and Marbach, unpublished]. These reports then are somewhat contradictory. Several reasons may account for the apparent contractions. Not always have intra- and extra-cellular enzymes been examined. The taxonomic groups to which the fungi concerned belong differ and hence their function and response to the environment may differ. The sequence of appearance of the enzyme during fungal development may also be different. In *Agaricus* laccase is present during vegetative development, but its level drops during fruiting [417]. However, in the pathogenic fungus *Botrytis cinerea*, laccase appears in the culture medium only when vegetative growth has ceased [Mayer and Marbach, unpublished]. In *Aspergillus nidulans* spore colouration is dependent on, or correlates with the appearance of laccase [380, 418] but in *Neurospora* conidiation is normal even in laccase-less mutants [412]. It seems fairly obvious that in different fungi, laccase may fulfill different functions.

The best documented role of laccase is in the wood rotting fungi. Laccase is present in the white rot fungi and absent from the soft rot ones [419, 420]. Nevertheless, the laccase content in them may be variable and seems to be of dubious taxonomic value [420, 421]. The function of laccase has long been assumed to be oxidation of lignin, at least in the wood rotting fungi. Quite an elegant confirmation for this view comes from the work of Ander and Eriksson [422]. These workers showed that a laccase-less mutant of *Sporotrichum pulverentum* is unable to degrade lignin. Addition of laccase to the lignin-containing culture medium of the laccase-less mutant enabled it to degrade the lignin. The conclusion reached by the authors was that oxidation products of lignin, produced by laccase action, may regulate formation of other lignin and polysaccharide degrading enzymes. This is apparently the first convincing proof of the function of laccase. Laccases may also be involved in demethoxylation of lignin during its degradation [423].

In the case of the pathogens, laccase may have an entirely different function. In these, laccase may act to detoxicate antifungal compounds produced by the host plant. No clear evidence is available for such a function, but there are some indications pointing to it in the literature [424, 425].

An entirely different function must be assigned to the laccase present in the secretory ducts of the Anacardiaceae. Surprisingly, this does not appear to have attracted attention and there are no speculations in the literature. At least two possibilities suggest themselves. One is

that laccase has a role in the hardening which the secretions undergo when the tissue is cut or damaged and the cut surface exposed to the atmosphere. Such a function would be primarily a sealing-off of the damaged tissue. A more subtle role might be as a defence mechanism against pathogens. In this case the laccase might oxidize endogenous phenols to the resultant toxic quinones. At present these must be regarded as speculations which deserve experimental investigation.

EVOLUTION OF THE POLYPHENOL OXIDASES

The evolution pathway of the so-called blue oxidases has been considered by Dawson *et al.* [426]. They suggest the existence of an early ancestral laccase, more primitive than the presently known enzymes. This enzyme diverged into ceruloplasmin and more advanced laccases. The latter were said to diverge again later to give the fungal laccase on the one hand and the higher plant *Rhus* laccase on the other. According to Dawson *et al.* [426]. at some time in evolutionary history ascorbic acid oxidase branched off from the laccases. This hypothesis takes into account primarily the molecular properties of the enzymes but ignores their distribution. No prokaryotic laccase is known nor is laccase apparently present in the majority of green plants except the *Rhus* laccase which occurs in the Anacardiceae. For the rest, laccase in higher plants is present only very sporadically. Furthermore, the higher plant laccase seems to have fundamentally different properties than that of the fungi. It seems, therefore, that laccase evolved first in the fungi and then independently in higher green plants. It also seems somewhat unlikely that ascorbic acid oxidase is derived from laccase, because its distribution is much wider in higher plants than that of laccase. Genetic control of laccase in fungi is extremely complex and a large number of genes, including structural ones are involved [427]. However, insufficient data are available for a comparison of ascorbic acid oxidase and laccase.

The catechol oxidases probably arose in a quite distinct manner. It obviously is a very ancient enzyme. It is clearly present in prokaryonts, e.g. *Streptomyces*, *Mycobacterium* and *Bacillus subtilis*, *Pseudomonas melanogenum* and *Vibrio tyrosinaticus* [29–31, 34–38]. Next catechol oxidase occurs almost universally in all fungi, and has been reported in various algae, e.g. *Nitella* and *Monostroma*, but not apparently in the unicellular green algae [39, 40, 42]. The enzyme has not been studied in the bryophytes or pteridophytes in any detail, but its presence seems probable, e.g. in *Riccia*, *Marchantia*, *Sphaerocarpus* and *Aspidium* [43–46]. In the gymnosperms [47] and certainly in the angiosperms the enzyme is almost ubiquitous and it is easier to list the few cases in which it is absent than to record its presence. In general the enzyme reported in bacteria [e.g. 35, 36] and fungi is very similar to that reported in higher plants. Molecular weight and copper content show the same kind of characteristics although substrate specificity seems to be very variable. We know too little about its subcellular location in the lower organisms to permit comparison with higher plants. The impression, however, is that in bacteria and fungi the enzyme is primarily soluble and in the higher plants much of it is particulate. It is difficult to draw evolutionary conclusions. A ubiquitous enzyme of unknown physiological function, present apparently in all stages of evolutionary development, must confer some some kind of survival benefit to the organism in which it occurs. We must also remember that the catechol oxidases are not confined to the plant kingdom. They are equally ubiquitous in the animal kingdom and even a cursory survey of the literature shows them to be present in crustaceans, insects, fish and mammals. Perhaps the only trend which can be observed in the enzyme's properties is the decrease in its hydroxylating activity in higher plants and animals. This perhaps is an indication of its changing physiological function during evolution.

PRACTICAL PROBLEMS AND APPLICATIONS

The laccases and catechol oxidases are as we have shown enzymes of very widespread distribution. As a result of their activity highly reactive coloured products are formed, often condensed molecules of higher MW than the original substrate of enzyme action. These compounds appear in many food products as the result of accidental oxidation reaction or due to deliberate processing designed to lead to such colour formation. The general problem of browning reactions has been discussed [254, 428], and the widespread occurrence of the substrates of this group of enzymes has been documented [429]. Generally the accidental browning reactions are undesirable and lead to a loss of palatability of the foodstuffs or at least make them less appealing visually. The degradation of anthocyanins caused by catechol oxidases [430] can also result in reduced quality of fruits, vegetables and their products. In contrast in the drying of tea, roasting of coffee or fermentation of cocoa the action of catechol oxidase is an initial step in obtaining the desired product [431–433]. The possible importance in vinification of catechol oxidase of the grapes and laccase from infecting *Botrytis* has also been discussed [434].

The importance of the phenol oxidizing enzymes in the chemistry of wood, in its degradation and in its resistance to various attacking organisms must also be stressed. Plant biochemists are well aware of the interference of catechol oxidase, its substrates and oxidation products in the isolation of enzymes and other proteins from plant tissues [131, 132].

The knowledge of phenol oxidases has been applied mainly in food processing, either to prevent browning or in facilitating enzyme reactions when desirable. Conformational changes occurring during processing of food products may have considerable practical implications [66, 143].

