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On the Biosynthesis of Toluquinones from *Aspergillus fumigatus*

III. Autoxidation of the Hydroquinone Forms of the Pigments*

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The oxidative activity of the medium of surface cultures of *Aspergillus fumigatus*, previously ascribed to the action of exo-cellular phenolases, was found to be due to the presence of the toluquinones produced by the mould. An investigation of the kinetics of the autoxidation of 4-hydroxy-2,5-toluhydroquinone and fumigatin hydroquinone (the major pigment in *A. fumigatus*) showed that these reactions are first order with respect to oxygen pressure and hydroquinone concentration, and three-halves order with respect to hydroxyl ion concentration. The rate of autoxidation of a hydroquinone was found to be exponentially related to the energy of the highest occupied molecular orbital of the hydroquinone, enabling an estimation of the non-enzymatic oxidation rates of the hydroquinone forms of all of the pigments from *A. fumigatus* to be made; 4-hydroxy-2,5-toluhydroquinone and fumigatin hydroquinone were used as reference compounds. A comparison of these non-enzymatic reactions and the natural oxidation process showed that no enzymes are required in the biological formation of the toluquinonoid pigments from the corresponding hydroquinones, the rate of quinone formation being limited by physical (oxygen diffusion) instead of chemical factors.

In an investigation[1] of certain strains of *Aspergillus fumigatus* and *Penicillium spinulosum*, known to produce toluquinonoid pigments, Küster and Little showed that endo-cellular phenolases with the same specificity were present in all of the strains studied. Exo-cellular phenolases were, in addition, found in some of the strains, for instance in *A. fumigatus*, L.S.H.T.M. A 46. The authors proposed that the endo-cellular enzymes, which showed laccase properties, were responsible for the formation of fumigatin and spinulosin (at that time the only toluquinones known to be produced by the moulds mentioned above) from the corresponding hydroquinones. The biological function of the exo-cellular phenolases was not established.

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More recently it has been confirmed that the toluquinones from *A. fumigatus*, L.S.H.T.M. A 46, are formed *via* the corresponding hydroquinones.[2] The pigments are, in fact, present in the reduced form during the entire active phase of production. A fairly slow conversion (of some day's duration) into the quinone forms takes place at a late stage in the development of the mould, concomitant with an increase in the pH of the culture medium from pH 3 to about pH 7. The oxidation process appears, however, not to require the participation of endo-cellular enzyme systems. On the contrary, the rate of quinone formation is considerably increased (50 % of the total amount of hydroquinones are oxidized within some hours) after removal of the mycelium. Consequently, if the oxidation process is enzymatically catalyzed exo-cellular (see above) rather than endo-cellular phenolases must be involved.

The presence of exo-cellular phenolases in *A. fumigatus*, L.S.H.T.M. A 46, does not appear to be definitely established; some rather important objections may be raised against the methods used by Küster and Little for measuring the phenolase activities (see experimental part). The oxidative effect of culture filtrates of the mould was, therefore, reinvestigated, using a slightly modified technique, and extending the observations over a wide range of pH-values. The results of these investigations, which are shown in Fig. 1, confirmed that

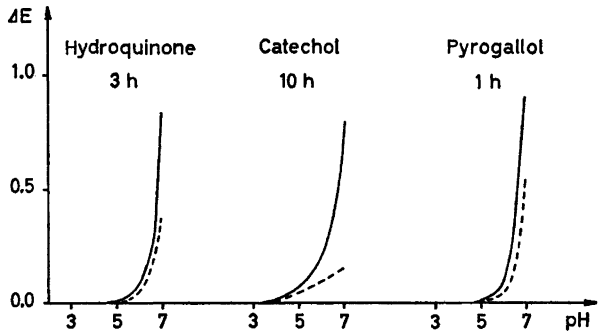

Fig. 1. Oxidative activity of culture filtrates of *A. fumigatus*. The dashed lines show the autoxidation of the substrates in phosphate buffer solutions.

the culture filtrates increased the rate of oxidation of hydroquinone, catechol, and pyrogallol (the substrates studied by Küster and Little). The catalytic effect was, however, fairly small and seemed not to be due to the presence of enzymes; no decrease in activity was obtained by boiling the culture filtrates, or by keeping them at pH 0, for several hours before incubation. These observations suggested that the catalytic activity could be caused by low-molecular compounds, and in that case probably by the toluquinonoid pigments produced by the mould; none of the components of the original Raulin-Thom medium has any oxidative effect on the substrates used.[3] Confirmatively, the activity disappeared after dialysis of the culture filtrates, but was restored by addition of a mixture of the pigments, obtained from the cultures by extraction. Further experiments showed that the catalytic activity of the culture filtrates

Table 1. Catalytic effect at pH 6.8 of various media on the oxidation of 0.3 % solutions of hydroquinone (I), catechol (II), and pyrogallol (III).

