Biochem. J. (1986) 235, 91–96 (Printed in Great Britain)

Effect of methimazole on the activity of mushroom tyrosinase

Andrawis ANDRAWIS and Varda KAHN
Department of Food Technology, Agricultural Research Organization, The Volcani Center, P.O.B. 6, Bet Dagan 50250, Israel

Methimazole (1-methyl-2-mercaptoimidazole) inhibits both the mono- and the *o*-dihydroxy-phenolase activities of mushroom tyrosinase when assayed spectrophotometrically. With DL-3,4-dihydroxyphenylalanine as substrate, the inhibition was found to be a mixed-type one with K_i 4.6×10^{-6} M. We found that methimazole can interact with the oxidation products of *o*-dihydroxyphenols, probably with *o*-quinones, to form a conjugate. The conjugate formed between methimazole and *o*-benzoquinone was separated by chromatography on Sephadex G-10 and was characterized by an absorption maximum at 248–260 nm. Our data suggest that methimazole inhibits mushroom tyrosinase activity in two ways: (i) by conjugating with *o*-quinones, thereby causing an apparent inhibition in pigmented product formation as judged by the spectrophotometric assay; and (ii) by chelating copper at the active site of the enzyme, as judged by assaying the release of ^3HHO from L-[3,5-^{3}H]tyrosine.

INTRODUCTION

Tyrosinase catalyses two reactions in which molecular oxygen is the hydrogen acceptor and phenols are the hydrogen donors:

$$\text{monohydroxyphenol} + O_2 + AH_2 \rightarrow o\text{-dihydroxyphenol} + H_2O + A \quad (1)$$

$$o\text{-dihydroxyphenol} + \tfrac{1}{2}O_2 \rightarrow o\text{-quinone} + H_2O \quad (2)$$

where AH_2 represents a hydrogen donor (reductant) (Mason, 1965). Reactions 1 and 2 are referred to as the monohydroxyphenolase and *o*-dihydroxyphenolase activities of tyrosinase, respectively.

Tyrosinases isolated from different sources were shown to be inhibited by thiol compounds such as diethyldithiocarbamate (Anderson, 1968), 2-mercaptobenzothiazole (Palmer & Roberts, 1967), cysteine (Pierpoint, 1966) and GSH (Seiji *et al.*, 1969).

Methimazole is a thiourea derivative which contains a thiol group; it was reported to be an inhibitor of mushroom tyrosinase (Hanlon & Shuman, 1975) and of dopamine β-hydroxylase (Stolk & Hanlon, 1973), both being copper-containing enzymes. The inhibition of mushroom tyrosinase and of dopamine β-hydroxylase by methimazole was attributed to the Cu^{2+}-chelating ability of methimazole (Hanlon & Shuman, 1975; Stolk & Hanlon, 1973).

According to Anderson (1968), some thiol compounds act as inhibitors of tyrosinase because of their ability to reduce *o*-quinones back to the parent *o*-dihydroxyphenols (e.g. thioglycollate and mercaptobenzothiazole) (Palmer & Roberts, 1967; Pierpoint, 1966) and cysteine at certain concentrations (Pierpoint, 1966), while other thiol compounds act as inhibitors because of their ability to form a stable conjugate with the *o*-quinones formed (GSH, cysteine and methionine) (Cowley & Palmer, 1979; Gupta & Vithayathil, 1982; Pierpoint, 1966; Roston, 1960; Sanada *et al.*, 1972; Seiji *et al.*, 1969).

Since methimazole is a thiol compound, we thought it was important to find out if it inhibits tyrosinase activity solely due to its ability to chelate Cu^{2+} (as reported by Hanlon & Shuman, 1975), or also due to its potential ability to reduce and/or to conjugate with the *o*-quinone formed in the course of the reaction.

EXPERIMENTAL

Monohydroxyphenolase activity

Monohydroxyphenolase activity of mushroom tyrosinase was assayed either spectrophotometrically, or by a radioassay.

Spectrophotometric assay. The reaction mixture included, in a total volume of 3.0 ml: 3 mM-L-tyrosine, 47 mM-sodium phosphate (pH 6.5) and 25 μg of mushroom tyrosinase (added last). The rate of formation of dopachrome as a function of time was followed at 475 nm. The lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the *x*-axis, as suggested by Pomerantz & Warner (1967).

Radioassay. The hydroxylation of tyrosine to dopa was measured by determining the amount of ^3HHO released from L-[3,5-^{3}H]tyrosine, essentially as described by Pomerantz (1964).

o-Dihydroxyphenolase activity

The *o*-dihydroxyphenolase activity of mushroom tyrosinase was assayed as described by Andrawis & Kahn (1985).

