Inhibition of Banana Polyphenoloxidase by 2-Mercaptobenzothiazole

Abstract. *2-Mercaptobenzothiazole is an exceptionally potent inhibitor of banana polyphenoloxidase; it significantly delays the onset of substrate oxidation at concentrations as low as 10^{-7}M and causes prolonged inhibition at 2×10^{-5}M or higher. Inhibition results from formation of a dissociable, mixed complex between the enzyme and the inhibitor.*

In an earlier screening of a series of potential inhibitors (*1*), 2-mercaptobenzothiazole (MBT) was the most effective inhibitor of banana polyphenoloxidase (PPO) (*2*). We have confirmed and amplified these findings and studied the mechanism of inhibition.

Aqueous solutions of the inhibitor were prepared daily from 3.5*M* stock solutions (*3*), and final concentrations were confirmed by spectral analysis (log $E = 4.30$ at 313 mμ). Soluble banana PPO was prepared and assayed (*4*). The MBT was normally added to the reaction mixture just prior to adding the substrate. We added Cu^{++} as the sulfate.

At concentrations of $10^{-7}M$ to about $10^{-5}M$, MBT completely inhibited banana PPO for a time; after this there was a spontaneous and abrupt return to 60 to 100 percent of the uninhibited rate (Fig. 1). Inhibition was usually just detectable at $5 \times 10^{-8}M$. At concentrations exceeding $10^{-5}M$, the inhibition was prolonged for hours or days. Oxygen uptake (measured with the oxygen electrode) was correspondingly inhibited.

Concentrations 100 to 500 times higher are required for comparable inhibition of banana PPO by even the best of other commonly used phenoloxidase inhibitors (*1*). In a few experiments, crude mushroom tyrosinase (*5*) was also severely inhibited by $10^{-5}M$ MBT.

These results and the observations that MBT inhibits potato tyrosinase (*6*) and tobacco leaf polyphenoloxidase (*7*) indicate that MBT is a potent, general inhibitor of phenoloxidases. At $10^{-4}M$, MBT will completely prevent the browning reactions in banana pulp homogenates, and we routinely incorporate this concentration into our enzyme extraction media to reduce the inactivation of enzymes by oxidized phenolic constituents. The MBT is preferable to cysteine, mercaptoethanol, or ascorbic acid for this purpose because the lower concentrations of MBT required are unlikely to have undesirable side effects. It is also potentially useful as a browning inhibitor in foods and, assuming reasonable specificity, could be useful for deciding whether phenoloxidases function as terminal oxidases in plant tissues.

We studied MBT inhibition of banana PPO in more detail to elucidate the inhibitory mechanism. The fact that pigment formation and oxygen uptake are simultaneously inhibited by MBT rules out a reducing mechanism. We assumed that banana PPO is a copper metalloenzyme, like other phenoloxidases, and looked for evidence of interactions between MBT and the enzymically bound copper.

Addition of Cu^{++} to the assay mixture reduced or completely overcame inhibition by MBT (Fig. 2). Banana PPO was neither inhibited nor activated by such concentrations of Cu^{++}.

Complex formation between copper and MBT was indicated by marked changes in the absorption spectrum of $10^{-4}M$ solutions of MBT on addition of Cu^{++}. The method of continuous variations (*8*) showed that complex formation involved 1 mole of copper and 2 moles of MBT.

Inhibition was instantaneous. Incubation of PPO with MBT for up to 30 minutes prior to addition of substrate had no effect on the inhibition. In the presence of substrate, the delay period was decreased by increasing amounts of PPO, but it was still observed when we used quantities of PPO four to five times higher than those used in the standard assay procedure. Doubling the substrate concentration had no effect on the inhibition.

Dilution tended to reverse the inhibition, although the data were complicated by the fact that the PPO concentration affects the delay period. In a typical case, 24 milliunits of PPO exhibited a delay of 11 minutes in the presence of $10^{-6}M$ MBT and a delay of 5.5 minutes in the presence of $2 \times 10^{-7}M$ MBT. When 120 milliunits of the enzyme were incubated for 10 minutes with $10^{-6}M$ MBT and then diluted so that the resulting solution contained 24 milliunits of PPO in $2 \times 10^{-7}M$ MBT, the delay period was 3.7 minutes. Thus, the delay period on dilution of a higher concentration of MBT was approximately that found on direct exposure of the enzyme to the lower MBT concentration.