Medium	Relative catalytic effect I	II	III
Culture filtrate *	1.0	1.0	1.0
Culture filtrate, boiled *	1.2	1.2	1.1
Culture filtrate, dialyzed	0.0	0.0	0.0
Toluquinone mixture solution *	0.9	1.1	1.0
Fumigatin solution *	0.8	0.9	0.9

* 0.002 M with respect to the total amount of quinones.

was due merely to the presence of the toluquinones (see Table 1). The conclusion must, therefore, be that there are, so far, no indications of the presence of exo-cellular phenolases in *A. fumigatus*, L.S.H.T.M. A 46. On the other hand, these negative results do not prove that such enzyme systems are absent, since they might be highly specific towards the natural substrates.

Quinones are not formed until the twelth to fifteenth day of growth in normally developed cultures. The hydroquinones present in the medium of younger cultures are, however, rapidly oxidized after removal of the mycelium. It was found that above pH 2.5 the rate of oxidation was essentially independent of the pH of the medium. Below pH 1.5 the formation of quinones was negligible, and at intermediate values the rate of oxidation varied regularly with pH, as indicated in Fig. 2. The non-enzymatic formation of quinones must, obviously, predominate in alkaline solutions, where hydroquinones act as strong reductants against oxygen. However, in order to decide whether the high oxidation rate at, for instance, pH 3 indicates that enzymes are involved in the natural process (taking place at pH 3—7), the non-enzymatic oxidation of the hydroquinone forms of the different pigments had to be more carefully studied.

The non-enzymatic oxidation of hydroquinone and a number of alkyl derivatives of hydroquinone has been the subject of several reports, partly due to the general interest in these compounds as components of photographic developers. The hydroquinones yield the corresponding quinones as the primary product, with the consumption of equimolar amounts of oxygen;

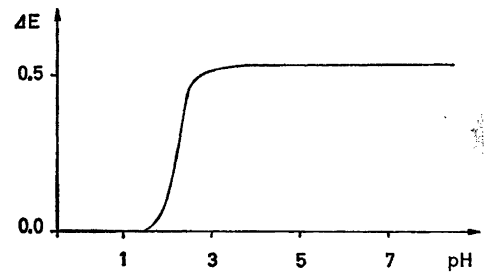

Fig. 2. Rate of quinone formation in the culture medium of *A. fumigatus* as a function of pH.

the formation of peroxide in such autoxidations has been known for a long time, and is now exploited for the large-scale production of hydrogen peroxide.[4] In alkaline solutions secondary reactions take place, leading to the formation of hydroxyquinones and polymeric products [5] (generally referred to as "quinone humic acids"). All reports on the kinetics of the process [6–9] agree that the rate of oxidation (v_1) is dependent on the oxygen pressure (p_{O_2}), the concentration of the hydroquinone (H_2Q), and the pH of the solution, in accordance with the following expression:

$$v_1 = -\frac{d[H_2Q]}{dt} = -\frac{d[O_2]}{dt} = kp_{O_2}[H_2Q][OH]^n ; n = 1.5-2 \quad (1)$$

Some confusion exists regarding the exact expression for the pH-dependence. Some investigators have found the reaction rate to be proportional to the second power of hydroxyl ion concentration, others to the three-halves power; the latter was claimed in the first and in the most recent report.[6,9]

Is is, however, not evident that eqn. (1) can be applied to the autoxidation of the hydroquinones from *A. fumigatus*, which contain other substituents (*e.g.* hydroxyl groups) than alkyl groups. The kinetics of the autoxidation of 4-hydroxy-2,5-toluhydroquinone were, therefore, investigated by the use of manometric (Warburg) techniques. The latter compound was chosen as a model substance for the natural hydroxytoluhydroquinones, since it (in contrast to these) is fairly stable and easy to prepare in a pure form. In preliminary runs the oxygen pressure and the concentration of 4-hydroxy-2,5-toluhydroquinone were varied. The oxygen uptake during the first 15 min was used as a measure of the reaction rate at the initial concentration of the hydroquinone. These experiments indicated that the reaction is first order with respect to the oxygen pressure and the hydroquinone concentration, in accordance with eqn. (1). When the oxidation process was carried out in oxygen, instead of in air, the initial rate of oxygen uptake increased by a factor of 5.0 (see Table 2), in good agreement with the theoretical value (4.8) if the rate is assumed to be proportional to the oxygen pressure. Similarly, variations of the hydroquinone concentration gave a linear change in the initial rate of oxygen absorption (see Fig. 3).