All the spectrophotometric measurements were conducted using a Varian 635 spectrophotometer equipped with a recorder.

Abbreviations used: mushroom tyrosinase, monophenol mono-oxygenase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), also known as phenolase, catecholase, polyphenoloxidase (PPO) and cresolase; methimazole, 1-methyl-2-mercaptimidazole (1-methyl-2-thiolimidazole); dopa, 3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenethylamine.

Chemical preparation of a solution of 4-methyl-*o*-benzoquinone

A 10 mM solution of 4-methylcatechol in 100 ml of anhydrous diethyl ether was oxidized with 0.65 g of Ag_2O (Cason, 1948), filtered, and the filtrate obtained was used as a stock of 4-methyl-*o*-benzoquinine. Aliquots of 0.1 ml of this filtrate were used as needed.

Non-enzymic synthesis of the catechol–methimazole conjugate and its separation by chromatography on Sephadex G-10

The conjugate of *o*-quinone of catechol (*o*-benzoquinone) with methimazole was prepared chemically by the method of Sanada *et al.* (1972). Catechol (440 mg) was oxidized by 4 g of Ag_2O in 200 ml of anhydrous diethyl ether, filtered and the ether layer was mixed by shaking with 100 ml of aqueous solution containing 1100 mg of methimazole. After incubation for 15 min, the aqueous layer was washed twice with 200 ml of diethyl ether to remove unreacted catechol. The aqueous layer was concentrated to 10 ml using the Speed Vac Concentrator (Savant, model RT 100A). The sample was then centrifuged at 27000 *g* for 15 min and the supernatant was saved for column chromatography.

A sample of the supernatant was applied to a column of Sephadex G-10 (2.5 cm × 60 cm) and the column was eluted with distilled water. Fractions (1.5 ml) were collected in an ISCO model 328 fraction collector attached to a UA-5 monitor, and the A_{280} was recorded.

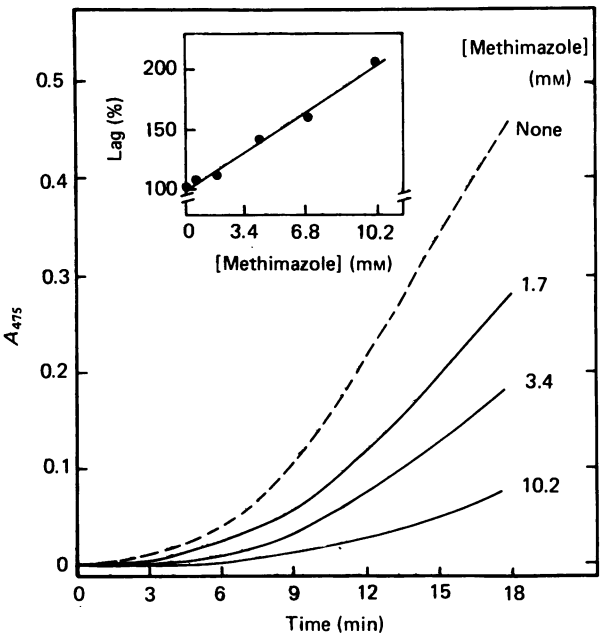


Fig. 1. Effect of methimazole on the rate of tyrosine hydroxylation by mushroom tyrosinase

The reaction mixture included, in a total volume of 3 ml, 3 mM-L-tyrosine, 47 mM-sodium phosphate buffer (pH 6.5), 25 μg of mushroom tyrosinase (added last) and methimazole, as indicated. The lag period in the absence of methimazole was 6.1 min.

Materials

Mushroom tyrosinase (grade III), DL-dopa, L-tyrosine, methimazole, dopamine, catechol and DL-cysteine hydrochloride were obtained from Sigma, 4-methylcatechol was obtained from Fluka, and L-[3,5-^{3}H]tyrosine was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). All other chemicals and biochemicals were reagent grade.

RESULTS AND DISCUSSION

Effect of methimazole on the monohydroxyphenolase activity of mushroom tyrosinase

The monohydroxyphenolase activity of mushroom tyrosinase (reaction 1 above), in the absence of an exogenous reductant, is characterized by an initial lag period.

Using tyrosine as the substrate, the data in Fig. 1 show the kinetics of tyrosine hydroxylation (dopachrome formation at 475 nm) in the presence and absence (control) of different concentrations of methimazole. The data in Fig. 1 show that methimazole extends the lag period of tyrosine hydroxylation by mushroom tyrosinase and also inhibits the rate of dopachrome formation following the lag period.