In view of the observations on inhibition of catechol oxidase by short chain peptides and by specific proteins (see section on inhibitors) and since oligopeptides are known to form complexes with copper [e.g. 193, 435], it may be suggested that short chain peptides should be surveyed with the aim of finding non-toxic inhibitors of the enzyme. The use of spraying with plant hormones to reduce catechol oxidase levels in intact plants has so far been successful only in one case [69]. The ability of catechol oxidase to hydroxylate monophenols has been used only for synthesizing dopa, either by an immobilized enzyme [436] or by a fermentation method [37]. Little attention has been paid to observations on the relationship between fungal fruiting body formation and the appearance of laccase or catechol oxidase from a practi-

although much attention has been devoted to the possibility that catechol oxidase is involved in defence mechanisms in plants, the possible participation of fungal phenol oxidases in the attack of plant pathogenes was not adequately considered.

In conclusion we may say that in addition to being an extremely fascinating group of enzymes of as yet uncertain biological function, polyphenol oxidases are also a group of enzymes of vital importance to man. This importance is due to the fact that the properties, palatability, resistance, and usability of most products derived from plants may be modified by the presence of catechol oxidases and laccases.

REFERENCES

1. Malmström, B. G. and Ryden, L. (1968) in *Biological Oxidations* (Singer, T. P., ed.). p. 415. Interscience, New York.
2. Mason, H. S. (1955) *Adv. Enzymol.* **16**, 105.
3. Bonner, W. D. (1957) *Annu. Rev. Plant Physiol.* **8**, 427.
4. Levine, W. G. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P. and Blumberg, W. E., eds.). p. 371. Academic Press, New York.
5. Malmström, B. G. (1970) *Pure Appl. Chem.* **24**, 393.
6. Brooks, D. W. and Dawson, C. R. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P. and Blumberg, W. E., eds.). p. 343. Academic Press, New York.
7. Vanneste, W. H. and Zuberbühler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.). p. 371. Academic Press, New York.
8. Fee, J. A. (1975) in *Structure and Bonding* (Dunitz, J. D., Hemmerich, P., Holm, R. H., Ibers, J. A. Jørgensen, C. K., Neilands, J. B., Reinen, D. and Williams. R. J. P., eds.). Vol. 23, p. 1. Springer, Berlin.
9. Holwerda, R. A., Wherland, S. and Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng.* **5**, 363.
10. Dawson, C. R. and Tarpley, W. B. (1951) in *The Enzymes* (Summer, J. B. and Myrbäck, K., eds.). Vol. 2, p. 454. Academic Press, New York.
11. Ingraham, L. L. (1959) in *Pigment Cell Biology* (Gordon, M., ed.). p. 609. Academic Press, New York.
12. Coleman, J. E. (1974) *Adv. Chem. Ser.* **136**, 267.
13. Wright, C. I. and Mason, H. S. (1946) *J. Biol. Chem.* **165**, 45.
14. Mason, H. S. and Wright, C. I. (1949) *J. Biol. Chem.* **180**, 235.
15. Mayer, A. M., Harel, E. and Ben-Shaul, R. (1966) *Phytochemistry* **5**, 783.
16. Pierpoint, W. S. (1966) *Biochem. J.* **98**, 580.
17. Kean, E. A. (1964) *Biochim. Biophys. Acta* **92**, 602.
18. Yamaguchi, M., Henderson, H. M. and Hwang, P. M. J. (1969) *Analyt. Biochem.* **32**, 178.
19. Pomeranz, S. H. (1966) *J. Biol. Chem.* **241**, 161.
20. Mazzocco, F. and Pifferi, P. G. (1976) *Analyt. Biochem.* **72**, 643.
21. Esterbauer, H., Schwarzl, E. and Hayn, M. (1977) *Analyt. Biochem.* **77**, 486.
22. Mayer, A. M., Harel, E. and Shain, Y. (1964) *Phytochemistry* **3**, 447.
23. van Loon, L. C. (1971) *Phytochemistry* **10**, 503.
24. Keilin, D. and Mann, T. (1939) *Nature* **143**, 23.
25. Lerner, H. R., Harel, E., Lehman, E. and Mayer, A. M. (1971) *Phytochemistry* **10**, 2637.
26. Bertrand, G. (1895) *C. R. Acad. Sci. Paris* **121**, 166.
27. Bertrand, G. (1896) *C. R. Acad. Sci. Paris* **122**, 1215.
28. Bertrand, G. (1896) *C. R. Acad. Sci. Paris* **123**, 463.
29. Chuck, C. M. (1965) *Mendel Bull.* p. 38.
30. Menduri, A. (1968) *Bull. Soc. Ital. Biol. Sper.* **44**, 1688.
31. Prabhakaran, K. (1968) *Nature* **218**, 473.
32. Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. (1972) *Microbios* **5**, 273.
33. Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. (1973) *Microbios* **8**, 151.
34. Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. (1975) *Infect. Immun.* **12**, 267.
35. Lerch, K. and Ettlinger, L. (1972) *Eur. J. Biochem.* **31**, 427.
36. Pomeranz, S. H. and Murthy, V. V. (1974) *Arch. Biochem. Biophys.* **160**, 73.
37. Yoshida, H., Tanaka, Y. and Nakayama, K. (1974) *Agric. Biol. Chem.* **38**, 627.
38. Mayles, B. A. (1975) *Chem. Abst.* **83**, 128494u.
39. Tocher, R. D. and Meeuse, B. J. D. (1966) *Can. J. Botany* **44**, 551.
40. Stom, D. I., Apartsin, M. S., Bobovskaya, L. P. and Ivanova, G. G. (1975) *Fiziol. Rast.* **22**, 227.
41. Yopp, J. H. (1975) *Phycologia* **14**, 299.
42. Holst, R. W. and Yopp, J. H. (1976) *Phycologia* **15**, 119.
43. van Poucke, M. (1967) *Physiol. Plant.* **20**, 932.
44. Rao, K. R., Malakondaiah, N. and Ramana, K. V. R. (1969) *Z. Pflanzenphysiol.* **61**, 168.
45. Babbel, G. R. (1974) *Bot. Gaz.* **135**, 297.
46. Malesset-Bras, M. (1962) *C. R. Acad. Sci. Paris* **254**, 914.
47. Cambie, R. C. and Bocks, S. M. (1966) *Phytochemistry* **5**, 391.
48. Mironyuk, V. I. (1969) *Ukr. Bot. Zh.* **26**, 54.
49. Corduan, G. (1976) *Z. Pflanzenauecht*, **76**, 47.
50. Asghar, S. S. and Sidiqi, M. (1970) *Enzymologia* **39**, 289.
51. Roberts, M. F. (1974) *Phytochemistry* **13**, 119.
52. El Khalifa, M. D. and Siddig, M. A. M. (1974) *Angew. Bot.* **48**, 281.
53. Maercker, U. (1965) *Protoplasma* **60**, 173.
54. Suzuki, Y. (1964) *Phyton* **21**, 7.
55. Mayer, A. M. and Friend, J. (1960) *J. Exp. Botany* **11**, 141.
56. McClendon, J. H. (1953) *Am. J. Botany* **40**, 260.
57. Harel, E., Mayer, A. M. and Shain, Y. (1964) *Physiol. Plant.* **17**, 921.
58. Harel, E., Mayer, A. M. and Shain, Y. (1965) *Phytochemistry* **4**, 783.
59. Weurman, C. and Swain, T. (1955) *J. Sci. Food Agric.* **6**, 186.
60. Heimer, Y. and Mayer, A. M. (1966) *Israel J. Botany* **15**, 1.
61. Harel, E., Mayer, A. M. and Shain, Y. (1966) *J. Sci. Food Agric.* **17**, 389.
62. Harel, E., Mayer, A. M. and Lerner, H. R. (1970) *J. Sci. Food Agric.* **21**, 542.
63. Takeo, T. and Baker, J. E. (1973) *Phytochemistry* **12**, 21.
64. Taneja, S. R., Abrol, Y. P. and Sachar, R. C. (1974) *Cereal Chem.* **51**, 457.
65. Singh, R. and Singh, D. (1974) *Biochem. Physiol. Pflanz.* **166**, 233.
66. Kidron, M., Harel, E. and Mayer, A. M. (1978) *Am. J. Enol. Vitic.* **29**, 30.
67. Mayer, A. M. (1965) *Israel J. Botany* **13**, 74.
68. Stafford, H. A. (1969) *Phytochemistry* **8**, 743.
69. Knapp, F. W., Hall, C. B., Buchanan, D. W. and Biggs, R. H. (1970) *Phytochemistry* **9**, 1453.
70. Griffith, T. and Conn, E. E. (1973) *Phytochemistry* **12**, 1651.
71. Forrest, G. I. (1969) *Biochem. J.* **113**, 765.
72. Stafford, H. A. and Galston, A. W. (1970) *Plant Physiol.* **46**, 763.
73. Vernon, S. L. and Straus, J. (1962) *Phytochemistry* **11**, 273.
74. Volk, R., Harel, E., Mayer, A. M. and Gan-Zvi, E. (1977) *J. Expt. Botany* **28**, 820.
75. Volk, R., Harel, E., Mayer, A. M. and Gan-Zvi, E. (1978) *J. Exp. Botany*, (in press).
76. Esser, K. (1963) *Naturwissenschaften* **17**, 576.
77. Esser, K. (1963) *Arch. Mikrobiol.* **46**, 217.
78. Leonard, T. J. (1971) *J. Bacteriol.* **106**, 162.
79. Parisot, D. (1976) *Physiol. Veg.* **14**, 339.
80. Horowitz, N. H., Fling, M., Macleod, H. L. and Sueoka, N. (1960) *J. Mol. Biol.* **2**, 96.