Two separate processes are involved in the uptake of oxygen; these are the solution of the gas in the reaction mixture (at the rate v_2) and the reaction

Table 2. Rate of initial oxygen uptake in 0.002 M solutions of 4-hydroxy-2,5-toluhydroquinone (I) and fumigatin hydroquinone (II) at different oxygen pressures.

Gas phase	p_{O_2} in atm	Reaction rate (μmoles/min) at pH			
		3.1 I	3.4 I	2.0 II	2.4 II
Air	0.209	0.027	0.078	0.029	0.115
Oxygen	1.000	0.136	0.390	0.143	0.589
Ratio	4.78	5.02	5.00	4.93	5.12

of the dissolved oxygen with the substance in the solution (at the rate v_1). v_2 is determined by the diffusion coefficient (D), the area (O) of the gas-solution surface, the mean length (d) of oxygen diffusion (from the surface to the place in the solution where it is consumed), and the difference between the actual concentration of oxygen in the solution (c) and the concentration of a saturated solution (c_0), in conformity with eqn. (2). Under steady state conditions

$$v_2 = D(O/d)(c_0 - c) \qquad (2)$$

v_2 equals v_1. Even though it is practically impossible to calculate the value of c, it is clear that when O is large, d small, and v_1 not to large, c can be put approximately equal to c_0 (the solution is saturated with respect to oxygen). With the technique used in the present investigation these conditions appeared to be fulfilled at a pH below 3.5 (absorption rates below 0.15 μmole/min),

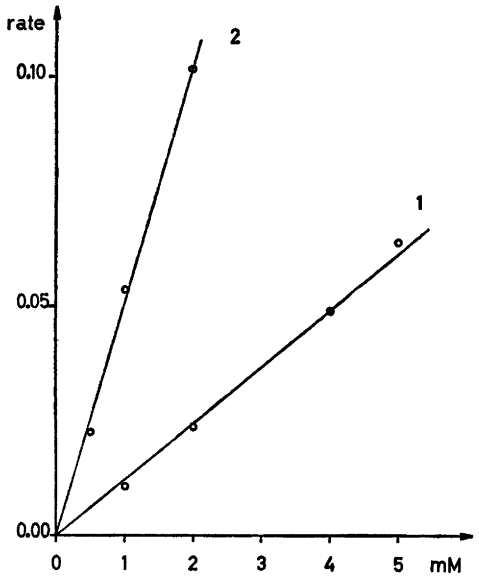

Fig. 3. Initial rate (in μmoles/min) of autoxidation of 4-hydroxy-2,5-toluhydroquinone (curve 1) and fumigatin hydroquinone (curve 2) at pH 3.05 and pH 2.32, respectively, as a function of the hydroquinone concentration.

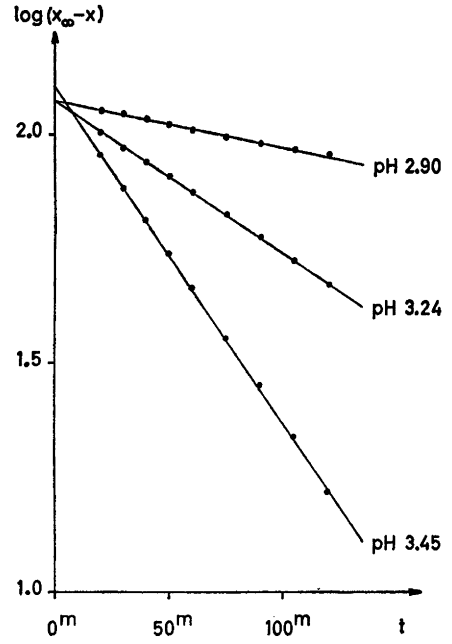

Fig. 4. Determinations of k_{10}' values for the autoxidation of 4-hydroxy-2,5-toluhydroquinone.

where alterations of the total volume of the reaction solution, or of the shaking frequency, had no influence on the reaction rate. At a pH above 4 the rate of autoxidation of 4-hydroxy-2,5-toluhydroquinone remained essentially constant throughout the greater part of the reaction. This constant (but non-repro-

ducible) rate varied from 0.2 to 0.3 μmole/min in different experiments, and was influenced by the total volume of the reaction mixture, the shaking frequency, and the oxygen pressure, but was not affected by pH changes (pH > 4). The solution can, obviously, not be assumed to be saturated with respect to oxygen at this maximal rate of oxygen uptake; the measurements had to be restricted to absorption rates below 0.15 μmole/min.