Effect of methimazole on the *o*-dihydroxyphenolase activity of mushroom tyrosinase

The data in Fig. 2 show that methimazole inhibits the *o*-dihydroxyphenolase activity of mushroom tyrosinase in the presence of each of the substrates tested. It can be seen that inhibition of the *o*-dihydroxyphenolase activity of mushroom tyrosinase by methimazole was more effective when DL-dopa or dopamine, rather than catechol or 4-methylcatechol, was used as the substrate.

Kinetic data obtained by using different concentrations of DL-dopa and different concentrations of methimazole were subjected to analysis by double-reciprocal plots of velocity versus methimazole concentration. The results shown in Fig. 3 indicate a mixed-type inhibition with K_i 4.6×10^{-6} M and K_m 0.64×10^{-3} M. These data confirm those obtained by Hanlon & Shuman (1975), who also found that methimazole inhibited mushroom tyrosinase in a mixed-type way with K_i 10^{-6} M.

The inhibition of mushroom tyrosinase activity by methimazole was reversed by exhaustive dialysis against 0.05 M-sodium phosphate buffer (pH 6.5), indicating that methimazole does not inactivate the enzyme but only inhibits it.

Does methimazole inhibit mushroom tyrosinase activity due to its ability to reduce *o*-quinones back to *o*-dihydroxyphenol?

The possibility that methimazole, being a thiol compound, can reduce *o*-quinone back to *o*-dihydroxyphenol was tested using DL-dopa as the substrate. The oxidation of dopa by mushroom tyrosinase in the presence of a compound that can reduce dopaquinone back to dopa, i.e. ascorbate, is characterized by an initial lag period in dopachrome formation (Pomerantz, 1966). As soon as the reductant is completely oxidized, the formation of dopaquinone, dopachrome and related pigmented oxidation products of DL-dopa can be detected.

Cysteine, at low concentrations, is known to reduce *o*-quinones back to *o*-dihydroxyphenols (Pierpoint, 1966;

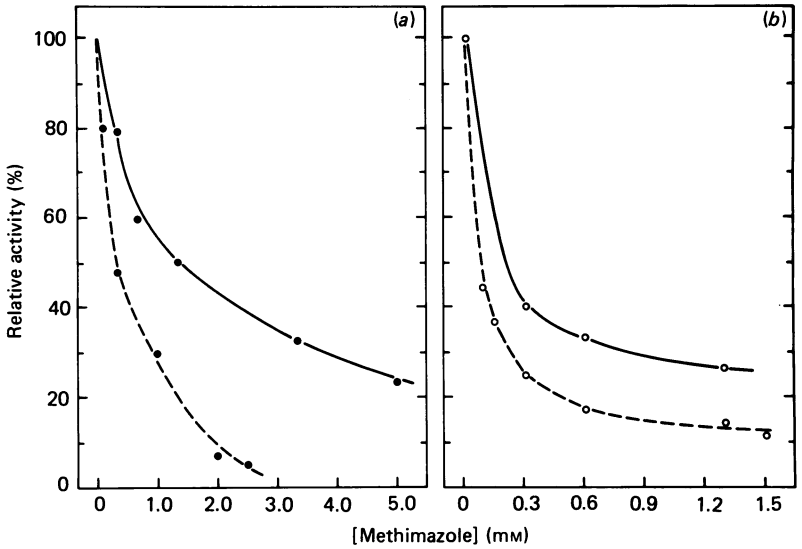


Fig. 2. Effect of methimazole on the *o*-dihydroxyphenolase activity of mushroom tyrosinase

The reaction mixture included, in a total volume of 3 ml, 6.6 mM of the indicated *o*-dihydroxyphenol, 47 mM-sodium phosphate (pH 6.5), methimazole as indicated and 10–20 μg of mushroom tyrosinase (added last). (*a*) Oxidation of 4-methylcatechol (—) and catechol (---) was followed at 410 nm; (*b*) oxidation of DL-dopa (—) and of dopamine (---) was followed at 475 nm.