81. Horowitz, N. H., Fling, M., Macleod, H. and Sueoka, N. (1961) *Genetics* **46**, 1015.
82. Horowitz, N. H., Fling, M., Macleod, H. and Watanabe, Y. (1961) *Cold Spring Harbor. Symp. Quant. Biol.* **26**, 233,
83. Sussman, A. (1961) *Arch. Biochem. Biophys.* **95**, 407.
84. Horowitz, N. H., Fling, M. and Asano, K. (1964) *Proc. 6th Int. Congr. Biochem.*
85. Horowitz, N. H., Fligh, M., Feldman, H. M., Pall, M. L. and Froehner, S. C. (1970) *Develop. Biol.* **21**, 147.
86. Horowitz, N. H., Feldman, H. M. and Pall, M. I., (1970) *J. Biol. Chem.* **245**, 2784.
87. Walker, J. R. L. (1962) *N.Z. J. Sci.* **5**, 316.
88. Azizov, I. M. and Mayeva, S. A. (1965) *Fiziol. Rast.* **12**, 342.
89. Kahn, V. (1977) *J. Food Sci.* **42**, 38.
90. Ben-Shalom, N., Harel, E. and Mayer, A. M. (1978) *J. Sci. Food Agric.* **29**, 398.
91. Bruchet, G. (1966) *C. R. Acad. Sci., Paris* **262**, 1940.
92. Kocher, H. P. and Ettlinger, L. (1975) *Pathol. Microbiol.* **42**, 248.
93. Palmer, J. K. (1963) *Plant Physiol.* **38**, 508.
94. Delon, R. (1974) *Phytopathol. Z.* **80**, 199.
95. Pruidze, G. N. (1972) *Soobshch, Akad. Nauk Gruz. SSR* **66**, 677.
96. Tolbert, N. E. (1973) *Plant Physiol.* **51**, 234.
97. Kato, C., Uritani, I., Saijo, R. and Kakeo, T. (1976) *Plant Cell Physiol.* **17**, 1045.
98. Mayer, A. M. (1961) *Physiol. Plant.* **14**, 322.
99. Sanderson, G. W. (1964) *Biochim. Biophys. Acta* **92**, 622.
100. Craft, C. C. (1966) *Am. Potato J.* **43**, 112.
101. Coombs, J., Baldry, C., Bucke, C. and Long, S. P. (1974) *Phytochemistry* **13**, 2703.
102. Alberghina, F. A. M. (1964) *Phytochemistry* **3**, 65.
103. Li. P. L. and Bonner, J. (1947) *Biochem. J.* **41**, 105.
104. Ruis, H. (1972) *Phytochemistry* **11**, 53.
105. Ben-Shalom, N., Kahn, V., Harel, E. and Mayer, A. M. (1977) *J. Sci. Food Agric.* **28**, 545.
106. Katz, Y. and Mayer, A. M. (1969) *Israel J. Botany* **18**, 11.
107. Parish, R. W. (1971) *Z. Pflanzenphysiol.* **66**, 176.
108. Parish, R. W. (1972) *Eur. J. Biochem.* **31**, 446.
109. Czaininski, Y. and Catesson, A. M. (1974) in *Electron Microscopy of Enzymes* (Hayat, M. A., ed.). Vol. 2, p. 66. Van-Nostrand Reinhold, New York.
110. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.
111. Harel, E. and Mayer, A. M. (1971) *Phytochemistry* **10**, 17.
112. Liebcrei, R. and Biehl, B. (1976) *Ber. Deutch. Bot. Ges.* **89**, 663.
113. Bonner, W. D. (1955) *Plant Physiol.* **30**, xxx.
114. Demenyuk, M. N., Nizharadze, A. N. and Salkova, E. G. (1974) *Prikl. Biokhim. Mikrobiol.* **10**, 659.
115. Hoffer, A. (1964) *Planta* **62**, 137.
116. Harel, E. and Mayer, A. M. (1968) *Phytochemistry* **7**, 199.
117. Walker, J. R. L. and Hulme, A. C. (1966) *Phytochemistry* **5**, 259.
118. Ben-Shalom, N., Kahn, V., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 1153.
119. Robb, D. A., Mapson, L. W. and Swain, T. (1965) *Phytochemistry* **4**, 731.
120. Stelzig, D. A., Akhtar, S. and Ribeiro, S. (1972) *Phytochemistry* **11**, 535.
121. Mayer, A. M. (1966) *Phytochemistry* **5**, 1297.
122. Kenten, R. H. (1958) *Biochem. J.* **68**, 244.
123. Barnell, H. R. and Barnell, F. (1945) *Ann. Botany* **9**, 77.
124. Reeve, R. M. (1959) *Am. J. Botany* **46**, 210.
125. Reeve, R. M. (1959) *Am. J. Botany* **46**, 645.
126. Mace, M. E. (1963) *Physiol. Plant.* **16**, 915.
127. Roberts, E. A. H. (1952) *J. Sci. Food Agric.* **5**, 193.
128. Bendall, D. S. and Gregory, R. P. F. (1963) in *Enzymic Chemistry of Phenolic Compounds* (Pridham. J. B., ed.). p. 7. Pergamon Press, Oxford.
129. Kertesz, D. and Zito, R. (1965) *Biochim. Biophys. Acta* **96**, 447.
130. Bouchilloux, S., McMahill, P. and Mason, H. S. (1963) *J. Biol. Chem.* **238**, 1699.
131. Loomis, W. D. and Battaile, J. (1966) *Phytochemistry* **5**, 423.
132. Anderson, J. W. (1968) *Phytochemistry* **7**, 1973.
133. Perera, K. P. W. C. and Wickremasinghe, R. L. (1972) *Tea Quart.* **43**, 153.
134. Benjamin, N. D. and Montgomery, M. W. (1974) *J. Food Sci.* **38**, 799.
135. Barthe, J. P. (1974) *C. R. Acad. Sci. Paris* **278**, 735.
136. Ramaswamy, S. and Rege, D. V. (1975) *Acta Aliment. Acad. Sci. Hung.* **4**, 355.
137. Walker, J. R. L. and Hulme, A. C. (1965) *Phytochemistry* 4. 677.
138. Herzfeld, F. and Esser, K. (1969) *Arch. Mikrobiol.* **65**, 146.
139. Dhar, S. C. and Bose, S. M. (1965) *Leather Sci.* p. 54.
140. Patil, S. S. and Zucker, M. (1965) *J. Biol. Chem.* **240**, 3938.
141. Fling, M. Horowitz, N. H. and Heinemann, S. F. (1963) *J. Biol. Chem.* **238**, 2045.
142. Katan, T. and Galun, E. (1975) *Analyt. Biochem.* **67**, 485.
143. Dubernet, M. and Ribereau-Gayon, P. (1974) *Phytochemistry* **13**, 1085.
144. Guttridge, S. and Robb, D. A. (1973) *Biochem. Soc. Trans.* **1**, 519.
145. O'Neil, S. P., Graves, D. J. and Ferguson, J. J., Jr. (1973) *J. Macromol. Sci. Chem.* **7**, 1159.
146. Shimoda, T., Yonekura, M. and Funatsu, M. (1975) *Agric. Biol. Chem.* **39**, 2423.
147. Smith, J. L. and Krueger, R. C. (1962) *J. Biol. Chem.* **237**, 1121.
148. Jolley, R. L. and Mason, H. S. (1965) *J. Biol. Chem.* **240**, PC 1489.
149. Jolley, R. L., Jr., Robb, D. A. and Mason, H. S. (1969) *J. Biol. Chem.* **244**, 1593.
150. Robb, D. A. (1967) *Adv. Biol. Skin* **8**, 283.
151. Bull. A. T. and Carter, B. L. A. (1973) *J. Gen. Microbiol.* **75**, 61.
152. Frieden, E. and Ottesen, M. (1959) *Biochim. Biophys. Acta* **34**, 248.
153. Yasunobu, K. T. (1959) in *Pigment Cell Biology* (Gordon, M., ed.), p. 583. Academic Press, New York.
154. Mallette, M. F. and Dawson, C. R. (1949) *Arch. Biochem.* **23**, 29.
155. Duckworth, H. W. and Coleman, J. E. (1970) *J. Biol. Chem.* **245**, 1611.
156. Kertesz, D. and Zito, R. (1957) *Nature* **179**, 1017.
157. Zito, R. and Kertesz, D. (1966) in *Biological and Chemical Aspects of Oxygenases* (Bloch, K. and Hayaishi, O., eds.), p. 290. Maruzen, Tokyo.
158. Guttridge, S. and Robb, D. A. (1975) *Eur. J. Biochem.* **54**, 107.
159. Nambudiri, A. M. D., Bhat, J. V. and Roa, P. V. S. (1972) *Biochem. J.* **130**, 425.
160. Schoot-Uiterkamp, A. J. M. and Mason, H. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 993.
161. Schoot-Uiterkamp, A. J. M., Van der Deen, H., Berendsen, H. C. J. and Boas, J. F. (1974) *Biochim. Biophys. Acta* **372**, 407.
162. Jolley, R. L., Jr., Evans, L. H., Makino, N. and Mason, H. S. (1974) *J. Biol. Chem.* **249**, 335.
163. Makino, N. and Mason, H. S. (1973) *J. Biol. Chem.* **248**, 5731.
164. Makino, N., McMahill, P., Mason, H. S. and Moss, T. H. (1974) *J. Biol. Chem.* **249**, 6062.
165. Strothkamp, K. G., Jolley, R. L. and Mason, H. S. (1976) *Biochem. Biophys. Res. Commun.* **70**, 519.
166. Lerch, K. (1976) *FEBS Letters* **69**, 157.
167. Madhosingh, C. (1970) *Life Sci.* **7**, 553.
168. Samorokova-Bianki, G. B., Strel'tina, S. A. and Zaprometov, M. N. (1977) *Biokhimiya (Moscow)* **42**, 443.
169. Buzin, G. A., Dzhemukhadze, K. M. and Mileshko, L. F. (1974) *Dokl. Akad. Nauk SSSR* **218**, 708.
170. Dizik, N. S. and Knapp, F. W. (1970) *J. Food Sci.* **35**, 282.
171. Balasingam, K. and Ferdinand, W. (1970) *Biochem. J.* **118**, 15.

