ACTA CHEMICA SCANDINAVICA 20 (1966) 657–665

# Hydroxyquinone:NADH Oxidoreductase from *Aspergillus fumigatus*

## I. Isolation and Properties of the Enzyme

GÖSTA PETTERSSON

*Institute of Biochemistry, University of Lund, Lund, Sweden*

A cell-free enzyme system with the ability of catalyzing the oxidation of NADH by a large number of hydroxy- or methoxyquinonoid compounds (see Table 3) has been isolated from the mould *Aspergillus fumigatus* and purified 70-fold. Unsubstituted or methyl substituted benzo- and naphthoquinones (*e.g.* menadione), and ubiquinones (except for the lowest homologue) were not substrates for the enzyme, which, therefore, appears to be distinct from all of the quinone reductases described previously. The enzyme seemed not to require any metal ions or cofactors for activity and was not inhibited by SH—reagents, flavine antagonists, or other compounds (dicoumarol, amytal, rotenone) known to inhibit diaphorases, ubiquinone or menadione reductases. The general properties of the enzyme have been studied and its biological function is discussed.

In 1938 Anslow and Raistrick isolated fumigatin (2-hydroxy-3-methoxy-6-methyl-1,4-benzoquinone) and its hydroquinone from a certain strain of *Aspergillus fumigatus*.[1] It was early recognized that fumigatin hydroquinone might function as a biogenetic precursor of fumigatin, and in 1963 Küster and Little showed that the mould contains endo-cellular phenolases, which were proposed to be responsible for the formation of fumigatin from the corresponding hydroquinone.[2] More recent investigations have confirmed that the hydroquinone forms of fumigatin and the related quinonoid pigments isolated from the mould are the primary metabolic products; the pigments were, in fact, found to be present in the hydroquinone form during the larger part of the production phase.[3] No endo-cellular enzyme systems appeared, however, to be required for the production of the corresponding quinones, which were shown to be formed by a non-enzymatic autoxidation process.[4] On the contrary, it was found that the mould mycelium prevented the different hydroquinones from being oxidized by the air and kept the pigments in the reduced state by some active metabolic process.[3] A cell-free enzyme system carrying out the reduction of fumigatin by NADH has now been obtained from *A.*

*fumigatus* mycelium. The preparation, partial purification, and general properties of this hydroxyquinone:NADH oxidoreductase are described in the present report.

## EXPERIMENTAL

*Culture conditions. Aspergillus fumigatus*, L.S.H.T.M. A 46, was used throughout this work. The mould was grown as surface cultures at 25° in 2000 ml Fernbach flasks holding 500 ml portions of the Raulin-Thom solution described by Anslow and Raistrick.[1] After 8—10 days of growth the cultures were harvested and the mycelium removed by filtration. The mycelium could be stored at −15° for several weeks without losing its enzymatic activity.