Determinations of the pH-dependence of the process were, therefore, performed in air below pH 3.5. Each set of data were analyzed by plotting $^{10}\log(x_\infty - x)$ against t, where x is the volume absorbed at time t, and x_∞ the maximal oxygen uptake. Since the preliminary experiments indicated that the rate of autoxidation is governed by the first order eqn. (3), the slope of the

$$-\frac{d[O_2]}{dt} = -\frac{d[H_2Q]}{dt} = k'[H_2Q] \qquad (3)$$

resulting line will be independent of x, and its value k_{10}' multiplied with 2.3 will give the first order reaction constant (k'). As shown in Fig. 4, the experimental data (below pH 3.5) gave straight lines in the $^{10}\log(x_\infty - x),t$ plot, confirming that the reaction is first order with respect to the hydroquinone concentration. The variation with pH of the k' values obtained, indicated that the autoxidation is three-halves order with respect to hydroxyl ion concentra-

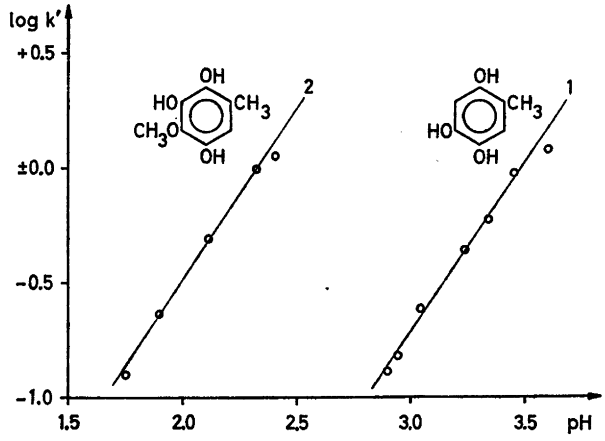

Fig. 5. pH-dependence of the autoxidation of 4-hydroxy-2,5-toluhydroquinone (curve 1) and fumigatin hydroquinone (curve 2). k' is given in reciprocal hours.

tion (*cf.* eqn. (1)). This is shown in Fig. 5, where the logarithm of the rate constant is plotted against pH. The points fall well along a line with the theoretical slope of a three-halves order reaction. A statistical analysis of the data gave a regression coefficient of 1.56. The deviation from the theoretical value 1.5 was statistically insignificant.

The kinetics of the non-enzymatic oxidation of fumigatin hydroquinone, the major secondary metabolite in *A. fumigatus*, L.S.H.T.M. A 46, were

studied in the same direct way. This reaction also was found to be first order with respect to oxygen pressure (Table 2) and hydroquinone concentration (Fig. 3), and three-halves order with respect to hydroxyl ion concentration (Fig. 5). Eqn. (1) with $n = 1.5$, therefore, appears to be of general validity as the kinetic expression for the autoxidation of hydroquinones. There is at least, little doubt that it governs the autoxidation of the toluhydroquinones produced besides fumigatin hydroquinone by *A. fumigatus*, which are all structurally closely related to fumigatin hydroquinone and 4-hydroxy-2,5-toluhydroquinone.

The autoxidation rates may be expected to correlate with the reducing properties of the different hydroquinones. These can be quantitatively estimated by the application of molecular orbital methods; the energy of the highest occupied molecular orbital (EHOMO) gives a measure of the electron-donor capacity (a low-lying HOMO corresponds to a poor electron donor).[10] The qualitative effect of the introduction of alkyl substituents into the hydroquinone nucleus is known from previous investigations, and the parallelism in the variation of rate of autoxidation and EHOMO is illustrated in Table 3,

Table 3. Molecular orbital data and relative autoxidation rates for a number of alkyl derivatives of hydroquinone.