172. Matheis, G. and Belitz, H. D. (1975) *Z. Lebensm. Unters. Forsch*, **157**, 221.
173. Heinaru, E., Janes, H. and Sarapuu, L. (1975) *Tartu Riikliku Ulik. Toim*, **362**, 68 (*Chem. Abst.* **84**, 132636 f).
174. Yunusov, T. S. and Yuldashev, P. Kh. (1975) *Khim. Prir. Soedin*, **11**, 409 (*Chem. Abst.* **83**, 203088 w).
175. Kidron, M., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 1050.
176. Vaughan, P. F. T., Eason, R., Paton, J. Y. and Ritchie, G. (1975) *Phytochemistry* **14**, 2383.
177. Hyodo, H. and Bando, S. (1965) *Agric. Biol. Chem.* **29**, 763.
178. Bokuchava, M. A., Soboleva, G. A., Datunashvilli, E. N. and Mindadze, R. K. (1974) *Prikl. Biokhim. Mikrobiol.* **10**, 275.
179. Gregory, R. P. F. and Bendall, D. S. (1966) *Biochem. J.* **101**, 569.
180. Harel, E., Mayer, A. M. and Lehman, E. (1973) *Phytochemistry* **12**, 2649.
181. Jolley, R. L., Jr., Nelson, R. M. and Robb, D. A. (1969) *J. Biol. Chem.* **244**, 3251.
182. Ashida, M. (1971) *Arch. Biochem. Biophys.* **114**. 749.
183. Burnett, J. B. (1971) *J. Biol. Chem.* **246**, 3079.
184. Yunusov, T. S. and Yuldashev, P. Kh. (1975) *Khim. Prir. Soedin*, **11**, 112. (*Chem. Abst.* **83**, 55237 b).
185. Yunusov, T. S. and Yuldashev, P. Kh. (1975) *Khim. Prir. Soedin*, **11**, 112., (*Chem. Abst.* **83**, 55238 c).
186. Nau, H., Lerch, K. and Witte, L. (1977) *FEBS Letters* **79**, 203.
187. Yunusov, T. S. and Yuldashev, P. Kh. (1975) *Tezisy Dokl.—Vses Simp. Bioorg. Khim.* (Rasskazov, V. A., ed.) p. 51. Akad. Nauk SSSR, Vladivostok, (*Chem. Abst.* **85**, 89233 d).
188. Deinum, J., Lerch, K. and Reinhammar, B. (1976) *FEBS Letters* **69**, 161.
189. Kertesz, D., Rotilio, G,. Brunori, M., Zito, R. and Antonini, E. (1972) *Biochem. Biophys. Res. Commun*, **49**, 1208.
190. Dressler, H. and Dawson, C. R. (1960) *Biochim. Biophys. Acta* **45**, 515.
191. Kubowitz, F. (1938) *Biochem. Z.* **299**, 32.
192. Kertesz, D. (1966) *in The Biochemistry of Copper* (Peisach, J., Aisen, P. and Blumberg, W. E., eds.), p. 359. Academic Press, New York.
193. Brill, A. S., Martin, R. B. and Williams, R. J. P. (1964) in *Electronic Aspects of Biochemistry* (Pullman, B., ed.), p. 519. Academic Press, New York.
194. Malkin, R. and Malmström, B. G. (1970) *Adv. Enzymol.* **32**, 177.
195. Pomeranz, S. H. (1963) *J. Biol. Chem.* **238**, 2351.
196. Lerner, A. B. (1953) *Adv. Enzymol.* **14**, 73.
197. Harrison, W. H., Whisler, W. W. and Ko, S. (1967) *J. Biol. Chem.* **242**, 1660.
198. Mayer, A. M. (1962) *Phytochemistry* **1**, 237.
199. Lanzarini, G., Pifferi, P. G. and Zamorani, A. (1972) *Phytochemistry* **11**, 89.
200. Donaldson, N. (1958) *The Chemistry and Technology of Naphthalene Compounds*, E. Arnold, London.
201. Mason, H. S. (1956) *Nature*, **177**, 79.
202. Aerts, F. E. and Vercauteren, R. E. (1964) *Enzymologia* **28**, 1.
203. Mason, H. S. (1966) in *Biological and Chemical Aspects of Oxygenases* (Bloch, K. and Hayaishi, O., eds.), p. 287. Maruzen, Tokyo.
204. Osaki, S. (1963) *Arch. Biochem. Biophys.* **100**, 378.
205. Shimao, K. (1962) *Biochim. Biophys. Acta* **62**, 205.
206. Macrae, A. R. and Duggleby, R. G. (1968) *Phytochemistry* **7**, 855.
207. Brown, F. C. and Ward, D. N. (1958) *J. Biol. Chem.* **233**, 77.
208. Long, T. J., Och, F. F. and Alben, J. O. (1971) *Arch. Biochem. Biophys.* **146**, 64.
209. Björkman, O. and Holmgren, P. (1960) *Physiol. Plant.* **13**, 582.
210. Clayton, R. A. (1959) *Arch. Biochem. Biophys.* **81**, 404.
211. Rivas, N. J. and Whitaker, J. R. (1973) *Plant Physiol.* **52**, 501.
212. Vaughan, P. F. T. and Butt, V. S. (1969) *Biochem. J.* **111**, 32P.
213. Matheis, G. and Belitz, H. D. (1977) *Z. Lebensm. Unters. Forsch.* **163**, 279.
214. Taneja, S. R. and Sachar, R. C. (1974) *Planta* **116**, 133.
215. Szarkowski, J. W. (1957) *Bull. Acad. Polon. Sci.* **5**, 1.
216. Rubin, B. A., Khandobina, L. M. and Geraskina, G. V. (1971) *Biol. Nauki* **14**, 78.
217. Taneja, S. R. and Sachar, R. C. (1974) *Phytochemistry* **13**, 1367.
218. Stafford, H. A. and Dresler, S. (1972) *Plant Physiol.* **49**, 590.
219. Legrand, G. (1966) *Ann. Sci. Univ. Besancon. Bot.* **3**, 21.
220. Vaughan, P. F. T. and Butt, V. S. (1970) *Biochem. J.* **119**, 89.
221. Lerner, H. R., Mayer, A. M. and Harel, E. (1974) *Phytochemistry* **13**, 397.
222. Nilova, V. P., Zarubina, M. A., Guseva, T. A., Naumov, G. P., Chernikov, S. L. and Kuznetsova, S. V. (1973) *Dokl. Akad. Nauk SSSR* **210**, 1467.
223. Challice, J. S. and Williams, A. H. (1970) *Phytochemistry* **9** 1261.
224. Baruah, P. and Swain, T. (1959) *J. Sci. Food Agric.* **10**, 125.
225. Takeo, T. (1965) *Agric. Biol. Chem.* **29**, 558.
226. Neumann, J., Legrand, G., Lchougre, G. and Lavollay, J. (1960) *C. R. Acad. Sci. Paris* **251**, 3091.
227. Wong, T. C., Luh, B. S. and Whitaker, J. R. (1971) *Plant Physiol.* **48**, 19.
228. Constantinides, S. M. and Bedford, C. L. (1967) *J. Food Sci.* **32**, 446.
229. Takeo, T. and Uritani, I. (1965) *Agric. Biol. Chem.* **30**, 155.
230. Abukharama, D. A. and Wollhouse, H. W. (1966) *New Phytol.* **65**, 477.
231. Coggon, P., Moss, G. A. and Sanderson, G. W. (1973) *Phytochemistry* **12**, 1947.
232. Walker, J. R. L. (1964) *Aust. J. Biol. Sci.* **17**, 360.
233. Singer, T. P. and Kearney, E. B. (1954) in *The Proteins* (Neurath, H. and Bailey, K., eds.). Vol. 2, p. 135. Academic Press, New York.
234. Frieden, E., Osaki, S. and Kobayashi, H. (1965) *J. Gen. Physiol.* **49**, 213.
235. Lerner, H. R. and Mayer, A. M. (1976) *Phytochemistry* **15**, 57.
236. Lerner, H. R. (1976) Ph.D. thesis, The Hebrew University of Jerusalem.
237. Volkmann, C. M. and Beersteder, E., Jr. (1956) *J. Biol. Chem.* **223**, 1021.
238. Swain, T., Mapson, L. W. and Robb, D. A. (1966) *Phytochemistry* **5**, 469.
239. Kubowitz, F. (1937) *Biochem. Z.* **292**, 221.
240. Warburg, O. and Negelein, E. (1928) *Biochem. Z.* **193**, 339.
241. Palmer, J. K. and Roberts, J. B. (1967) *Science* **157**, 200.
242. Suzuki, Y. (1959) *Sci. Rep. Tōhoku Univ. Ser. IV (Biol.)* **25**, 119.
243. Robb, D. A., Swain, S. and Mapson, L. W. (1966) *Phytochemistry* **5**, 665.
244. Pifferi, P. G., Baldassari, L. and Cultrera, R. (1974) *J. Sci. Food Agric.* **25**, 263.
245. Walker, J. R. L. (1975) *J. Sci. Food Agric.* **26**, 1825.
246. Fourche, J., Jensen, H., Neuzil, E. and Bellegarde, B. (1977) *C. R. Acad. Sci. Paris*, **284**D, 2163.
247. Jellinck, P. H. and Smith, G. (1971) *Life Sci.* **10**, 791.
248. Hulme, A. C., Jones, J. D. and Wolltorton, L. S. C. (1964) *Nature* **201**, 795.
249. Bossi, R. (1959) *Phytopathol. Z.* **37**, 273.
250. Braun, R. (1960) *Phytopathol. Z.* **39**, 197.
251. Sharon, M. and Mayer, A. M. (1967) *Israel J. Chem.* **5**, 275.
252. Weser, U. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 982.
253. Walker, J. R. L. (1968) *Phytochemistry* **7**, 1231.
254. Mathew, A. G. and Parpia, H. A. B. (1971) *Adv. Food Res.* **19**, 75.
255. Rich, P. R. and Bonner, W. D., Jr. (1977) *Plant Physiol.* **59**, (Suppl.) 60, abstr. 329.