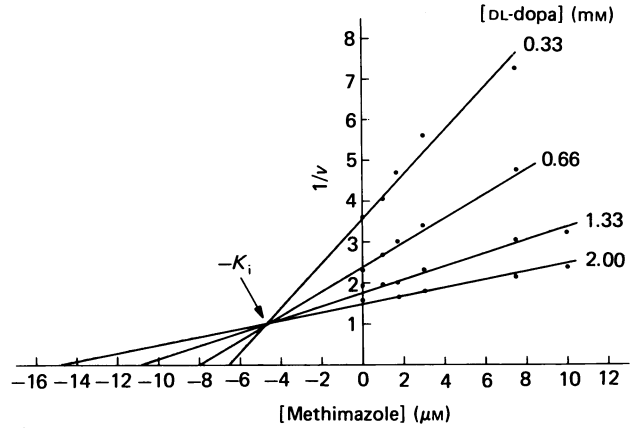


Fig. 3. Inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase by different concentrations of methimazole

The reaction mixture included, in a total volume of 3 ml, 47 mM-sodium phosphate buffer (pH 6.5), 50 μg of mushroom tyrosinase (added last), different concentrations of methimazole and DL-dopa as indicated. Velocity (v) refers to initial *o*-dihydroxyphenolase activity (ΔA_{475}/min).

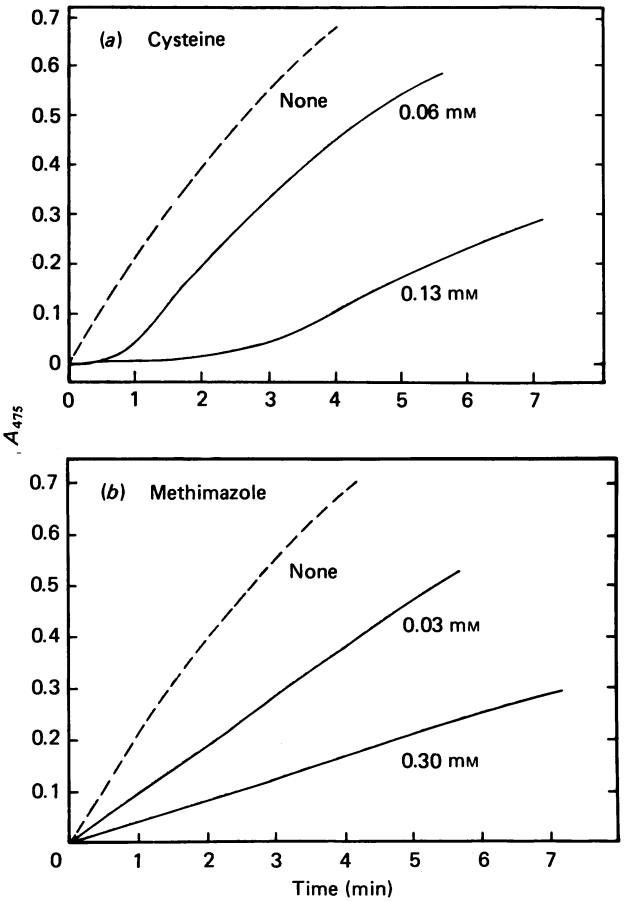


Fig. 4. Comparison between the effect of cysteine (*a*) and of methimazole (*b*) on the rate of DL-dopa oxidation by mushroom tyrosinase

The reaction mixture included, in a total volume of 3 ml, 0.66 mM-DL-dopa, 47 mM-sodium phosphate buffer (pH 6.5), cysteine or methimazole as indicated, and 20 μg of mushroom tyrosinase (added last)

Kahn, 1985). A comparison was therefore made between the effect of low concentrations of cysteine and of methimazole on the rate of DL-dopa oxidation by mushroom tyrosinase. The concentration of DL-dopa used as the substrate in this experiment was particularly low in order to enable better detection of an initial lag period, if such exists. The data in Fig. 4 show that an initial lag period in dopachrome formation (A_{475}) was detected when cysteine (at 0.06 or 0.13 mM), but not when methimazole (at 0.03 or 0.3 mM), was included in the reaction mixture.

It can therefore be concluded that the inhibition exerted by methimazole on mushroom tyrosinase activity (seen above) is not due to the ability of methimazole to reduce *o*-quinones back to *o*-dihydroxyphenols.

Does methimazole form a conjugate with *o*-quinone or with related oxidation products of *o*-dihydroxyphenols?

Several instances are known in which thiol compounds form non-enzymically a stable conjugate with *o*-quinone (Anderson, 1968; Cowley & Palmer, 1979; Pierpoint, 1966, 1969; Roston, 1960; Sanada *et al.*, 1972; Seiji *et al.*, 1969).