No.	Derivative of hydroquinone	Relative autoxidation rate *	log rate	$\frac{\text{EHOMO}-\alpha}{\beta}$
1	Unsubstituted	1.0	0.00	0.882
2	Methyl-	3.9	0.59	0.856
3	2,3-Dimethyl-	10.5	1.02	0.839
4	2,5-Dimethyl-	17.0	1.23	0.824
5	2,6-Dimethyl-	18.2	1.26	0.827
6	Trimethyl-	31.0	1.49	0.812

* values according to James, Snell, and Weissberger.[8]

on the example of the hydroquinones studied by James, Snell, and Weissberger.[8] A linear relationship is, in fact, obtained in a plot of the logarithm of the relative reaction rate against EHOMO, as shown in Fig. 6. Even though this linear relationship cannot be assumed to be of general applicability (following the general feature of the molecular orbital calculations it is preferable to utilize the theoretical data for the comparison of the electron-donor properties within a series of related compounds) it seems to be a good approximation over limited ranges of EHOMO, and within a group of structurally related compounds. Thus the rate constants (k) for the different toluhydroquinones from *A. fumigatus* can be roughly estimated by interpolation from the line defined by the experimentally determined oxidation rates for fumigatin hydroquinone and 4-hydroxy-2,5-toluhydroquinone (see Fig. 7). With the assumption that the rate constants are proportional to the three-halves power of hydroxyl ion concentration, the corresponding half-times ($t_{½} = {}^{e}\log 2/k'$) have been computed for pH 3 and pH 4 ($p_{O_2} = 0.21$ atm). The results

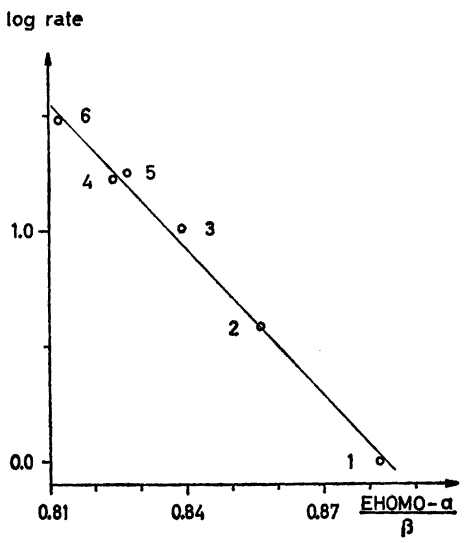

Fig. 6. Logarithm of the relative autoxidation rates (log rate) of alkyl derivatives of hydroquinone as a function of EHOMO. Numbers refer to compounds in Table 3.

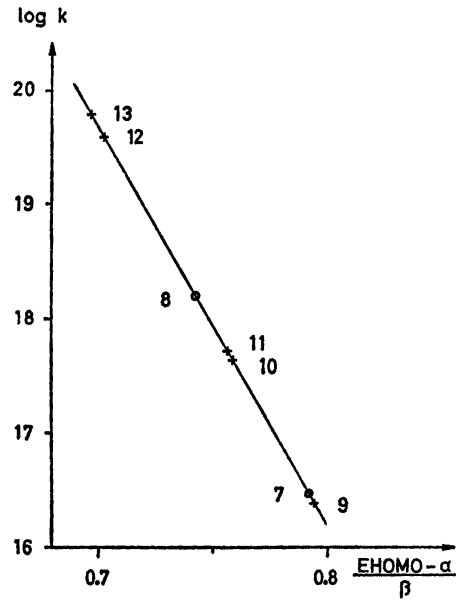

Fig. 7. Linear estimation of autoxidation rate constants (h^{-1} atm^{-1} $M^{-1.5}$) of toluhydroquinones from *A. fumigatus*, using molecular orbital data. Numbers refer to compounds in Table 4. 4-Hydroxy-2,5-toluhydroquinone (7) and fumigatin hydroquinone (8) have been used as reference compounds.

of these calculations are listed in Table 4. Similar calculations of the reaction rates above pH 4 will be less reliable, partly since it is uncertain whether eqn. (1) is valid over such a wide range of pH values, and partly since the molecular orbital calculations have been performed on the undissociated molecules. Ionization of the hydroquinones will, however, increase the electron-donor capacity, and the autoxidation of the reduced forms of the pigments can be considered as taking place very rapidly (half-times in the order of or less than 1 sec) above pH 5.

The biological application of the results obtained is complicated by the fact that the different quinone-hydroquinone systems, present in the culture medium of *A. fumigatus* during the oxidation process, are in rapid redox equilibrium with each others. It is, however, clear that the total course of the process will be essentially determined by the two major pigments, fumigatin hydroquinone and 3,4-dihydroxy-2,5-toluhydroquinone, which constitute about 90 % of the total amount of pigments. The time required to attain 50 % oxidation of the hydroquinone mixture would thus be in the order of 15 min at pH 3 and 30 sec at pH 4, if the solution is assumed to be saturated with respect to air. As previously mentioned, the natural oxidation process

Table 4. Molecular orbital data and calculated autoxidation rates for the toluhydroquinones from *A. fumigatus*. The rate constants (k) are given in h^{-1} atm^{-1} $M^{-1.5}$. The half-times ($t_{½}$) refer to $p_{O_2} = 0.21$ atm.