256. Walker, J. R. L. (1970) *Nature* **227**, 298.
257. Harel, E., Mayer, A. M., Markus, Z. and Avigad, G. (1967) *Israel J. Botany* **16**, 38.
258. Avigad, G. and Markus, Z. (1965) *Israel J. Chem.* **3**, 193.
259. Madhosingh, C. and Sundberg, L. (1974) *FEBS Letters* **49**, 156.
260. Madhosingh, C. (1975) *Can. J. Microbiol.* **21**, 2108.
261. Karahanis, Y. and Frieden, E. (1961) *J. Biol. Chem.* **236**, PC 1.
262. Enselme, J. and Petat, J. M. (1949) *Bull. Soc. Chim. Biol.* **31**, 179.
263. Biedermann, W. (1956) *Mitt. Lebensm. Hyg.* **47**, 86.
264. Pifferi, P. G., Riva, A. and Zamorani, A. (1975) *Agrochimia* **19**, 499.
265. Jen, J. J. and Kahler, K. R. (1974) *Hortic. Sci.* **9**, 590.
266. Lerner, H. R., Mayer, A. M. and Harel, E. (1972) *Phytochemistry* **11**, 2415.
267. Kenten, R. H. (1957) *Biochem. J.* **67**, 300.
268. Jolley, R. L., Jr. (1967) *Adv. Biol. Skin.* **8**, 269.
269. Arnaud, Y. (1965) *J. Polym. Sci.* **16**, 4103.
270. Sato, M. (1976) *Phytochemistry* **15**, 1665.
271. Van Holde, K. E. (1966) in *Symposium on Molecular Architecture in Cell Physiology* (Hayashi, T. and Szent-Györgyi, A. G., eds.), p. 81. Prentice-Hall, New Jersey.
272. Tanford, C. (1962) *J. Am. Chem. Soc.* **84**, 4240.
273. Bigelow, C. C. (1967) *J. Theor. Biol.* **16**, 187.
274. Kahn, V. (1976) *Phytochemistry* **15**, 267.
275. Hyodo, H. and Uritani, I. (1964) *J. Biochem.* **57**, 161.
276. Hyodo, H. and Uritani, I. (1966) *Plant Cell Physiol.* **7**, 137.
277. Taneja, S. R. and Sachar, R. C. (1977) *Phytochemistry* **16**, 867.
278. Taneja, S. R. and Sachar, R. C. (1977) *Phytochemistry* **16**, 871.
279. Sato, M. and Hasegawa, M. (1976) *Phytochemistry* **15**, 61.
280. Mayer, A. M. and Friend, J. (1960) *Nature* **185**, 464.
281. Roesch, R. (1966) *Arch. Mikrobiol.* **54**, 80.
282. Yamaguchi, M., Hwang, P. M. and Campbell, J. D. (1970) *Can. J. Biochem.* **48**, 198.
283. Lerner, H. R. and Mayer, A. M. (1975) *Phytochemistry* **14**, 1955.
284. Rossier, C. and Turin, G. (1976) *Arch. Mikrobiol.* **108**, 317.
285. Katan, T., Arnon, R. and Galun, E. (1975) *Eur. J. Biochem.* **59**, 387.
286. Feldman, J. F. and Thayer, J. P. (1974) *Biochem. Biophys. Res. Commun.* **61**, 977.
287. Bastín, M. (1968) *Can. J. Botany* **46**, 1339.
288. Hyodo, H. and Uritani, I. (1966) *Agric. Biol. Chem.* **30**, 1083.
289. Batra, G. K. and Kuhn, C. W. (1975) *Physiol. Plant. Pathol.* **5**, 239.
290. Balasubramani, K. A., Deverall, B. J. and Murphy, J. V. (1971) *Physiol. Plant Pathol.* **1**, 105.
291. Matta, A. and Abbattista, G. I. (1970) *Phytopathol. Mediterr.* **9**, 168.
292. Cheung, K. W. K. and Henderson, H. M. (1972) *Phytochemistry* **11**, 1260.
293. Hori, K. (1973) *Appl. Entomol. Zool.* **8**, 103.
294. Onslow, M. W. and Robinson, M. E. (1928) *Biochem. J.* **22**, 1327.
295. Kertesz, D. and Zito, R. (1962) in *Oxygenases* (Hayaishi, O., ed.), p. 307. Academic Press, New York.
296. Jolley, R. L. Jr., Evans, L. H. and Mason, H. S. (1972) *Biochem. Biophys. Res. Commun.* **46**, 878.
297. Guttridge, S. L., Dicksen, G. and Robb, D. A. (1977) *Phytochemistry* **16**, 517.
298. Strothkamp. K. G. and Mason, H. S. (1974) *Biochem. Biophys. Res. Commun.* **61**, 827.
299. Vaughan, P. F. T. and McIntyre, R. J. (1975) *Biochem. J.* **151**, 759.
300. McIntyre, R. J. and Vaughan, P. F. T. (1975) *Biochem. J.* **149**, 447.
301. Kim, K. H. and Tchen, T. T. (1962) *Biochim. Biophys. Acta* **59**, 569.
302. Ingraham, L. L. (1957) *J. Am. Chem. Soc.* **79**, 666.
303. Matheis, G. and Belitz, H. D. (1977) *Z. Lebensm. Unters. Forsch.* **163**, 191.
