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2,3-NAPHTHALENEDIOL, A SPECIFIC COMPETITIVE INHIBITOR OF PHENOLASE

A. M. MAYER, E. HAREL and Y. SHAIN

Botany Department, Hebrew University of Jerusalem, Israel

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Abstract—2,3-Naphthalenediol is shown to be a competitive inhibitor of phenolases from a number of plant species, including those in chloroplasts, mitochondria and soluble fractions prepared from apples. A soluble laccase-like enzyme present in peaches however is not inhibited by the diol. The inhibitory properties of other naphthalene derivatives were investigated. The inhibition of phenolase by the diol is discussed on the basis of its inability to undergo keto-enol tautomerism and the results related to the possible reaction mechanism of the oxidation of phenols by phenolase.

VERY few specific inhibitors of phenolase are known. Most of those that are used act by complexing the copper in the enzyme and these also inhibit iron-containing enzymes to a greater or lesser extent.[1-4] However, a few compounds are known, such as *p*-nitrophenol and salicylaldoxime, which show a fairly good specificity for phenolase.

During studies on the phenolase from apples it was noted that 2,3-naphthalenediol was not a substrate. This compound is one of the few ortho dihydric phenols which do not form quinones, and this is ascribed to a fixation of the double bonds near the hydroxyl groups.[5] It seemed possible therefore that 2,3-naphthalenediol might inhibit phenolase and in fact we have shown that the compound is a highly specific competitive inhibitor, but that it does not affect laccase activity.

RESULTS AND DISCUSSION

The effect of 2,3-naphthalenediol on the phenolase activity of a chloroplast and a mitochondrial fraction prepared from apples, variety "Grand Alexander", was first studied. From Fig. 1 it can be seen that typical curves, showing competitive inhibition, are obtained when catecholase activity is studied. With the chloroplast enzyme preparation and 4-methylcatechol as a substrate a value of $K_m = 5{\cdot}64 \times 10^{-3}$ M and $K_i = 1{\cdot}01 \times 10^{-3}$ M was obtained for 2,3-naphthalenediol at pH 5·1. For mitochondria under the same conditions $K_m = 3{\cdot}6 \times 10^{-3}$ M and $K_i = 8{\cdot}54 \times 10^{-4}$ M, (Fig. 1). Cresolase activity was inhibited by 100% at pH 5·1 when substrate and inhibitor concentrations were both 5×10^{-3} M.

It was noted that inhibition of phenolase activity showed a slight lag phase both with chloroplast and mitochondrial preparations. This might be due to the fact that inhibition is

[1] C. R. DAWSON and W. B. TARPLEY, in *The Enyzmes* (Edited by J. B. SUMNER and K. MYRBÄCK), Vol. II, p. 454, Academic Press, N.Y. (1951).
[2] W. O. JAMES, *Plant Respiration*, Oxford (1953).
[3] J. D. BONNER, Jr., *Ann. Rev. Plant Physiol.* **8**, 427 (1957).
[4] D. HACKETT, *Ann. Rev. Plant Physiol.* **10**, 113 (1959).
[5] N. DONALDSON, *The Chemistry and Technology of Naphthalene Compounds*, Edward Arnold Publishers, London (1958).

caused by the semiquinone form of 2,3-naphthalenediol which is said to exist.[5,6] However, addition of ascorbic acid to the reaction mixture, which would maintain the inhibitor in the fully reduced form, did not abolish the lag phase which is apparently due to slow penetration of the inhibitor into the particles. When the particulate fraction was pre-incubated with the inhibitor the lag period was abolished entirely.

It seemed important to determine how general is the effect of 2,3-naphthalenediol on phenolase activity, and its action on the enzymes from a number of sources was investigated (Table 1). With one exception, the soluble fraction of peaches, 2,3-naphthalenediol very