We tested the possible formation of a conjugate between methimazole and oxidation products of an *o*-dihydroxyphenol in a non-enzymic system as follows. *o*-Quinones can be prepared chemically by oxidizing *o*-dihydroxyphenols with an oxidant such as Ag_2O (Cason, 1948). The data in Fig. 5 show that 4-methyl-*o*-benzoquinone (prepared chemically) is characterized by a peak at around 400 nm and that the spectrum is stable for at least 5 min. When 4-methyl-*o*-benzoquinone was mixed with methimazole, the spectrum scanned after 1 min had the same shape as that of the *o*-quinone alone, but of lower absorption, whereas after 5 min of incubation the peak at 400 nm disappeared and a new one in the visible range was not detected.

Since we showed above that methimazole does not reduce *o*-quinone back to *o*-dihydroxyphenol (Fig. 4), the data in Fig. 5 strongly suggest the formation of a conjugate between 4-methyl-*o*-benzoquinone and methimazole.

Additional experiments were made in an attempt to synthesize and isolate an *o*-quinone–methimazole conjugate. The conjugate between *o*-benzoquinone and methimazole was synthesized as described in the Experimental section and its isolation was achieved by chromatography on a column of Sephadex G-10.

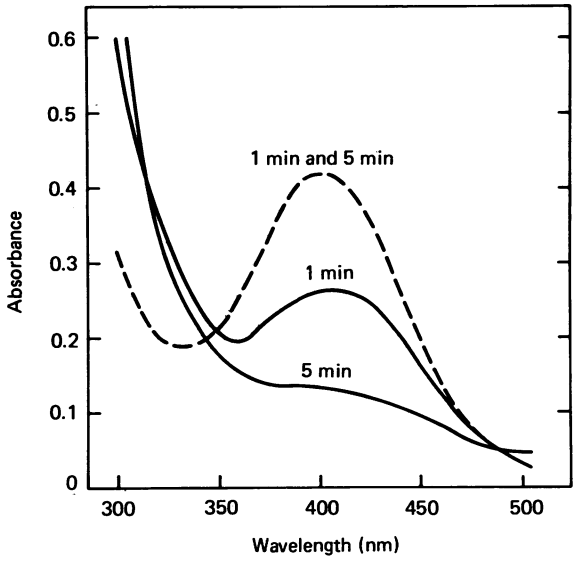


Fig. 5. Effect of methimazole on the absorption of 4-methyl-*o*-benzoquinone

The reaction mixture included, in a total volume of 3 ml, 0.33 mM-4-methyl-*o*-benzoquinone (added last) (0.1 ml of filtrate prepared chemically as described in the Experimental section), 6.6 mM-sodium phosphate buffer (pH 6.5), and either no methimazole (---) or 0.33 mM-methimazole (—). Absorbance of each mixture was recorded after 1 or 5 min (as indicated) after adding the 4-methyl-*o*-benzoquinone, using water as the blank.

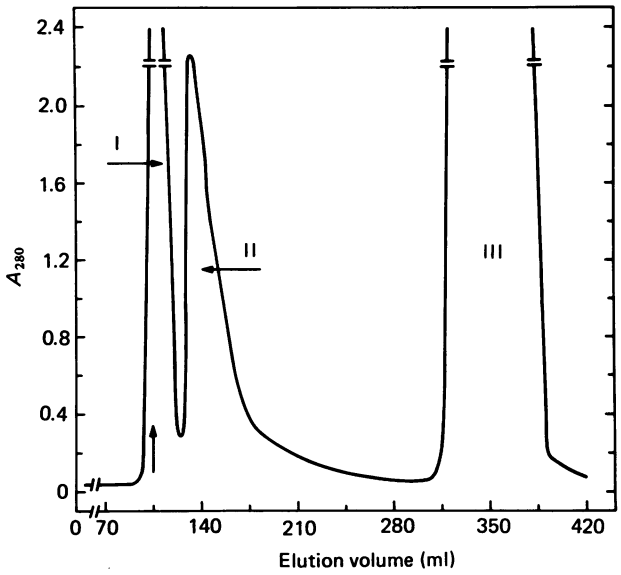


Fig. 6. Separation of the benzoquinone–methimazole conjugate by chromatography on Sephadex G-10 column

A sample of the supernatant obtained when preparing the conjugate of *o*-benzoquinone–methimazole (see the Experimental section) was applied to a Sephadex G-10 column (2.5 cm × 60 cm) and the column was eluted with water. Fractions (1.5 ml) were collected in an ISCO model 328 fraction collector attached to a UA-5 monitor and A_{280} was recorded (as an arbitrary wavelength to aid in locating the position of elution of the various components, rather than to aid in their identification).

Three regions were resolved during chromatography: I, II and III, with I being eluted at the void volume of the column (V_0) (105 ml) and II and III being eluted at volumes of 132 ml and 318 ml, respectively (Fig. 6).