304. Wood, B. J. B. and Ingraham, L. L. (1965) *Nature* **205**, 291.
305. Wilson, D. M. (1968) *Phytopathol.* **58**, 1697.
306. Leonard, T. J. and Phillips, L. E. (1973) *J. Bacteriol.* **114**, 7.
307. Wood, D. A. and Goodenough, P. W. (1977) *Arch. Mikrobiol.* **114**, 161.
308. Martinelli, S. D. and Bainbridge, B. W. (1974) *Trans. Br. Mycol. Soc.* **63**, 361.
309. Chet. I. and Henis, Y. (1975) *Annu. Rev. Plant Pathol.* **13**, 169.
310. Chet, I., Retig, N. and Henis, Y. (1972) *J. Gen. Microbiol.* **72**, 451.
311. Kuo, M. J. and Alexander, M. (1967) *J. Bacteriol.* **94**, 624.
312. Bull, A. T. (1970) *Arch. Biochem. Biophys.* **137**, 345.
313. Podojil, M., Ricicova, A., Suchy, J. and Vanek, Z. (1965) in Proc. Congr. on *Advances in Antibiotics Research*, p. 552, Prague.
314. Westermark, U. and Eriksson, K. E. (1974) *Acta Chem. Scand.* **B28**, 209.
315. Vaughan, P. F. T., Butt, V. S., Grisebach, H. and Schill, L. (1969) *Phytochemistry* **8**, 1373.
316. Schill, L. and Grisebach, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1555.
317. Jindra, A., Kovacs, P., Pittnerova, Z. and Mikulas, P. (1966) *Phytochemistry* **5**, 1303.
318. Roberts, M. F. (1971) *Phytochemistry* **10**, 3021.
319. Elstner, E. F., Konze, J. R., Selman, B. R. and Stoffer, C. (1976) *Plant Physiol.* **58**, 163.
320. Arnon, D. I. (1950) in *Copper Metabolism* (McElroy, D. and Glass, B., eds.). p. 89. John Hopkins Press, Baltimore.
321. Hill, R. and Hartree, E. F. (1953) (*Annu. Rev. Plant Physiol.* **4**, 115.
322. Crawford, R. M. M. (1967) *Nature* **214**, 427.
323. Gordon, S. A. and Paleg, L. G. (1961) *Plant Physiol.* **36**, 838.
324. Tomaszewski, M. and Thimann, K. V. (1966) *Plant Physiol.* **41**, 1443.
325. Briggs, J. A. and Ray, P. M. (1956) *Plant Physiol.* **31**, 165.
326. Tomaszewski, M. (1959) *Bull. Acad. Polon. Sci.* **7**, 127.
327. Sondheimer, E. and Griffin, D. H. (1960) *Science* **131**, 672.
328. Gaspar, Th. and Dinant, M. (1966) *Wiss. Z. Univ. Rostock Math.—Natur Wiss. Reihe* **16**, 441 (*Chem. Abst.* **69**, 66320 k).
329. Shinshi, H. and Nogudri, M. N. (1975) *Phytochemistry* **14**, 1255.
330. Habaguchi, K. (1977) *Plant Cell Physiol.* **18**, 181.
331. Habaguchi, K. (1977) *Plant Cell Physiol.* **18**, 191.
332. Marbach, I. and Mayer, A. M. (1974) *Plant Physiol.* **54**, 817.
333. Marbach, I. and Mayer, A. M. (1975) *Plant Physiol.* **56**, 93.
334. Marbach, I. and Mayer, A. M. (1978) *J. Exp. Botany* **29**, 69.
335. Farkas, G. L. and Kiraly, Z. (1962) *Phytopathol. Z.* **44**, 105.
336. Hare, R. C. (1966) *Bot. Rev.* **32**, 95.
337. Wood, R. K. S. (1967) *Physiological Plant Pathology.* Blackwells Scientific Publications, Oxford.
338. Schaefer, H. (1972) *Phytopathol. Z.* **75**, 285.
339. Kosuge, T. (1969) *Annu. Rev. Phytopathol.* **7**, 195.
340. Brueske, C. H. and Drapkin, V. H. (1973) *Phytopathology* **63**, 329.
341. Stephens, G. J. and Wood, R. K. S. (1974) *Nature* **251**, 358.
342. Pitt, D. (1975) *Trans. Br. Mycol. Soc.* **65**, 91.
343. Pollock, C. and Drysdale, R. B. (1976) *Phytopathol. Z.* **86**, 56.
344. Weaver, M. L., Hautala, E. and Reeve, R. M. (1970) *Am. Potato J.* **47**, 479.
345. Roeber, K. C. (1976) *Tagungsber. Akad. Landwirtschaftswiss. DDR* **140**, 191 (*Chem. Abst.* **85**, 156656 j).
346. Moustafa, F. A. and Whittenbury, R. (1970) *Phytopathol. Z.* **67**, 214.
347. Mukherjee, P. K. and Gosh, J. J. (1975) *Sci. Cult.* **41**, 433.
348. Trajkovski, V. (1976) *Swed. J. Agric. Res.* **6**, 215.
349. Hildebrand, D. C. and Schroth, M. N. (1964) *Phytopathology* **54**, 59.