*Isolation of the enzyme.* All operations involved in the preparation of the enzyme were (unless otherwise stated) performed at 4°. The mycelium from 8 flasks was briefly washed with distilled water and carefully dried between filter papers. The cell mass (9.4 g dry weight) was frozen to −15° and was desintegrated in the frozen state by means of a BIOX X-press.[5] After thawing the homogenate was extracted with 400 ml of distilled water, and unbroken cells and cell debris were eliminated by centrifugation (10 000 *g*; 10 min). The brownish-purple supernatant solution (pH 6.2—6.4) was stored at −15° for at least two weeks, and was then allowed to stand at 4° for another 24 h. During this treatment (aging) a fairly large amount of enzymatically inactive protein material separated, and this precipitate was removed by centrifugation (20 000 *g*; 20 min). To each ml of the supernatant solution were slowly added with stirring 30 mg (dry weight) of CM-cellulose, and the pH of the mixture was adjusted to pH 6.0. After 10 min the cellulose was removed by centrifugation and discarded, and the supernatant solution, which contained the enzymatic activity, was buffered with sodium phosphate to a final concentration of 0.2 M, pH 7.0. To each ml of the buffered enzyme solution 270 mg of ammonium sulphate were added, and the precipitate obtained was removed by centrifugation (10 000 *g*; 5 min) and discarded. The addition of a further 120 mg of ammonium sulphate per ml enzyme solution (from 45 to 60 % saturation) yielded a precipitate carrying the larger part of the enzyme activity. This precipitate was removed from the solution by centrifugation (10 000 *g*; 5 min), and was dissolved in 55 ml of 0.1 M phosphate buffer, pH 6.5; ammonium sulphate precipitations were carried out quickly. The enzyme solution was then fractionated with acetone at about −3°. The precipitate formed after addition of 35 ml of acetone was discarded and a further 30 ml (from 40 to 55 %) of acetone were added. The enzymatically active precipitate obtained was removed by centrifugation (5000 *g*; 10 min) and was dissolved in 30 ml of 0.03 M phosphate buffer, pH 5.5. In the next step the enzyme was adsorbed on a column (2 × 8 cm) of DEAE-cellulose, which had been treated with the same phosphate buffer. The DEAE-cellulose was then washed with 50 ml of 0.03 M phosphate buffer, pH 5.5, and with 150 ml of 0.03 M phosphate buffer, pH 6.5. The enzyme was finally eluted with 50 ml of 0.05 M phosphate buffer, pH 6.5.

The colourless enzyme solution prepared in this way contained 0.2—0.4 mg of protein per ml, and the specific activity was usually higher than 50 units per mg; one enzyme

*Table 1.* Purification of hydroxyquinone:NADH oxidoreductase.

| Procedure | Vol. ml | Conc. U/ml | Protein mg/ml | Spec.act. U/mg | Yield % |
|---|---|---|---|---|---|
| Initial extract | 400 | 15.0 | 16.8 | 0.89 | 100 |
| After aging | 400 | 13.8 | 10.4 | 1.33 | 92 |
| After CM-cellulose | 380 | 13.8 | 6.5 | 2.10 | 87 |
| 45—60 % ammonium sulphate ppt. | 55 | 52.4 | 8.8 | 5.96 | 48 |
| 40—55 % acetone ppt. | 30 | 42.0 | 2.6 | 16.0 | 21 |
| After DEAE-cellulose | 50 | 21.6 | 0.35 | 60.6 | 18 |

unit (U) is defined as the amount that catalyzes the oxidation of 1 μmole of NADH (the reduction of 1 μmole of fumigatin) per min under the specified assay conditions. The typical fractionation scheme given in Table 1 shows that the over-all purification achieved was about 70-fold. Investigations of the general properties of the enzyme were performed using preparations that had been purified at least 50-fold. The enzyme solution could be stored at −15° for several weeks with only minor loss of activity. For some experiments (those in which the substrate specificity and the effect of metal ions were studied) the enzyme preparation was further purified by being passed over a column (2 × 20 cm) of Sephadex G-25 gel to remove low-molecular material; this treatment did not result in any loss of activity.

*Assay method.* Hydroxyquinone:NADH oxidoreductase was assayed spectrophotometrically by measurement of the disappearance of the quinonoid substrate (generally fumigatin) at 20°. 1−5 U of enzyme solution (50−500 μl) with pH adjusted to 5.3 were diluted to a volume of 2.9 ml with 0.05 M phosphate buffer, pH 5.3, and 0.5 ml of a 14 mM solution of the quinonoid substrate in the same phosphate buffer were added. The reaction was initiated by the addition of 100 μl of a 70 mM solution of NADH in the above phosphate buffer, and was followed by measurement of the decrease in extinction of the reaction mixture at the wave-length of maximum absorption of the quinone in the visible region (550 mμ for fumigatin). The reduction of quinones with $\lambda_{max} < 450$ mμ (*e.g.* aurantiogliocladin and juglone) was measured at 450 mμ, where the absorption of NADH ($\lambda_{max} = 350$ mμ) is negligible. The initial reaction rates were obtained graphically from plots of the extinction against the reaction time (see Fig. 1).