The spectrum of each of the fractions eluted from Sephadex G-10 (Fig. 6) was scanned and it was found that they absorb in the u.v. region but not in the visible region of the spectrum. *o*-Benzoquinone is characterized by a peak in the visible range (388 nm) (Duckworth & Coleman, 1970). The observation that none of the fractions eluted absorb in the visible range indicates that *o*-benzoquinone itself was not present.

The u.v. spectra of fractions eluted from Sephadex G-10 in region I were characterized by a high peak at 262 nm and a shoulder at 224 nm (Fig. 7, I). Since these fractions were eluted at V_0, they must be of relatively high M_r, probably representing a polymer of *o*-benzoquinone.

The u.v. spectra of various fractions eluted from the column in region II were not identical: fractions eluted at volumes of 130–145 ml (II-I) were characterized by a peak at 260 nm (Fig. 7, II-I) and those eluted at volumes of 148–161 ml (II-2) were characterized by a peak at 248 nm and a shoulder at 310 nm (Fig. 7, II-2).

The u.v. spectra of fractions eluted from the column in region III were all the same and were characterized by a high peak at 250 nm and a small one at 210 nm (Fig. 7, III), identical with the spectrum of untreated methimazole.

The observation that the u.v. spectrum of fractions of region II was different from that of methimazole alone (a peak at 250 nm and 210 nm) and of catechol alone

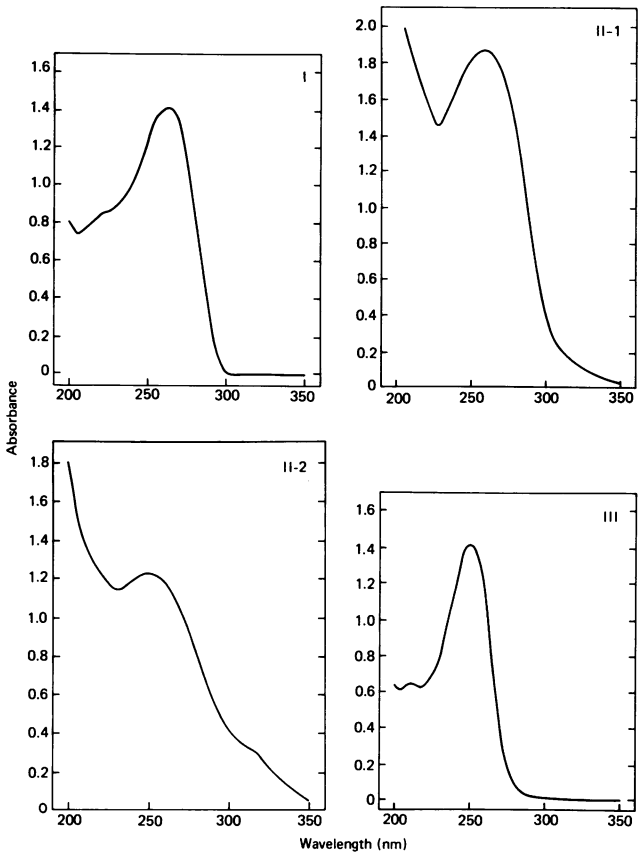


Fig. 7. U.v. spectra of the peak fractions eluted from Sephadex G-10 in regions I, II-1, II-2 and III (see Fig. 6)

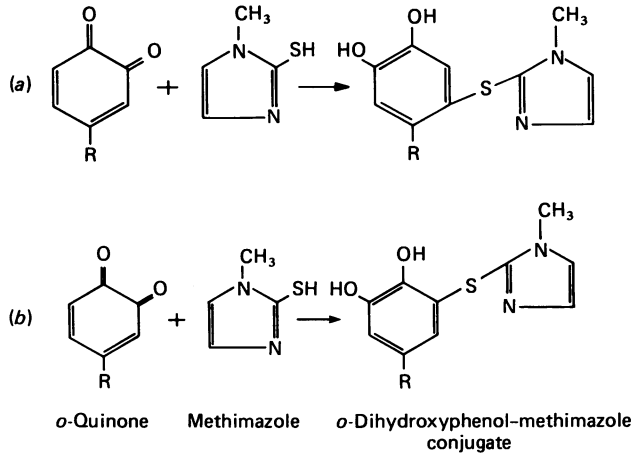
The peak fraction from region I of Fig. 6 was diluted 1:4 with water before scanning.

(278 nm and a shoulder at 212 nm) suggests that region II of Fig. 7 contained a new compound, probably the conjugate.