350. Benfield, G., Bocks, S. H., Bromley, K. and Brown, B. R. (1964) *Phytochemistry* **3**, 79.
351. Sijpesteijn, A. K. and Pluijgers, C. W. (1962) *Mededel. Landbouwhogeschool Opzoekingssta Staat Gent* **27**, 1199.
352. Konig, D. and Niehaus, F. (1970) *Phytopathol. Z.* **68**, 193.
353. Ersek, T., Barna, B. and Kiraly, Z. (1973) *Acta Phytopathol.* **8**, 3.
354. Blakeman, J. P., Mokahel, M. A. and Hadley, G. (1976) *New Phyto.* **77**, 697.
355. Vidhyasekaran, P. (1975) *Phytopathol. Z.* **82**, 88.
356. Patil, S. S. and Dimond, A. E. (1968) *Phytopathology*, **57**, 492.
357. Addy, S. K. and Goodman, R. N. (1972) *Indian J. Phytopathol.* **25**, 575.
358. Vidhyasekaran, P. and Parambaramani, C. (1973) *Indian J. Exp. Biol.* **11**, 254.
359. Retig, N. (1974) *Physiol. Plant Pathol.* **4**, 145.
360. Friend, J. and Thornton, J. D. (1974) *Phytopathol. Z.* **81**, 56.
361. Maraite, H. (1973) *Physiol. Plant Pathol.* **3**, 29.
362. Walker, J. R. L. (1969) *Phytochemistry* **8**, 561.
363. Yoshida, H. (1883) *J. Chem. Soc.* **43**, 472.
364. Franke, W. (1960) *Handbuch der Pflanzen Physiologie* Vol. 12/1, p. 401, Springer, Berlin.
365. Laborde, J. (1897) *C. R. Acad. Sci. Paris* **125**, 248.
366. Kéilin, D. and Mann, T. (1940) *Nature* **145**, 304.
367. Nakamura, T. and Ogura, Y. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P. and Blumberg, W. E., eds.), p. 389. Academic Press, New York.
368. Joel, D., Marbach, I. and Mayer, A. M. (1978) *Phytochemistry*, **17**, 796.
369. Lehman, E., Harel, E. and Mayer, A. M. (1974) *Phytochemistry* **13**, 1713.
370. Mayer, A. M. and Harel, E. (1968) *Phytochemistry* **7**, 1253.
371. Wosilait, W. D., Nason, A. and Terrell, A. J. (1954) *J. Biol. Chem.* **206**, 271.
372. Harkin, J. M., Larsen, M. J. and Obst., J. R. (1974) *Mycologia* **66**, 469.
373. Froehner, S. C. and Eriksson, K. E. (1974) *J. Bacteriol.* **120**, 458.
374. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 191.
375. Lindeberg, G. and Fähraeus, G. (1952) *Physiol. Plant.* **5**, 277.
376. Omura, T. (1961) *J. Biochem.* **50**, 264.
377. Harkin, J. M. and Obst, J. R. (1973) *Science* **180**, 296.
378. Fåhraeus, G. and Ljunggren, H. (1961) *Biochim. Biophys. Acta* **46**, 22.
379. Brown, R. and Bocks, S. M. (1963) in *Enzyme Chemistry of Phenolic Compounds* (Pridham, J. B., ed.), p. 129. Pergamon Press, Oxford.
380. Clutterbuck, A. J. (1972) *J. Gen. Microbiol.* **70**, 423.
381. Iwasaki, H., Matsubara, T. and Mori, T. (1967) *J. Biochem.* **61**, 814.
382. Hoffmann, P. and Esser, K. (1977) *Arch. Mikrobiol.* **112**, 111.
383. Molitoris, H. P. and Esser, K. (1970) *Arch. Mikrobiol.* **72**, 267.
384. Malmström, B. G., Reinhammar, B. and Vanngard, T. (1970) *Biochim. Biophys. Acta* **205**, 48.
385. Dubernet, M. and Ribereau-Gayon, P. (1973) *C. R. Acad. Sci. Paris* **277**, 975.
386. Lalitha, H. and Sirsi, M. (1972) *Arch. Mikrobiol.* **84**, 350.
387. Reinhammar, B. (1970) *Biochim. Biophys. Acta* **205**, 35.
388. Wherland, S., Goldberg, M. and Pecht, I. (1978) *Biochim. Biophys. Acta*, (submitted).
389. Fåhraeus, G. and Reinhammar, B. (1967) *Acta Chem. Scand.* **21**, 2367.
390. Mayer, A. M., Marbach, I., Marbach, A. and Sharon, A. (1977) *Phytochemistry* **16**, 1050.
391. Esser, K. and Minuth, W. (1971) *Eur. J. Biochem.* **23**, 484.
392. Matsubara, T. and Iwasaki, H. (1972) *Bot. Mag.* **85**, 71.
393. Molitoris, H. P., van Breeman, J. F. L., van Bruggen, E. F. J. and Esser, K. (1972) *Biochim. Biophys. Acta* **271**, 286.
394. Jonsson, M., Petterson, E. and Reinhammar, B. (1968) *Acta Chem. Scand.* **22**, 2135.
395. Cheung, D. S. M. and Marshal, K. C. (1969) *Biochim. Biophys. Acta* **178**, 177.
396. Siiman, O., Young, N. M. and Corey, P. R. (1976) *J. Am. Chem. Soc.* **98**, 744.
