Phytochemistry, 1971, Vol. 10, pp. 2637 to 2640. Pergamon Press. Printed in England.

SHORT COMMUNICATION

PHENYLHYDRAZINE, A SPECIFIC IRREVERSIBLE INHIBITOR OF CATECHOL OXIDASE

H. R. LERNER, E. HAREL, E. LEHMAN and A. M. MAYER
Department of Botany, The Hebrew University, Jerusalem, Israel

(*Received* 22 *March* 1971)

Abstract—Phenylhydrazine is shown to specifically inhibit a number of catechol oxidases from plant tissues. A laccase-like enzyme from peaches is not inhibited while ascorbic oxidase is only partly inhibited by relatively high concentrations of the inhibitor. Inhibition is non-competitive and irreversible and increases with time of incubation. Inhibition is dependent on the presence of oxygen during incubation of the enzyme with inhibitor but independent of the presence of the phenolic substrate.

INTRODUCTION

A CONSIDERABLE number of inhibitors of catechol oxidase (E.C. 1.10.3.1) are known. Most of these inhibitors are chelators of copper and are therefore quite non-specific. Derivatives of phenyl thiourea are known to inhibit catechol oxidase, and although it is not certain that they react with copper in the enzyme, they are also fairly unspecific. 2-Mercaptothiazole has been shown to inhibit catechol oxidase from bananas, but although fairly specific in its action, inhibition is released on incubation of the inhibitor with the enzyme, apparently due to its partial oxidation.[1] Other rather specific inhibitors of catechol oxidase are *p*-nitrophenol and 2,3-naphthalenediol. The latter has been shown to be a competitive inhibitor of the enzyme.[2] However, both *p*-nitrophenol and 2,3-naphthalendiol are active only at relatively high concentrations, 10^{-3} M and above. Thus there is a lack of specific, highly active inhibitors of catechol oxidase. In this paper we show that phenylhydrazine might be such an inhibitor.

RESULTS AND DISCUSSION

The inhibitory action of phenylhydrazine was examined on a number of enzymes of the catechol oxidase type. The dependence of percentage inhibition on length of preincubation with inhibitor is shown in Fig. 1. The catechol oxidases from grape chloroplasts (Fig. 1a) apple chloroplasts (Fig. 1b) and potatoes (Fig. 1d) are inhibited almost completely by 5×10^{-4} M phenylhydrazine within 20 min. In contrast the mushroom tyrosinase is only inhibited by 50% at this concentration and requires 10^{-3} M phenylhydrazine for effective inhibition (Fig. 1c). A similar situation was observed for the catechol oxidase prepared from sugar beet chloroplasts (Fig. 1f). Inhibition of catechol oxidase from apricots was also time dependent—60% inhibition was obtained after 20 min incubation with 5×10^{-5} M phenylhydrazine.

In order to determine whether other copper enzymes are inhibited by phenylhydrazine we examined its effect on a laccase-like enzyme from peaches and on cucumber ascorbic acid oxidase. The laccase-like enzyme from peaches was not inhibited at all by phenylhydrazine concentrations up to 10^{-3} M. In fact, phenylhydrazine appeared to be a substrate

[1] J. K. PALMER and J. B. ROBERTS, *Science* **157**, 200 (1967).
[2] A. M. MAYER, E. HAREL and Y. SHAIN, *Phytochem.* **3**, 447 (1964).