No.	Derivative of 2,5-toluhydroquinone	$\frac{EHOMO-a}{\beta}$	k	$t_{½}$ in min pH 3	pH 4
7	4-Hydroxy- *	0.792	2.95×10^{16}	212	7
8	3-Hydroxy-4-methoxy-**	0.742	1.55×10^{18}	4	0.1
9	3-Hydroxy-	0.794	2.45×10^{16}	250	8
10	3,4-Dihydroxy-	0.758	4.35×10^{17}	14	0.5
11	3-Methoxy-4-hydroxy-	0.756	5.00×10^{17}	12	0.4
12	3,6-Dihydroxy-	0.702	3.80×10^{19}	0.2	0.005
13	3,6-Dihydroxy-4-methoxy-	0.696	6.15×10^{19}	0.1	0.003

* Reference compound; not produced by the mould.
** Fumigatin hydroquinone.

is much slower, even though it partly takes place above pH 4. The explanation for this is evident from the observation that the oxidation rate was considerably increased after removal of the mycelium, or by aerating the culture medium; the rate of quinone formation must be determined by the availability of oxygen, and not by the reactivity of the hydroquinones. Similarly, the results indicated in Fig. 2 can be completely explained as a consequence of the pH-dependence of the autoxidation rate (below pH 3) and of the rate-limiting effect of the oxygen diffusion process (above pH 3).

For these reasons it can be concluded that no phenolases are required in *A. fumigatus* for the formation of the toluquinonoid pigments from the corresponding hydroquinones. The oxidation rate is limited by physical (rather than chemical) factors, which are not affected by the presence of enzymes.

EXPERIMENTAL

Aspergillus fumigatus Fresenius, L.S.H.T.M. A 46, was used throughout this work. The culture conditions have been described previously.[11]

Oxidative activity of the culture filtrates. In their investigation of exo-cellular phenolases in *A. fumigatus*,[1] Küster and Little incubated the crude culture filtrates overnight at pH 6.8 with 0.5 % solutions of phenolic substances (hydroquinone, catechol, and pyrogallol). The increase in extinction at 500 mμ was assumed to give a measure of the phenolase activity, but no separate experiments were undertaken to establish that enzymes participate in the reactions.[3] Incubations seem to have been carried out under such conditions (possibly without shaking the mixtures) that the solutions may not have been saturated with respect to oxygen. Otherwise the rate of autoxidation of pyrogallol, at least, is too high to permit any reliable determinations of the increase in extinction (see below).

In the present investigation the oxidative activity of the medium from 12 days old surface cultures of *A. fumigatus*, L.S.H.T.M. A 46, was studied by a similar, spectrophotometric, technique. Freshly separated culture filtrates contain fairly large quantities of the hydroquinone forms of the pigments, the autoxidation of which would contribute to the increase in extinction at 500 mμ. The filtrates were, therefore, vigorously aerated at pH 5.0 for a few minutes, leading to a quantitative oxidation of the toluhydroquinones. Definite amounts (final concentration 0.3 %) of the phenolic substrates were then added

from concentrated stock solutions, and the pH of the incubation mixtures was adjusted by the addition of dilute hydrochloric acid or sodium hydroxide. Incubations were carried out, with shaking, in air at 28° for a suitable time, according to the rate of oxidation of the substrates. Blank solutions (without phenols) were prepared and incubated in the same way; above pH 5 these also showed a considerable increase in extinction at 500 mμ, due to the autoxidation of the quinone forms of the pigments (probably leading to the formation of polymeric products). The difference in increase in extinction at 500 mμ between the complete incubation mixtures and the blank solutions was used as a measure of the rate of oxidation of the phenolic substrates. The corresponding rates of autoxidation were determined in a similar manner, using 0.3 % phosphate buffer solutions of the phenols. Incubations overnight at pH 6.8 (see above) were not generally feasible, not even in the measurements of autoxidation rates; when pyrogallol was used as the substrate the extinction of the incubation solution exceeded 3 already after 2 h, and after a further 2 h a black precipitate was formed. Incubations for 3, 10, and 1 h were found suitable for hydroquinone, catechol, and pyrogallol, respectively. The results of these investigations are shown in Fig. 1.