397. Briving, C. and Deinum, J. (1975) *FEBS Letters* **51**, 43.
398. Mondovi, B., Graziani, M. T., Mims, W. B., Oltzik, R. and Peisach, J. (1977) *Biochemistry* **16**, 4198.
399. Pecht, I., Farver, O. and Goldberg, M. (1977) *Adv. Chem. Ser.* **162**, 179.
400. Malkin, R., Malmström, B. G. and Vanngard, T. (1969) *Eur. J. Biochem.* **7**, 253.
401. Graziani, M. T., Morpurgo, L., Rotilio, G. and Mondovi, B. (1976) *FEBS Letters* **70**, 87.
402. Broman, L., Malmström, B. G., Aasa, R. and Vanngard, T. (1963) *Biochim. Biophys. Acta* **75**, 365.
403. Tang, S. P. and Coleman, J. E. (1968) *J. Biol. Chem.* **243**, 4286.
404. Nakamura, T. (1960) *Biochem. Biophys. Res. Commun.* **2**, 111.
405. Nakamura, T. (1961) in *Free Radicals in Biological Systems* (Blois, M. S., Brown, H. W., Lemmon, R. M., Lindblom, R. O. and Weissbluth, M., eds.), p. 169. Academic Press, New York.
406. Aasa, R., Branden, R., Deinum, J., Malmström, B. G., Reinhammar, B. and Vanngard, T. (1976) *FEBS Letters* **61**, 115.
407. Andreasson, L.-E. and Reinhammar, B. (1976) *Biochim. Biophys. Acta* **445**, 579.
408. Farver, O., Goldberg, H., Lancet, D. and Pecht, I. (1976) *Biochem. Biophys. Res. Commun.* **73**, 494.
409. Farver, O., Goldberg, M., Wherland, S. and Pecht, I. (1978) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
410. Branden, R. and Deinum, J. (1977) *FEBS Letters* **73**, 144.
411. Bocks, S. M. (1967) *Phytochemistry* **6**, 777.
412. Froehner, S. C. and Eriksson, K.-E. (1974) *J. Bacteriol.* **120**, 450.
413. Fåhraeus, G., Tullander, V. and Ljunggren, H. (1958) *Physiol. Plant.* **11**, 631.
414. Johansson, M. and Hagerby, E. (1974) *Physiol. Plant.* **32**, 23.
415. Grabbe, K., Koenig, R. and Haider, K. (1968) *Arch. Mikrobiol.* **63**, 133.
416. Leonowicz, A. and Trojanowski, J. (1965) *Acta Biochim. Pol.* **22**, 291.
417. Turner, E. M., Wright, M., Ward, T., Osborne, D. J. and Self, R. (1975) *J. Gen. Microbiol.* **91**, 167.
418. Martinelli, S. D. (1972) *Biochem. J.* **127**, 16P.
419. Haider, K. and Trojanowski, J. (1975) *Arch. Mikrobiol.* **105**, 33.
420. Blaich, R. and Esser, K. (1975) *Arch. Mikrobiol.* **103**, 271.
421. Ander, P. and Eriksson, K-E. (1977) *Physiol. Plant.* **41**, 239.
422. Ander, P. and Eriksson, K-E. (1976) *Arch. Mikrobiol.* **109**, 1.
423. Kirk, T. K., Connors, W. J. and Zeikus, J. G. (1977) *Recent Adv. Phytochem.* **11**, 369.
424. Ward, E. W. B. and Stoessl, A. (1973) *Phytopathology* **62**, 1186.
425. Rubin, B. A. and Artsikovskaya, E. V. (1964) *Annu. Rev. Phytopathol.* **2**, 157.
426. Dawson, C. R., Strothkamp, K. G. and Krul, K. G. (1975) *N.Y. Acad. Sci.* **258**, 209.
427. Prillinger, H. and Esser, K. (1977) *Mol. Gen. Genet.* **156**, 333.
428. Joslyn, M. and Ponting, J. (1951) *Adv. Food Res.* **3**, 1.
429. Swain, T. (1962) in *The Chemistry of the Flavonoids* (Geissman, T. A., ed.), p. 513. Pergamon Press, Oxford.
430. Sakamura, S., Shibusa, S. and Obata, Y. (1966) *J. Food Sci.* **31**, 317.
431. Amorim, H. V. and Amorim, V. L. (1977) in *Enzymes in Food and Beverage Processing* (ACS Symposium Series 47), p. 27.

432. Sanderson, G. W. and Coggon, P. (1977) in *Enzymes in Food and Beverage Processing* (ACS Symposium Series 47), p. 12.
433. Holden, M. (1961) *Scientific and Technical Surveys*, No. 38. p. 31. The British Food Manufacturing Industries Research Association, Leatherhead.
434. Mayer, A. M. (1978) *Proc. III^e Symp. International d'Oenologie*, Bordeaux, 21–25 June, 1977.
435. Freeman, H. C. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P. and Blumberg, W. E., eds.), p. 77. Academic Press, New York.
436. Wykes, J. R., Dunhill, P. and Lilly, M. D. (1971) *Nature New Biol.* **230**. 187.