A control containing *o*-benzoquinone alone (prepared chemically) without methimazole was also chromatographed on a column of Sephadex G-10 and eluted with distilled water (results not shown). A major region was eluted at V_0 and a very small region was eluted at an elution volume of 280 ml. A region equivalent to region II of Fig. 6 was not detected in chromatography of the control on Sephadex G-10.

The observation that the elution profile of *o*-benzoquinone treated with methimazole (data in Fig. 6) was different from that of *o*-benzoquinone alone (control) strongly suggests that region II eluted from Sephadex G-10 (Fig. 6) represents the catechol–methimazole conjugate.

Tentatively, two structures of the conjugate likely to be formed between *o*-quinone and methimazole can be suggested; one is analogous to that suggested by Pierpoint (1966) to be formed between *o*-quinone and cysteine or that suggested by Gupta &Vithayathil (1982) to be formed between dopaquinone and methionine (Scheme 1*a*); the other is analogous to that suggested by Sanada *et al.* (1972) to be formed between *o*-benzoquinone and cysteine (Scheme 1*b*).



Scheme 1. Proposed structures of methimazole–*o*-quinone conjugate

Does methimazole inhibit mushroom tyrosinase activity also due to its ability to chelate Cu^{2+}?

Methimazole is known to be a copper chelator (Hanlon & Shuman, 1975; Stolk & Hanlon, 1973; Dobry-Duclaux & Perichon, 1976). Dobry-Duclaux & Perichon (1976) reported that methimazole and $CuSO_4$ form different complexes with different molar ratios of methimazole/Cu^{2+}.

Hanlon & Shuman (1975) compared the effect of methimazole on several copper-containing enzymes. With L-dopa as the substrate and using spectrophotometric assays, Hanlon & Shuman (1975) found that methimazole changed both the K_m and $V_{max.}$ of mushroom tyrosinase, and that methimazole behaved chiefly as a potent competitive inhibitor with K_i 10^{-6} M. They concluded that this inhibition of mushroom tyrosinase by methimazole was due to the ability of methimazole to chelate Cu^{2+}.

The data presented above showing that methimazole inhibits mushroom tyrosinase activity (Figs. 1 and 2) were obtained by assaying the rate of formation of oxidation products of mono- or *o*-dihydroxyphenols spectrophotometrically. In view of our finding that *o*-dihydroxyphenol–methimazole conjugate is formed (Figs. 5–7), it was important to determine if methimazole is only an apparent inhibitor of mushroom tyrosinase (in the sense that it decreases the extent of pigmented product formation due to its ability to conjugate with *o*-quinone), and/or if methimazole can directly inhibit tyrosinase activity by chelating to the Cu^{2+} at the active site of the enzyme.

These alternatives could best be tested by using the method of Pomerantz (1964) to assay for tyrosinase activity. The data in Fig. 8 show that methimazole extended the lag period in the release of ^3HHO from L-[3,5-^{3}H]tyrosine, and that it also inhibited the rate of tyrosine hydroxylation following the lag period.

This finding indicates that methimazole, in addition to its ability to cause an apparent inhibition, also inhibits mushroom tyrosinase directly, probably by complexing with Cu^{2+} at the active site of the enzyme. Comparison between the extent of inhibition exerted by methimazole on the rate of tyrosine hydroxylation by mushroom

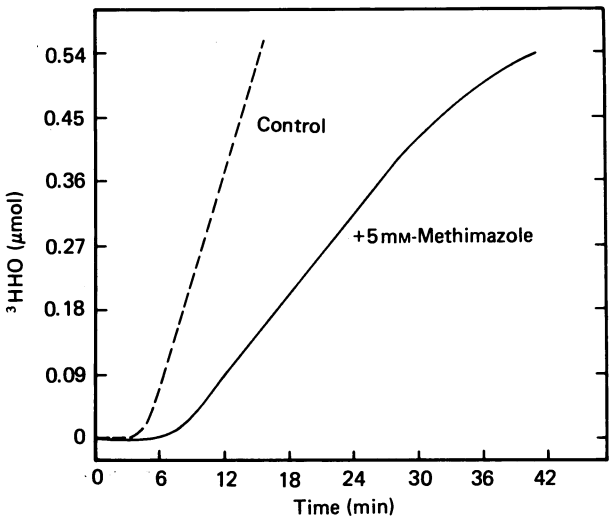


Fig. 8. Effect of methimazole on the rate of tyrosine hydroxylation by mushroom tyrosinase

The reaction mixture included, in a total volume of 6 ml, 2 mM-L-[3,5-^{3}H]tyrosine (1×10^5 c.p.m./μmol), 47 mM-sodium phosphate buffer (pH 6.5), 60 μg of mushroom tyrosinase (added last) (control) and 5 mM-methimazole where indicated.

tyrosinase, when assayed spectrophotometrically or by radioassay, showed that 5 mM-methimazole caused 80% inhibition when assayed spectrophotometrically and only 60% inhibition when assayed by the release of ^{3}HHO from L-[3,5-H^3]tyrosine.