The detergent in the PPO preparation had no effect on the inhibition in view of the fact that comparable results were obtained when the enzyme was freed of detergent by the purification procedure (*4*).

When we assume that banana PPO is a copper metalloenzyme and reason from the work of Felber *et al.* (*9*), these results strongly suggest that MBT inhibits by the formation of an en-

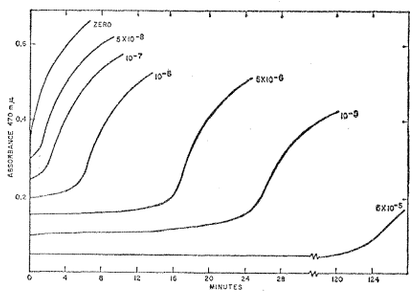

Fig. 1. Inhibition of banana polyphenoloxidase (PPO) (*4*) by sodium mercaptobenzothiazole (MBT). Initially, each spectrophotometer cell contained 100 international milliunits of PPO, 0.033*M* potassium phosphate (*p*H 7.0), and MBT at the concentrations shown on the curves. At zero time, $5 \times 10^{-3}M$ dopamine was added. Final volume, 3.0 ml. Curves have been displaced on the absorbance axis for clarity of presentation.

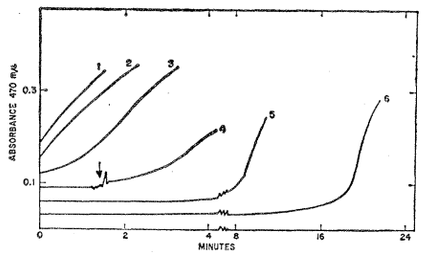

Fig. 2. Effect of Cu^{++} on MBT inhibition of banana PPO. Procedure as in Fig. 1, with 154 international milliunits of PPO, $10^{-5}M$ MBT, and Cu^{++} added as follows: control (no Cu^{++} or MBT), curve 1; reaction mix incubated 10 minutes with $10^{-4}M$ Cu^{++} prior to adding substrate, curve 2; $10^{-4}M$ Cu^{++} added just prior to substrate, curve 3; $10^{-4}M$ Cu^{++} added at arrow, curve 4; $10^{-5}M$ Cu^{++} added just prior to substrate, curve 5; no Cu^{++} added, curve 6. Time scale changes after 4 minutes; hence, rate of pigment formation for curves 5 and 6 appears greater than that for curves 1 and 2. Actually, the rate of 5 and 6 was about 70 percent of that of 1 and 2. Curves have been displaced on the absorbance axis for clarity of presentation.

zymatically inactive, dissociable, mixed complex:

$$\text{PPO-Cu} + n\,\text{MBT} \longrightarrow (\text{PPO-Cu})(\text{MBT})_n$$

Active enzyme with attached copper — Mixed complex of unknown composition

The exceptionally low concentrations of MBT made it difficult to obtain a conclusive explanation for the abrupt reversal of inhibition. The oxidation of dopamine to dopamine quinone probably proceeds slowly throughout the inhibitory period, catalyzed by trace concentrations of active enzyme in equilibrium with the inactive complex. The quinone formed then reacts with the free MBT to form the disulfide. We prepared MBT disulfide by chemical oxidation (*10*) and established that it is noninhibitory. Thus, the free MBT in the system is continually oxidized throughout the inhibition period and effectively removed from the system as the noninhibitory disulfide. Alternatively, the quinone could react with MBT to form a noninhibitory compound of thiazole and quinone (*7, 11*). In either case, as the free MBT concentration approaches zero, the equilibrium in the reaction shown will be displaced to the left, increasing the concentration of active enzyme. This further increases the rate of MBT oxidation (or reaction) and accelerates the dissociation of the inactive complex of enzyme and MBT, accounting for the abrupt reversal of inhibition. Failure to return entirely to the uninhibited rate at higher MBT concentrations may be explained by the partial inactivation of PPO by the quinone products. Phenoloxidases are notorious for such product inactivation.

Further studies of this unique inhibition, particularly the establishment of the precise structure and properties of the complex of enzyme and MBT could well provide the key to the mechanism of phenolase action.