Oxidative activity of the toluquinonoid pigments. The medium of 12 days old cultures of *A. fumigatus* was vigorously aerated at pH 5.0, and carefully extracted with ether. After removal of the ether in vacuum a phosphate buffer solution of the pigment mixture, containing the same concentration of quinones (about 2 mM) as the original culture filtrates, was prepared. The catalytic effect of this solution on the oxidation of hydroquinone, catechol, and pyrogallol was studied as described above. Similarly, the catalytic properties of a 2 mM phosphate buffer solution of fumigatin (the major pigment) were investigated. As shown in Table 1, there is little difference between the catalytic activity of these toluquinone solutions and that of the original culture filtrates.

pH-Dependence of the natural oxidation process. The metabolism solution from a great number of 10 days old surface cultures of *A. fumigatus* (in which the pigments are present almost exclusively in the reduced form) was filtered and transferred into empty culture flasks. The pH of the solutions was adjusted over a wide range of pH-values by the addition of dilute hydrochloric acid or sodium hydroxide. All these operations were performed quickly, and as far as possible in the absence of air. The formation of quinones in air at 25° was then followed by measuring the increase in extinction at 480 mμ of the solution in each flask (at each pH). A typical extinction/time curve is shown in Fig. 8. The increase

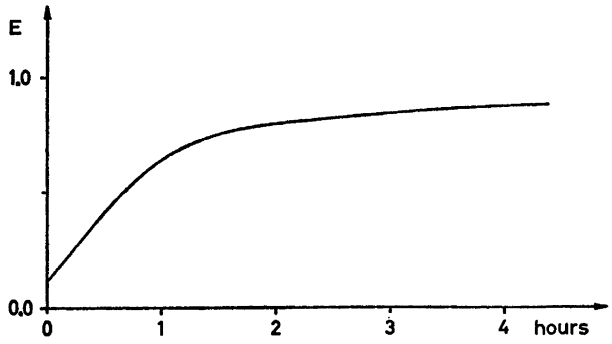

Fig. 8. Toluhydroquinone oxidation in the medium of young cultures of *A. fumigatus* (pH 3.0) after removal of the mycelium.

in extinction during the first hour was used as a measure of the rate of oxidation, and in Fig. 2 this quantity has been plotted against the pH of the culture filtrates (correction has been made for the difference in extinction at 480 mμ which is due to the dissociation of the hydroxytoluquinones above pH 5).

Preparation of 4-hydroxy-2,5-toluhydroquinone. 4-Hydroxy-2,5-toluhydroquinone was prepared from toluquinone by means of the Thiele-Winter reaction and hydrolysis of the resulting tricetate.[12]

Preparation of fumigatin hydroquinone. Fumigatin (0.8 g), isolated from *A. fumigatus* as previously described,[11] was shaken for a few moments with an excess of sodium dithionite (5 g) in water (100 ml) when an almost colourless solution was obtained. This was extracted twice with an equal volume of ether and the ether removed, leaving a syrupy residue (0.8 g) which was dried *in vacuo* in a sublimation tube (when it crystallized), and sublimed in a high vacuum. The sublimate, a slightly brown semisolid material, was dissolved in ether and washed with freshly prepared aqueous sodium bicarbonate, which removed traces of fumigatin. The ethereal solution was evaporated to dryness and resublimed. The sublimate was washed with a little carbon tetrachloride and resublimed, giving almost colourless micro-crystals, m.p. 100°. The preparation was fairly rapidly oxidized in the air, and had to be used immediately.

Autoxidation of 4-hydroxy-2,5-toluhydroquinone and fumigatin hydroquinone. The oxygen absorption was measured by the usual Warburg technique. Definite quantities (generally 6 μmoles) of 4-hydroxy-2,5-toluhydroquinone, dissolved in 0.2 ml of 0.01 M hydrochloric acid, were supplied to the side-arm; the main compartment contained 2.8 ml of 0.1 M citrate buffer solution. To eliminate the induction period (see below) 1.5 μmoles of 4-hydroxy-2,5-toluquinone were added to the buffer solution. After temperature equilibration at 25° the reactions were started by mixing the solutions, and were carried out with rapid shaking at a constant oxygen pressure; the consumption of oxygen was negligible (less than 1 %) compared with the total volume present in the reaction vessels. The measurements of oxygen uptake were made at intervals of 5 to 15 minutes, according to the rate of the reaction. Measurements of pH were made on the completed reaction solutions by means of a calibrated glass electrode; preliminary experiments showed that the buffers used kept the pH constant within 0.02 units throughout the entire course of the reaction. The pH range (2.9 – 3.5) was such that no appreciable change in the concentration of undissociated 4-hydroxy-2,5-toluhydroquinone was produced by reaction with the buffer. Except in some preliminary runs (where oxygen was used) the experiments were performed in the presence of air. The technique described gave reproducible results over a limited range of absorption rates (0.015 – 0.150 μmoles/min).