Methimazole is not only a copper chelator but is also a powerful OH$^\cdot$ scavenger (Cohen *et al.*, 1976), and one can be tempted to think that the inhibition exerted by methimazole on the *o*-dihydroxyphenolase activity of mushroom tyrosinase could be due, in part, to this property of methimazole. However, it is very unlikely that the inhibition of mushroom tyrosinase by methimazole is due to the latter's ability to scavenge OH$^\cdot$, since it was reported that OH$^\cdot$ radicals were not detected during the enzyme reaction (Hamilton, 1974; Tomita *et al.*, 1980). Hamilton (1974) did not detect a free radical intermediate in the course of tyrosinase activity, and Tomita *et al.* (1980) concluded also that OH$^\cdot$ radicals were not detected in the course of mouse melanoma tyrosinase action on either tyrosine or on DL-dopa.

The results presented in the present paper suggest that methimazole inhibits mushroom tyrosinase activity in two ways: (i) by conjugating with *o*-quinones to form *o*-dihydroxyphenol–methimazole conjugate, which has no absorption in the visible range and probably cannot be converted to pigmented products; and (ii) by chelating Cu^{2+} at the active site of the enzyme.

This research was supported by a grant from the United States–Israel Binational Agricultural Research and Development Fund (BARD). This is contribution no. 1401-E, 1985 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

REFERENCES

Anderson, J. W. (1968) Phytochemistry **7**, 1973–1988
Andrawis, A. & Kahn, V. (1985) Phytochemistry **24**, 397–405
Cason, J. (1948) in Organic Reactions (Adams, R., Backman, W. E., Blatt, A. H., Fieser, L. F., Johnson, J. R. & Snyder, H. K., eds.), vol. 4, pp. 305–317, John Wiley, New York
Cohen, G., Heikkila, R. E., Allis, B., Cabbat, F., Dembiec, D., MacNamee, D., Mytilineou, C. & Winston, B. (1976) J. Pharmacol. Exp. Ther. **199**, 336–352
Cowley, R. C. & Palmer, J. M. (1979) Phytochemistry **18**, 729–733
Dobry-Duclaux, A. & Perichon, P. (1976) J. Chim. Phys. **73**, 1058–1067
Duckworth, H. W. & Coleman, J. E. (1970) J. Biol. Chem. **245**, 1613–1625
Gupta, M. N. & Vithayathil, P. J. (1982) Bio-Org. Chem. **11**, 101–107
Hamilton, G. A. (1974) in Molecular Mechanisms of Oxygen Activation (Hayaishi, O., ed.), pp. 405–451, Academic Press, New York
Hanlon, D. P. & Shuman, S. (1975) Experientia **31**, 1005–1006
Kahn, V. (1985) J. Food Sci. **50**, 111–115
Mason, H. S. (1965) Annu. Rev. Biochem. **34**, 595–634
Palmer, J. K. & Roberts, J. B. (1967) Science **157**, 200–201
Pierpoint, W. S. (1966) Biochem. J. **98**, 567–580
Pierpoint, W. S. (1969) Biochem. J. **112**, 619–629
Pomerantz, S. H. (1964) Biochem. Biophys. Res. Commun. **16**, 188–194
Pomerantz, S. H. (1966) J. Biol. Chem. **241**, 161–168
Pomerantz, S. H. & Warner, M. C. (1967) J. Biol. Chem. **242**, 5308–5314
Roston, S. (1960) J. Biol. Chem. **235**, 1002–1004
Sanada, H., Suzue, R., Nakashima, Y. & Kawada, S. (1972) Biochim. Biophys. Acta **261**, 258–266
Seiji, M., Yoshida, T., Itakura, H. & Irimajiri, T. (1969) J. Invest. Dermatol. **52**, 280–286
Stolk, J. M. & Hanlon, D. P. (1973) Life Sci. **12**, 417–423
Tomita, Y., Siji, M. & Igarashi, A. (1980) J. Dermatol. **7**, 377–380

Received 21 May 1985/11 October 1985; accepted 22 November 1985