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References and Notes

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2. Nomenclature in this report: phenoloxidase, generic term to include all enzymes which catalyze the oxidation of phenols; tyrosinase, enzyme which catalyzes the oxidation of both mono- and diphenols; polyphenol oxidase, enzyme which catalyzes the oxidation of orthodiphenols only.
3. The stock solutions were supplied by R. T. Vanderbilt Co., New York, in approximately 50 percent solution (trademark *Nacap*). The exact concentration was determined by potentiometric titrations with 0.05*N* $AgNO_3$ (silver versus calomel reference electrode). This stock solution is stable for at least a year in the dark; dilute solutions tend to become cloudy 1 or 2 days after dilution.
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12. We thank Miss Leslie Short for technical assistance.
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Photoperiodic Control of the Cloacal Gland of the Japanese Quail

Abstract. *Changes in photoperiod cause correlated changes in testes, cloacal glands, cloacal foam, and reproductive behavior of male Japanese quail. The cloacal protrusion may serve as a convenient external index of androgen, permitting repeated measurement without operation on or killing of the animal.*

The usefulness of Japanese quail (*Coturnix coturnix japonica*) for many types of biologic research has been demonstrated repeatedly; several studies have been made of their reproductive system and its response to various photoperiods (*1*). When males have been studied, the size of their testes has been the standard criterion of reproductive condition. However, the reproductive system of the quail has a distinctive feature, the cloacal gland, which has not been described in any other species or subspecies, or been systematically studied in this subspecies (*2*). This unique structure, present in both sexes of *C. c. japonica*, is especially noticeable in the male because in the reproductively active individual the gland swells, causing a large protrusion just posterior to the cloacal vent.

The cloacal protrusion of many male passerines, in breeding condition, appears similar in relative size and external appearance, but its cause is quite different. In passerines the protrusion results from increased convolution of the sperm ducts (*3*).

In Japanese quail there is no such increase; rather there is considerable growth of the glandular tissue lining the wall of the cloaca just posterior to the cloacal opening. Microscopically the gland appears as columnar epithelial cells lining a network of tubules or lumina. When the enlarged cloacal protrusion (Fig. 1, left) is gently compressed, a white foam, having the appearance and consistency of meringue, is expressed from the cloaca (Fig. 1, right (*4, 5*). During copulation the foam is transferred into the female's cloaca, but little else is known about it; there is still no clear evidence showing that the

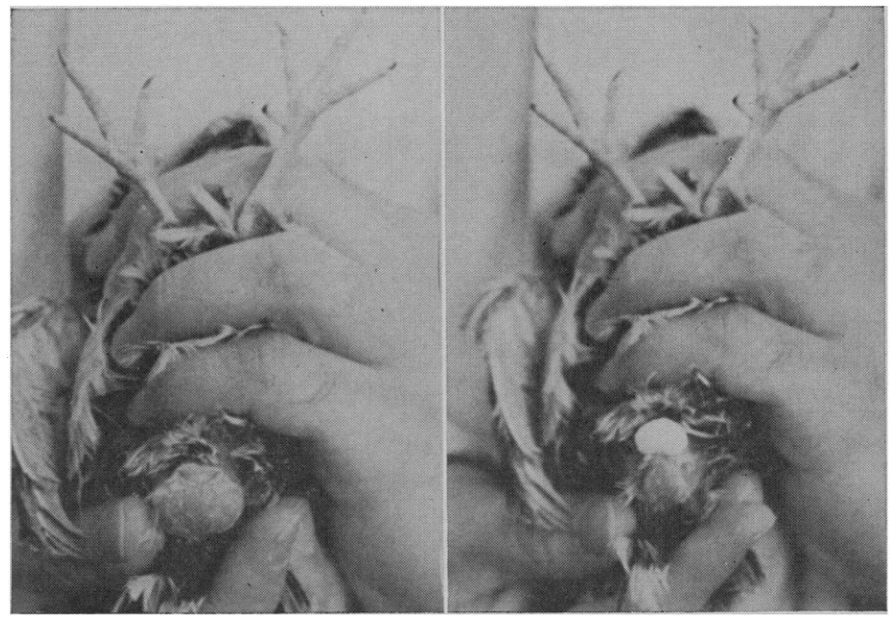

Fig. 1. (Left) External appearance of the plucked cloacal protrusion. (Right) Squeezing of the protrusion expresses the cloacal foam.