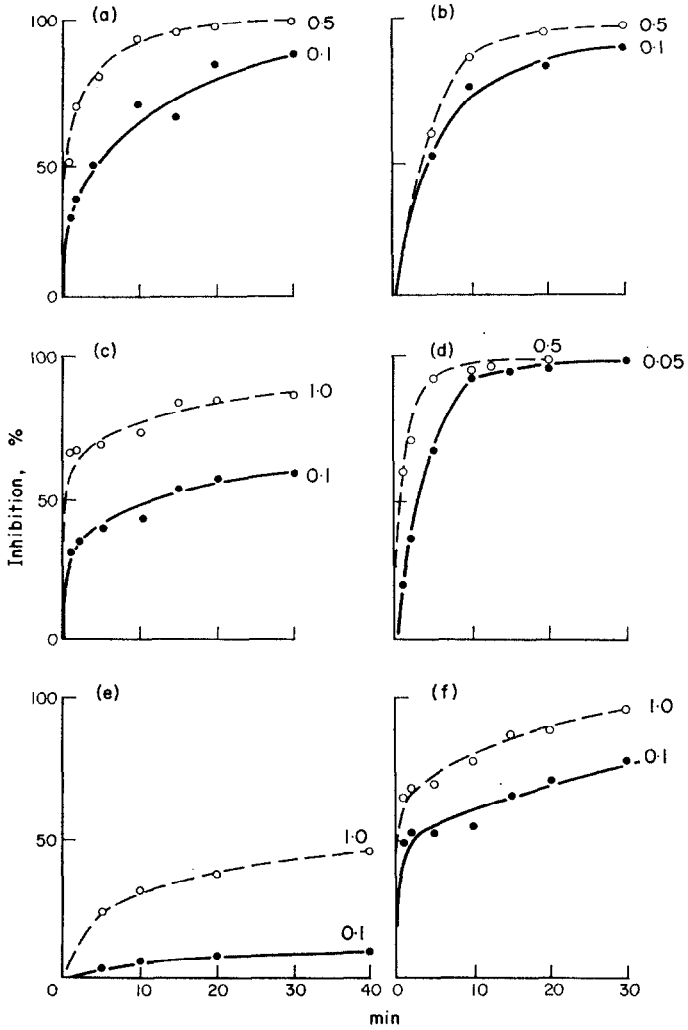

FIG. 1. INHIBITION OF CATECHOL OXIDASES AND ASCORBIC ACID OXIDASE AFTER PREINCUBATION WITH PHENYLHYDRAZINE.

Enzyme was preincubated with inhibitor in buffer for various lengths of time after which substrate was added and activity measured using oxygen electrode. The amounts of enzymes from the various sources were adjusted to give approximately the same rate of oxygen consumption per reaction mixture. Catechol oxidases from grape (a) apple (b) mushroom (c) potato (d) and sugarbeet (f). Ascorbic oxidase from cucumber (e). Numbers near curves indicate phenlhydrazine concentration in mM.

for this enzyme. In the case of cucumber ascorbic oxidase—even after incubation for 1 hr with 5×10^{-4} M phenylhydrazine, less than 30% inhibition was obtained and 10^{-3} M phenylhydrazine inhibited by less than 50% after 40 min (Fig. 1e). Cytochrome c reductase from lettuce was also not inhibited by 5×10^{-4} M phenylhydrazine. It may also be recalled that isocitric lyase is assayed in the presence of phenylhydrazine,[3] and therefore provides an additional example of an enzyme not inhibited by phenylhydrazine.

In all cases inhibition was not reversed by dialysis against suitable buffer for 24 hr. This was true both for the catechol oxidases and ascorbic acid oxidase from cucumber.

Lineweaver and Burk plots showed that inhibition of catechol oxidase by phenylhydrazine was non-competitive in the case of the grape and apricot enzymes using 4-methylcatechol as substrate. In the case of the apple enzyme there was an indication for a mixed type inhibition.

Inhibition of catechol oxidase increases with time of incubation of enzyme with the inhibitor (Fig. 1). This time dependent inhibition by phenylhydrazine might be due to slow formation of a new inhibitory compound. Phenylhydrazine was therefore incubated in buffer for various lengths of time and its inhibitory action tested. No significant change in inhibition with time was noted. Time dependent inhibition might also be due to the reaction of the inhibitor during a later stage of the enzymic reaction, for example with an enzyme substrate complex.[4]

Since the increasing inhibition with time of incubation occurred in the absence of phenolic substrate, it cannot be due to interaction of the inhibitor with enzyme-phenol complex. However, it might be dependent on the presence of oxygen which is also a substrate in the reaction. This was tested directly. Enzyme was incubated with phenylhydrazine

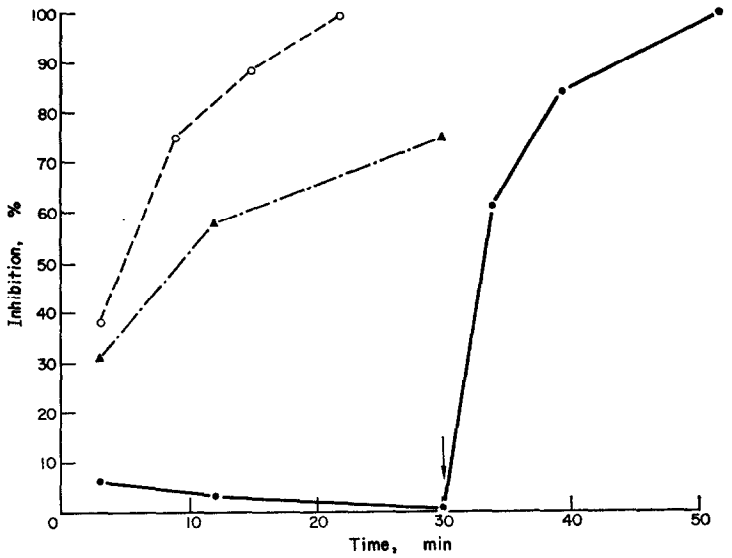

FIG. 2. INHIBITION OF CATECHOL OXIDASE FROM GRAPES UNDER VARIOUS CONDITIONS OF PREINCUBATION WITH PHENYLHYDRAZINE.

In all cases after various lengths of preincubation, the enzyme-inhibitor mixture was injected into substrate in the reaction vessel and O_2 uptake determined at pH 5·0 in phosphate-citrate buffer 5×10^{-2} M. Substrate was 4-methylcatechol, 5×10^{-3} M. Phenylhydrazine concentrations during incubation 2×10^{-4} M. Final phenylhydrazine concentration 5×10^{-5} M.

▲——▲ Preincubation of phenylhydrazine and enzyme in air.

○——○ Preincubation of phenylhydrazine and enzyme while bubbling air through reaction mixture.