The autoxidation of fumigatin hydroquinone was studied by the same technique (pH 1.7 – 2.4).

Products of the autoxidation reactions. The autoxidation of 4-hydroxy-2,5-toluhydroquinone yielded the corresponding quinone as the only aromatic product; no "quinone humic acids"[7] were obtained. Previous investigations have established that equimolar amounts of hydrogen peroxide are formed in the autoxidation of hydroquinones, and this was confirmed in the present work; the maximal oxygen uptake corresponded to the

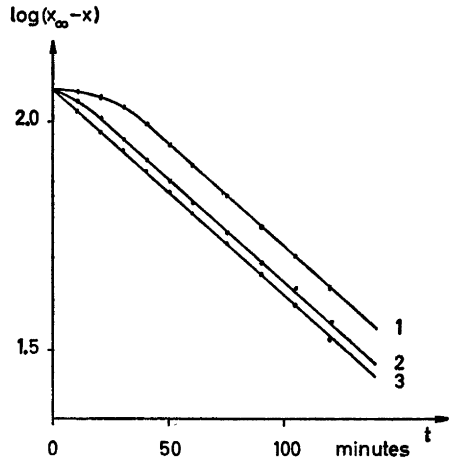

Fig. 9. Effect of 4-hydroxy-2,5-toluquinone on the induction period exhibited in the autoxidation of the corresponding hydroquinone (pH 3.34). Curve 1: No addition of 4-hydroxy-2,5-toluquinone. Curve 2: 0.5 μmoles of 4-hydroxy-2,5-toluquinone added. Curve 3: 1.5 μmoles of 4-hydroxy-2,5-toluquinone added.

consumption of one mole of oxygen per mole of 4-hydroxy-2,5-toluhydroquinone oxidized. Furthermore, approximately 3 μmoles of oxygen were evolved after addition of 1 ml of saturated aqueous ferric chloride (which catalyzes the decomposition of hydrogen peroxide) to the completed reaction mixtures, while no oxygen was produced when a citrate buffer solution of 4-hydroxy-2,5-toluquinone was treated in a similar manner.

Similarly, fumigatin hydroquinone yielded fumigatin as the only aromatic product. The maximal oxygen uptake was usually 5 – 10 % lower than calculated; some oxidation of the preparation of fumigatin hydroquinone seemed to take place during the experimental procedures before the start of the reactions.

Preliminary experiments showed that the rate of decomposition of 0.001 M hydrogen peroxide was negligible in citrate buffer solutions at pH 1 – 4. The addition of hydrogen peroxide (3 μmoles) to the reaction solutions had no influence on the reaction rates (k_{10}' values obtained).

Induction period of the autoxidation of 4-hydroxy-2,5-toluhydroquinone. In the absence of the corresponding quinone the autoxidation of 4-hydroxy-2,5-toluhydroquinone exhibited a pronounced induction period. This is shown in Fig. 9, where the slope of the curve in the $\log(x_\infty - x)$, t plot attains a maximal value, which is maintained throughout the remainder of the oxidation process, in conformity with the kinetics of a first order reaction. The induction period was shortened in the presence of 4-hydroxy-2,5-toluquinone and eliminated completely by the addition of about 1.5 μmoles of the latter compound to the reaction solution (3.0 ml). The presence of the quinone had no influence on the k_{10}' values obtained (see Fig. 9). Several other quinones (*e.g.* fumigatin and indophenol) were found to eliminate the induction period, but in these cases both the reaction rate and the first order kinetics of the reaction were affected (no straight lines were obtained in the $\log(x_\infty - x)$, t plot).

The autoxidation of fumigatin hydroquinone did not exhibit any induction period, possibly due to the presence of fumigatin (5 – 10 %; see above) in the solutions at the start of the reaction.

Molecular orbital calculations. Molecular orbital calculations were performed on the undissociated forms of the different hydroquinones. A detailed description of the methods used (parameter values *etc.*) has been given elsewhere.[2]

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