The effect of metal ions and of other substances mentioned below on the enzymatic reaction rate was tested by addition of appropriate amounts of the compounds (metal chlorides or sulphates) dissolved in 0.05 M phosphate buffer, pH 5.3, to the reaction solution. The pH-dependence of the enzymatic reduction of fumigatin by NADH was tested over a range from pH 3 to pH 9, using various buffer systems (pH 3.0−5.0, 0.05 M acetate; pH 5.0−7.5, 0.05 M phosphate; pH 7.5−9.0, 0.05 M Tris).

*Stoichiometry of the reaction.* The stoichiometry of the enzymatic reaction was tested by carrying out the oxidation of definite amounts of NADH in the presence of excess of fumigatin and *vice versa*; the amounts of fumigatin reduced or NADH oxidized were calculated from the observed decrease in extinction at 550 and 350 mμ, respectively. The results of these studies, which are given in Table 2, indicated that equimolar amounts

*Table 2.* Stoichiometry in the enzymatic reduction of fumigatin by NADH. The amounts of the substrates are given in μmoles.

| NADH oxidized | Fumigatin reduced |
|---|---|
| 1.7 | 1.7 * |
| 3.5 | 3.5 * |
| 7.0 | 6.9 * |
| 5.0 * | 5.0 |
| 10.5 * | 10.0 |
| 19.3 * | 20.0 |

of $NAD^+$ and fumigatin hydroquinone were formed during the reaction. Similar experiments indicated that all of the quinonoid substrates tested were reduced to the corresponding hydroquinones. In a few cases (fumigatin, aurantiogliocladin, 2-hydroxy-1,4-benzoquinone, 2-hydroxy-6-methyl-1,4-benzoquinone, 2-hydroxy-3,6-dimethyl-1,4-benzoquinone, and 2-hydroxy-3,5-dimethyl-1,4-benzoquinone) the hydroquinones formed were identified on thin-layer chromatograms of ethereal extracts of the completed reaction mixtures; the techniques and solvent systems used for chromatographic separation and identification of hydroquinones have been described previously.[3]

* Substrate present in excess.

## RESULTS AND DISCUSSION

The general properties of the hydroxyquinone:NADH oxidoreductase were studied using fumigatin (2 mM) as the electron acceptor and NADH (2 mM) as the electron donor. The initial reaction rate was determined graphically from extinction/time curves (some typical assays are shown in Fig. 1), and was found to be strictly proportional to the enzyme concentration (Fig. 2).

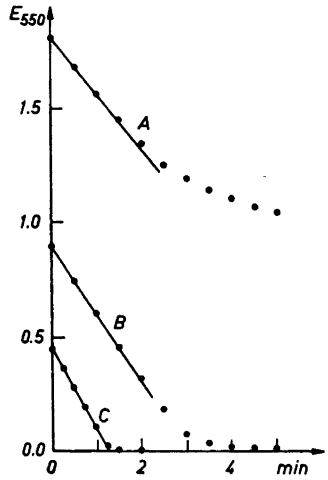


*Fig. 1.* Enzymatic (2.4 U) oxidation of NADH (2 mM) by fumigatin (4, 2, and 1 mM for curve A, B, and C, respectively).

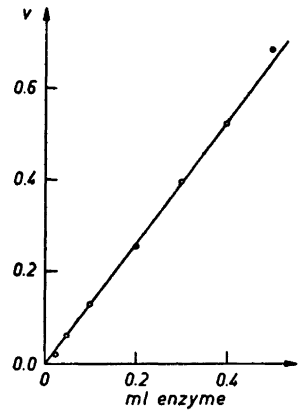


*Fig. 2.* Effect of protein concentration on the rate ($v = E_{550}$/min) of reduction of fumigatin by NADH. The reaction mixtures were 2 mM with respect to fumigatin and NADH, and contained the respective volume of enzyme solution (0.18 mg/ml; 11 U/ml) from the graph diluted to 3.5 ml with 0.05 M phosphate buffer, pH 5.3.