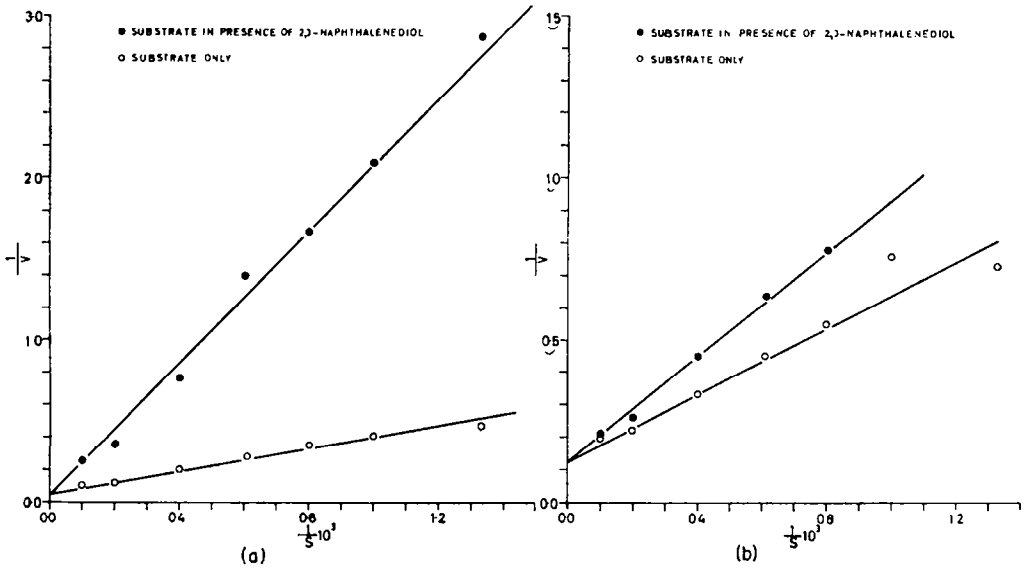

FIG. 1. COMPETITIVE INHIBITION OF 2,3-NAPHTHALENEDIOL IN THE OXIDATION OF 4-METHYLCATECHOL IN (a) CHLOROPLAST and (b) MITOCHONDRIAL FRACTIONS ISOLATED FROM APPLES.

pH = 5·1, inhibitor concentration 5×10^{-3} M.
● Substrate + naphthalenediol.
○ Substrate alone.

effectively inhibited the enzymic oxidation of 4-methylcatechol. It appeared likely that the soluble enzyme preparation from peaches did not contain a phenolase, and a number of experiments were made to confirm this. Table 2 shows a comparison of the effect of a number of inhibitors on two of the peach enzyme preparations, and it will be seen that they are entirely different in their response. The possibility that the peach soluble enzyme preparation contained a peroxidase was excluded by the fact that oxygen uptake in the presence of 4-methylcatechol with or without the addition of catalase and ethanol to the reaction mixture (10 mg of ethanol and 15, 75 or 150 catalase units were added per flask) did not in any way effect oxygen uptake. It was also shown that the soluble enzyme preparation did not oxidize ascorbic acid, thus excluding the involvement of ascorbic acid oxidase in the reaction.

The nature of the active enzyme in the soluble peach fraction can be deduced from the fact that it readily oxidized quinol and *p*-phenylenediamine. It is clear from this that the enzyme in the soluble fraction of peaches is laccase-like in nature. The possibility that this enzyme

[6] A. VON TREBST and H. ECK, *Z. Naturforsch.* **18b**, 105 (1963).

TABLE 1. INHIBITION OF PHENOLASE ACTIVITY FROM VARIOUS PLANT SOURCES BY 2,3-NAPHTHALENEDIOL

Plant source	Sub-cellular fraction	pH of reaction	% Inhibition
Apple, var. "Grand Alexander"	Chloroplasts	5·1	64
	Chloroplasts	7·3	93
	Mitochondria	5·1	73
	Mitochondria	7·3	95
	Soluble	5·1	69
Peach, var. "Salvey"	Chloroplasts	7·3	77
	Mitochondria	7·3	79
	Soluble	7·3	0
Lettuce seeds, var. "Grand Rapids"	Mitochondria	6·3	83
	Soluble	7·3	88
Sugar beet leaves, var. "Zwaanesse III"	Soluble	7·3	67
Potato tubers, var. "Up to Date"	Soluble	7·3	78

The Warburg flasks contained 0·4 ml enzyme preparation, 0·4 ml, 0·4 M sucrose–0·1 M phosphate buffer, at the desired pH, and 0·4 ml 2,3-naphthalenediol, 2×10^{-2} M or water. 0·4 ml 4-methylcatechol 2×10^{-2} M was added from the side-arm at zero time. Temperature 25°.

originates from fungal infection of the fruit was excluded by testing the flesh of peaches for the presence of fungi which were shown to be absent by conventional mycological techniques.

It can therefore be concluded that 2,3-naphthalenediol inhibits phenolase activity with a high degree of specificity, without affecting laccase activity. The inhibition of phenolase activity by 2,3-naphthalenediol is completely reversible as shown by the fact that apple chloroplasts incubated with inhibitor (1×10^{-2} M) in buffer, centrifuged, and resuspended in pure buffer showed full activity.