●——● Preincubation of phenylhydrazine with enzyme under N_2. Arrow at point when air was bubbled through the mixture.

[3] H. L. KORNBERG, *Regulation chez les micro-organismes. Colloq. Intern. Centre. Nat. Rech. Sci.* 124, p. 193 (1963).

[4] M. DIXON and E. C. WEBB, *The Enzymes*, Longmans, London (1964).

under nitrogen and inhibition then tested (Fig. 2). Under these conditions no inhibition was observed. When air was now passed through the enzyme-inhibitor mixture, inhibition rapidly resulted (Fig. 2). In addition, if air was bubbled through the incubation mixture of enzyme and phenylhydrazine the increase in inhibition with time was more rapid than in the control (Fig. 2).

We also attempted to preincubate the enzyme with phenylhydrazine under nitrogen in the presence of 4-methylcatechol, in order to determine whether the phenolic substrate could substitute for oxygen in the reaction involving enzyme and inhibitor. No inhibition was obtained under these conditions. It is quite clear therefore that effective inhibition of catechol oxidase by phenylhydrazine is dependent on the presence of O_2.

An attempt was also made to study these effects spectrophotometrically by following the changes in the absorption spectrum of phenylhydrazine at 275 nm with time. However, although such changes were observed, they were non-specific and could also be obtained by incubating solutions of bovine serum albumen with phenylhydrazine in buffer.

These findings open up new possibilities of investigating the sequence of events in the oxidation of phenolic substrates by catechol oxidases. It should also be possible to use inhibition by phenylhydrazine in order to study the nature of the groups at or near the active center of the enzyme. This may help in understanding the reaction mechanism of oxidation of phenolic compounds by catechol oxidase.

EXPERIMENTAL

Catechol oxidase from apricots was prepared from the soluble cell fraction of ripe destoned fruits. The fruit was ground in 0·2 M phosphate buffer pH 7·3, containing 20% polyclar AT and 0·1% Na ascorbate. The supernatant, obtained after centrifugation at 10,000 *g* for 30 min was treated with 1½ vols of cold acetone (−15°). The precipitate was collected and freeze-dried. The ground powder was used as enzyme source. Laccase from peaches was prepared as described by Mayer and Harel,[5] up to and including acetone precipitation followed by lyophilization.

Catechol oxidase from grapes was obtained by grinding the fruit with 0·1 M phosphate buffer pH 7·3 containing 0·01 M Na ascorbate using 1 l. buffer/2 kg fruit. The homogenate was filtered through gauze and centrifuged at 4000 *g* for 15 min. The precipitate was extracted with 1·5% Triton X-100 in buffer pH 7·3 and the mixture centrifuged at 15,000 *g* for 1 hr. The precipitate was re-extracted and the extracts combined. An $(NH_4)_2SO_4$ fractionation was carried out and the fraction precipitating between 45% and 95% saturation collected and redissolved. This solution after dialysis was used as enzyme source.

Catechol oxidase from apples chloroplasts was prepared according to Harel *et al*,[6] up to and including Triton X-100 extraction and $(NH_4)SO_4$ fractionation.

Catechol oxidase from potatoes was prepared by grinding the peeled tubers in phosphate 0·1 M, sucrose 0·4 M buffer pH 7·3 and centrifuging at 25,000 *g* for 1 hr. The resultant supernatant was used as enzyme source. Sugarbeet chloroplasts were prepared as described by Mayer and Friend.[7] Ascorbic acid oxidase from cucumbers was prepared by grinding cucumber peel with 0·1 M phosphate buffer pH 7·7 and centrifuging at 20,000 *g* for 30 min. The supernatant served as enzyme source. Mushroom tyrosinase Grade III was obtained from Sigma.

Enzyme activity in all cases was determined using an oxygen electrode;[8] grape and apple catechol oxidase were examined at pH 5·0, mushroom tyrosinase at pH 6·5, sugarbeet chloroplasts at pH 6·3, potato catechol oxidase at pH 6·0, and ascorbic acid oxidase at pH 7·5. Substrate concentration for 4-methylcatechol was 5×10^{-3} M and 10^{-3} M for ascorbic acid. Cytochrome c reductase was determined by following the reduction of cytochrome c spectrophotometrically.[9] Phenylhydrazine A.R. was obtained from B.D.H., and purified by decolourization of an aqueous solution with active charcoal, followed by precipitation of the chloride with HCl.

Acknowledgements—This work has been supported in part by Grant No. FG-267 from the United States Department of Agriculture to A. M. Mayer.

[5] A. M. MAYER and E. HAREL, *Phytochem.* **7**, 1253 (1968).
[6] E. HAREL, A. M. MAYER and Y. SHAIN, *Phytochem.* **4**, 783 (1965).
[7] A. M. MAYER and J. FRIEND, *J. Expt. Botl.* **11**, 141 (1960).
[8] A. M. MAYER, E. HAREL and R. BEN-SHAUL, *Phytochem.* **5**, 783 (1966).
[9] L. W. ROBINSON and A. M. MAYER, *Plant Cell Physiol.* **4**, 361 (1963).