The stoichiometry of the reaction is shown in Table 2; there was a good molar equivalence between the amounts of NADH disappearing and the amounts of fumigatin reduced. The products formed (fumigatin hydroquinone and $NAD^+$) had no influence on the reaction rate, even when being present in large excess (10 mM). The pH-dependence of the reaction was tested over a range from pH 3 to pH 9, and the pH-optimum was found to be between 5.0 and 5.5 (Fig. 3, curve A). The effect of pH on the stability of the enzyme was tested by exposing the enzyme to a range of pH-values (pH 3—9) for 15 min, and then measuring the activity after readjusting the pH to 5.3. As seen from curve B in Fig. 3 the enzyme was found to be irreversibly inactivated below pH 5 (rapidly) as well as above pH 7 (slowly).

The purified enzyme was best stored in 0.05 M phosphate buffer solution, pH 6.5, in the frozen state at −15°. Such preparations showed little loss in

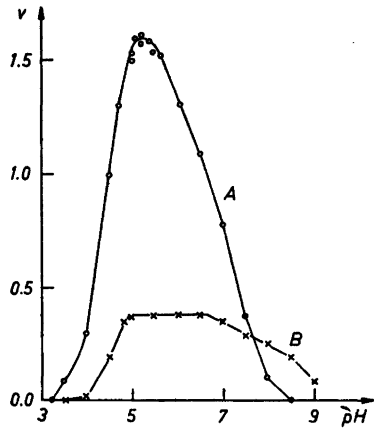


*Fig. 3.* Effect of pH on hydroxyquinone: NADH oxidoreductase. For curve A the activity ($v$ μmoles fumigatin reduced per min) was tested at the pH values given; for curve B the enzyme was exposed for 15 min to the pH values given and the activity then tested at pH 5.3.

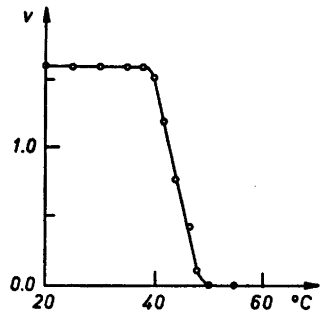


*Fig. 4.* Temperature inactivation of hydroxyquinone:NADH oxidoreductase. The enzyme was treated for 10 min at the temperatures given before the activity ($v$ μmoles fumigatin reduced per min) was tested under the usual assay conditions.

activity during several weeks of storage. At 0° they were stable for several hours, while at 20° half the enzymatic activity was destroyed in 8 h. The enzyme solution (pH 5.3) did not show any significant loss of activity by 10 min treatment at different temperatures up to 38° prior to incubations, but was rapidly inactivated at temperatures above 40° (Fig. 4).

Crude enzyme preparations (before ammonium sulphate precipitation) were capable of carrying out the reduction of fumigatin by NADPH, as well as by NADH; at corresponding concentrations of the two electron donors the reaction rate with NADH was 5—10 times higher than that with NADPH. More purified preparations were, however, specific for NADH as substrate. Since NADPH in the presence of $NAD^+$ could not replace NADH the presence of transhydrogenase activity was ruled out.