TABLE 2. THE EFFECT OF VARIOUS INHIBITORS ON THE OXIDATION OF 4-METHYLCATECHOL BY THE PHENOLASE IN THE CHLOROPLAST AND SOLUBLE FRACTIONS FROM PEACHES VAR. "SALVEY"

Inhibitor	Concentration, M	% Inhibition	
		Chloroplasts	Soluble fraction
Phenylthiourea	5×10^{-4}	87	3
Salicylaldoxime	5×10^{-3}	7	0
Dieca	5×10^{-5}	48	24
CO/O_2*	90/10	55	0
KCN	$1{\cdot}5 \times 10^{-5}$		+10 enhancement
NH_2OH	5×10^{-3}		0

pH of the reaction mixtures 7·3. Other conditions as in Table 1.

* As control for the reaction in presence of 90% CO/10% O_2 an atmosphere of 90% N_2/10% O_2 was used. The gas mixtures were prepared according to Umbreit *et al.*[7] Illumination with 45 lx (in the Warburg flasks) had no effect either in chloroplasts or soluble fraction.

[7] W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, *Manometric Techniques*, Burgess Pub. Co. (1957).

In order to establish further the nature of the inhibition caused by 2,3-naphthalenediol a number of other naphthalene derivatives were tested (Table 3). As can be seen from these results, 2-amino-3-naphthol and 2,3-diaminonaphthalene both inhibited, although they were less effective than the diol. However, 6,7-dihydroxynaphthalene-2-sulphonic acid did not inhibit phenolase at pH 7·3. At pH 5·1, however, the sulphonic acid derivative shows a clear-cut inhibitory effect of the soluble fraction (Table 3). Since sulphonic acid has a low pH it seemed possible that the lack of inhibition by this compound at the higher pH is in fact due to a repulsion between the negative charge of the inhibitor and a negative group on the enzyme surface which becomes increasingly ionized above pH 5·1. In the chloroplasts at the lower pH lack of entry of the inhibitor might account for the lack of inhibition.

The inhibition of phenolase by 2,3-naphthalenediol is relevant to the mechanism of attack of phenolase on phenolic substances. It was suggested[8] that the attack is electrophilic and

TABLE 3. THE EFFECT OF VARIOUS NAPHTHALENE DERIVATIVES ON THE OXIDATION OF 4-METHYLCATECHOL

		% Inhibition					
Plant source	Sub-cellular fraction	2,3-Naphthalenediol	6,7-Dihydroxy, 2-naphthalenesulfonic acid*	2-Naphthol-3,6-disulfonic acid	3-Hydroxy, 2-naphthoic acid	Naphthalene-2,3-diamine	2-Amino-3-naphthol
Apples	Chloroplasts	94	0	+9	8	39	42
	Mitochondria	95	0	+12	34	37	38
Sugar beet, leaves	Soluble	67	0	+13	0	18	21

pH 7·3. Other conditions as in Table 1.

* This compound showed no inhibition to the apple chloroplasts at pH 5·1, but inhibited the soluble fraction 25%. With *p*-cresol as the substrate it showed no inhibition of the chloroplast fraction at pH 7·3, and 76% at pH 5·1.

that the phenol must be able to undergo ketonization. The latter suggestion is amply supported by the fact that the naphthalenediol is not oxidized. This lack of oxidation can be ascribed directly to its inability to undergo keto-enol tautomerism.

EXPERIMENTAL

Apples were divided into peel and flesh. They were macerated on a kitchen shredder and each part was disintegrated in sucrose–0·4 M, phosphate–0·1 M buffer, pH 7·3 in the presence of 0·01 M sodium ascorbate in a Waring blender. The homogenate was filtered through cotton gauze and the pH readjusted to 7·3. The homogenate was then centrifuged at 400 *g* for 5 min. The crude supernatant so obtained served as a source for the various fractions.

The *chloroplast fraction* was obtained by centrifuging the supernatant for 10 min at 1000 *g*. The precipitate, here called chloroplasts, was washed and resuspended in the required buffer. The supernatant from this separation was then centrifuged for 5 min at 5000 *g* to remove chloroplast fragments and other ill-defined particles and the precipitate discarded. The supernatant was next centrifuged for 30 min at 20,000 *g*, and the precipitate washed and

[8] A. M. MAYER, *Phytochemistry* **1**, 237 (1962).

recentrifuged for 20 min at 20,000 *g*, and finally resuspended in the required buffer. This fraction is termed the mitochondrial fraction.

The first supernatant of the last separation was centrifuged at 100,000 *g* for 1 hr. The resultant supernatant is referred to as the 'soluble' fraction. This fraction was dialysed against buffer for 24 hr before use.

The enzymes from the other plant tissues were prepared in essentially the same way.

Phenolase activity was determined by following O_2 uptake, using conventional Warburg techniques at 25°. Activity was calculated from the linear part of the curve, lasting 6–12 min.

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