The substrate specificity of the enzyme with respect to the electron acceptor is shown in Table 3. It is seen that the enzyme preparations catalyzed the oxidation of NADH by a large variety of benzoquinonoid compounds, as well as by dibenzoquinones such as phoenicin and the naphthoquinone juglone. The presence of one hydroxyl or methoxyl group in the quinonoid nucleus appeared to be a minimal requirement for activity; unsubstituted 1,4-benzoquinone, methyl substituted benzoquinones, 1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone (menadione) were not substrates for the enzyme. Dihydroxy-, hydroxymethoxy-, and dimethoxy-1,4-benzoquinones were generally found to function as substrates, but no reaction occurred with benzoquinones (*e.g.* spinulosin) and dibenzoquinones (oosporein) containing a 2,5-dihydroxyquinonoid configuration. Among the ubiquinone (UQ) homologues

*Table 3.* Substrate specificity of hydroxyquinone:NADH oxidoreductase with respect to the electron acceptor. The reaction mixtures contained 35 μmoles of NADH, 1—2 μmoles of the respective electron acceptor tested, 0.05 mg of protein (1.7 U) diluted to a final volume of 3.5 ml with 0.05 M phosphate buffer, pH 5.3. The reaction rate is given in μmoles of NADH oxidized per min.

| Electron acceptor | Reaction rate |
|---|---|
| 1,4-Benzoquinone (BQ) | 0.0 |
| Methyl-BQ (toluquinone) | 0.0 |
| 2,6-Dimethyl-BQ | 0.0 |
| Tetramethyl-BQ | 0.0 |
| Hydroxy-BQ | 1.9 |
| 2-Hydroxy-5-methyl-BQ | 2.0 |
| 2-Hydroxy-6-methyl-BQ | 2.2 |
| 2-Hydroxy-3,5-dimethyl-BQ | 2.1 |
| 2-Hydroxy-3,6-dimethyl-BQ | 2.3 |
| 2-Hydroxy-5,6-dimethyl-BQ | 2.0 |
| 2-Hydroxy-3,5,6-trimethyl-BQ | 2.2 |
| 2,5-Dihydroxy-BQ | 0.0 |
| 2,5-Dihydroxy-3-methyl-BQ | 0.0 |
| 2-Hydroxy-5-methoxy-BQ | 0.0 |
| 2,3-Dihydroxy-5-methyl-BQ | 2.2 |
| 2-Hydroxy-3-Methoxy-5-methyl-BQ | 2.2 |
| 2-Hydroxy-3-methoxy-6-methyl-BQ (fumigatin) | 2.2 |
| 2-Hydroxy-6-methoxy-5-methyl-BQ | 2.1 |
| 2-Hydroxy-3-methoxy-5,6-dimethyl-BQ | 2.0 |
| 2-Hydroxy-5,6-dimethoxy-BQ | 0.8 |
| 2,5-Dihydroxy-3-methoxy-6-methyl-BQ (spinulosin) | 0.0 |
| 2-Methoxy-BQ | 2.0 |
| 2,6-Dimethoxy-BQ | 2.4 |
| 2,3-Dimethoxy-5-methyl-BQ ($UQ_0$) | 1.9 |
| Ubiquinones ($UQ_1$, $UQ_6$, $UQ_8$, and $UQ_{10}$) | 0.0 |
| 2,3-Dimethoxy-5,6-dimethyl-BQ | 2.3 |
| 2,5-Dimethoxy-3,6-dimethyl-BQ | 2.3 |
| 2,6-Dimethoxy-3,5-dimethyl-BQ | 2.2 |
| 2-Hydroxy-5-(2,4-dihydroxy-6-methylphenyl)-6-methyl-BQ | 2.0 |
| 4,4′-Dihydroxy-2,2′-dimethyl-3,6,3′,6′-dibenzoquinone | 1.9 |
| 2,2′-Dihydroxy-4,4′-dimethyl-3,6,3′,6′-dibenzoquiuone (phoenicin) | 2.0 |
| 2,2′,5,5′-Tetrahydroxy-4,4′-dimethyl-3,6,3′,6′-dibenzoquinone (oosporein) | 0.0 |
| 1,4-Naphthoquinone | 0.0 |
| 2-Methyl-1,4-naphthoquinone (menadione) | 0.0 |
| 5-Hydroxy-1,4-naphthoquinone (juglone) | 1.5 |
| Indophenol | 0.0 |
| 2,6-Dichlorophenol indophenol | 0.0 |
| Methylene blue | 0.0 |
| Potassium ferricyanide | 0.0 |

only the lowest one (not those containing (poly-)isoprenoid chains) was reduced. Oxidation-reduction dyes such as methylene blue, indophenol, 2,6-dichlorophenol indophenol, and ferricyanide could not function as electron acceptors.

An interesting feature of the enzymatic process is that the rate of oxidation of NADH (under the assay conditions specified in Table 3) is essentially the same for all of the quinonoid substrates (see Table 3). The reason for this will

be considered in the following paper, where the effect of the substrate concentrations on the enzymatic reaction velocity are determined and discussed in view of possible enzymatic mechanisms.

Some of the benzoquinone derivatives that could not function as substrates were found to affect the enzymatic reduction of fumigatin in a characteristic manner. When incubations were carried out in the presence of, for instance, toluquinone (< 5 mM) the reduction rate was essentially unchanged. The reaction showed, however, a pronounced initial lag period, the duration of which was found to be approximately proportional to the toluquinone concentration (Fig. 5). The reason for this is fairly evident. Since toluquinone

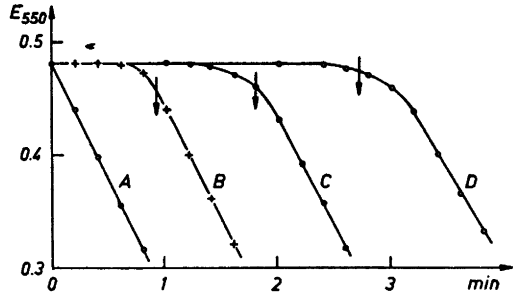


*Fig. 5.* Effect of toluquinone on the enzymatic reduction of fumigatin by NADH. Reaction conditions: 3.5 μmoles of fumigatin, 100 μl of enzyme solution (14 U/ml), and 0, 50, 100, and 150 μl of a 25 mM solution of toluquinone for curve A, B, C, and D, respectively, diluted to a final volume of 3.5 ml with 0.05 M phosphate buffer, pH 5.3. The arrow at each curve indicates the time calculated for the complete reduction of the toluquinone added.

has a more positive oxidation-reduction potential than fumigatin it immediately oxidizes the fumigatin hydroquinone formed, and the concentration of fumigatin remains almost constant until toluquinone has been reduced completely (this time is indicated by arrows for each curve in Fig. 5). The enzymatic reaction did not exhibit any similar lag period when incubations were carried out in the presence of spinulosin, which has a lower oxidation-reduction potential than fumigatin.

The enzyme did not exhibit any cofactor or metal ion requirement. No activity was lost when the enzyme preparations were passed over a Sephadex G-25 column to remove low-molecular material, and the addition of 0.01 M EDTA to the reaction mixtures had no effect on the reduction rate. Investigation of the ion effects on Sephadex G-25 treated enzyme preparations showed that the following ions did not affect the enzyme activity at a concentration of 1 mM: $K^+$, $Na^+$, $NH_4^+$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Pb^{2+}$, $Sn^{2+}$, $Zn^{2+}$, $Fe^{3+}$, and $Al^{3+}$. On the other hand, a pronounced inhibition was obtained with $Ag^+$, $Hg^{2+}$, and $Cu^{2+}$ (Table 4). The enzyme activity was not inhibited by preincubation with (or carrying out incubations in the presence of) SH-reagents such as iodocetate ($10^{-2}$ M) and *p*-chloromercuribenzoate ($10^{-3}$ M), or with $CN^-$ ($10^{-1}$ M), $N_3^-$ ($10^{-1}$ M), amytal ($10^{-3}$ M), dicoumarol ($10^{-4}$ M), rotenone

*Table 4.* Metal ion inhibition of hydroxyquinone:NADH oxidoreductase.

| Metal ion concentration (M) | Inhibition in % $Ag^+$ | $Hg^{2+}$ | $Cu^{2+}$ |
|---|---|---|---|
| $10^{-2}$ | 100 | — | — |
| $10^{-3}$ | 81 | 83 | 45 |
| $10^{-4}$ | 24 | 48 | 24 |
| $10^{-5}$ | 0 | 0 | 17 |
| $10^{-6}$ | 0 | 0 | 0 |

($10^{-3}$ M), and antimycin A (10 $\mu$g/ml). The enzyme appears not to be a flavoprotein, since FMN ($10^{-3}$ M), FAD ($10^{-3}$ M), and the flavine antagonist chloropromazine ($10^{-3}$ M) had no effect on purified preparations; crude preparations were, in fact, activated 120—150 % by the latter compound at $3 \times 10^{-4}$ M. Furthermore, purified preparations that had been passed over Sephadex G-25 gel showed no absorption attributable to flavines (or to cytochromes) in the visible region.

The quinone reductases described in previous reports, which all appear to be flavoproteins, can be divided into three groups with respect to the structure of the quinonoid substrate: Diaphorases, catalyzing the reduction of 1,4-benzoquinone and toluquinone,[6,7] ubiquinone reductases, acting on various homologues of the ubiquinone series,[8,9] and menadione reductases, for which 2-methyl-1,4-naphthoquinone (Vit. $K_3$) appears to be the natural substrate.[10,11] Common properties of the three groups are that NADH (sometimes also NADPH) functions as the electron donor and that a variety of oxidation-reduction dyes (methylene blue, indophenol, and ferricyanide) may function as electron acceptors. Hydroxyquinone:NADH oxidoreductase from *A. fumigatus* is distinct from all of the above enzymes. It does not catalyze the reduction of 1,4-benzoquinone, ubiquinones, menadione, or oxidation-reduction dyes, it cannot utilize NADPH as the electron donor, it is not inhibited by amytal, dicoumarol, rotenone, or chloropromazine, and it seems not to be a flavoprotein. Hydroxyquinone:NADH oxidoreductase appears, therefore, not to be involved in the ordinary electron transport chain,[12] but may nevertheless play an important role as a catalyst of the electron transport from NADH to oxygen. This is evident since the hydroquinone forms of the pigments (*e.g.* fumigatin) produced by *A. fumigatus* have been shown to be rapidly and non-enzymatically oxidized by oxygen from the air.[4] It is also possible that the biological function of the enzyme merely is to keep the pigments (or hydroquinonoid precursors of the pigments) in the reduced state during the production phase. This might be necessary for the biosynthesis of, for instance, fumigatin and spinulosin,[13,14] and might also prevent the formation of toxic amounts of quinones within the cells; the quinone form of the pigments would be expected to interact with amino and sulfhydryl groups present in biological systems.

## REFERENCES

1. Anslow, W. K. and Raistrick, H. *Biochem. J.* **32** (1938) 687.
2. Küster, E. and Little, B. T. *Biochim. Biophys. Acta* **67** (1963) 288.
3. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1428.
4. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1839.
5. Edebo, L. *J. Biochem. Microbiol. Technol. Eng.* **2** (1960) 453.
6. Ernster, L., Ljunggren, M. and Danielson, L. *Biochem. Biophys. Res. Commun.* **2** (1960) 88.
7. Walker, A. G. and Kilgour, G. L. *Arch. Biochem. Biophys.* **111** (1965) 529.
8. Hatefi, Y., Haavik, A. G. and Griffiths, D. B. *J. Biol. Chem.* **237** (1962) 1676.
9. Pharo, R. L. and Sanari, D. R. *Biochim. Biophys. Acta* **85** (1964) 346.
10. Martius, C. and Märki, F. *Biochem. Z.* **329** (1957) 450.
11. Misaka, E. and Nakanishi, K. *J. Biochem.* **58** (1965) 1.
12. Green, D. E. *CIBA Foundation Symposium on Quinones in Electron Transport*, London 1961, p. 130.
13. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 335.
14. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 543.

Received November 26, 1965.