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The Oxidases of *Gelasinospora tetrasperma* in relation to the Endogenous Respiration

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SUMMARY: The active growth of *Gelasinospora tetrasperma* in a chemically defined liquid medium was completed in 5 days. Cytochrome was detected at all ages (spectroscopically and by manometric assay) and a cytochrome-cytochrome oxidase system was mainly responsible for O₂ uptake. Phenol oxidase was not detected until the cultures had been growing 48 hr., and even then the proportion of the O₂ uptake of the mycelium due to this enzyme was small, since light reversed carbon monoxide inhibition of the O₂ uptake almost completely.

Whereas the importance of cytochrome *c* oxidase as a terminal oxidase of plant respiration is now firmly established, the role, if any, of phenol oxidases in plant respiration is uncertain (James, 1953*a*). In the Basidiomycete *Poly-stictus versicolor*, which produced both cytochrome *c* oxidase and a phenol oxidase, laccase, only the former enzyme was involved in oxygen uptake (Boulter & Burges, 1955). The present work was undertaken to assess the relative importance of cytochrome *c* oxidase and the phenol oxidase, tyrosinase, in the endogenous respiration of *Gelasinospora tetrasperma*, an Ascomycete, which produces both these enzymes.

METHODS

Culture medium. *Gelasinospora tetrasperma* Dowding, obtained from Dr Dowding, was grown in a liquid medium of the following composition: D(+) glucose, 10 g.; asparagin, 5 g.; KH₂PO₄, 0.5 g.; Mg₂SO₄·7H₂O, 0.5 g.; NaCl, 0.25 g.; biotin, 50 µg.; thiamine, 100 µg.; distilled water to 1 l. (glucose autoclaved separately; thiamine sterilized by filtration).

Inoculum. Mycelial suspensions from 48 hr. cultures, grown in 40 ml. medium at 25°, were used for inoculations. Mycelium from two flasks was washed and transferred with 40 ml. water to the metal cup of an Atomix blender (M.S.E., London) and blended at full speed for 10 sec. This agitation gave a fine mycelial suspension which was washed twice by centrifugation and resuspended in water; 1 ml. of suspension was used to inoculate each 250 ml. flask containing 40 ml. medium.

Culture conditions. Cultures were incubated at 25° on a reciprocal shaker (stroke 4 in., 75 oscillations/min.).

Chemicals. Substrates and inhibitors were obtained from the British Drug Houses Ltd., and were of analytical grade when available. Acid and alkaline compounds were neutralized; unstable substrates were recrystallized and

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fresh solutions of them made up just before use. O_2 and N_2 (O_2 free) were obtained in cylinders from the British Oxygen Co. Carbon monoxide was made by the action of concentrated sulphuric acid on formic acid and oxygen was removed from the evolved gas and from nitrogen by passage through alkaline pyrogallol triacetate and then through an acid and a water wash. Gas mixtures were made in 10 l. bottles by displacement of water.

Spectroscopic examinations were made with a Hilger micro-spectroscope, using a 500 W. metal filament lamp and allowing light to pass through thick suspensions of the mycelium to which sodium dithionite had been added.

Preparation of extracts. All operations were carried out between 0° and 4° . Fungal cultures of various ages were harvested and washed on a Buchner funnel with 150 vol. (w/v) of distilled water. Mycelium to be extracted for the examination of cytochrome *c* oxidase activity was ground with phosphate buffer in a mortar (1 part moist mycelium to $\frac{1}{3}$ part (w/w) quartz and 5 parts (w/v) 0.05 M-phosphate buffer (pH 7.5) containing 0.001 M-ethylenediamine-tetraacetic acid (Versene)). The suspension was centrifuged at 1000 *g* for 10 min. to remove cell debris and quartz and the supernatant fluid (H) then centrifuged at 5000 *g* for 20 min. This supernatant fluid (SN) was discarded and the residue (P) resuspended in 0.05 M-phosphate buffer (pH 7.5) containing Versene and centrifuged again for 20 min. at 5000 *g* to give a pellet which was suspended in one-third the original volume of 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene (P_1).

Extracts to be tested for Dopa oxidase activity were prepared by washing the mycelium as before and grinding it in a mortar with a minimum of quartz and buffer (1 part moist mycelium to 9 parts (w/v) 0.05 M-phosphate buffer to give pH 7.0). The cloudy suspension was centrifuged for 10 min. at 1000 *g* and the residue of quartz, cell debris and unbroken cells re-extracted with half the previous volume of buffer, allowed to stand 10 min. and centrifuged again at the same speed for 10 min. The two supernatant fluids were combined and dialysed against 0.01 M-phosphate buffer (pH 7.0) overnight at 2° . The extracts were adjusted to pH 7.0 before testing.

To determine the intracellular localization of Dopa oxidase activity, cell fractions were prepared from 4-day mycelium by grinding with quartz and phosphate buffer. The ground mycelium was centrifuged gently to give a cell-wall fraction (W_0) and a supernatant (SN_0). (SN_0) was centrifuged at 5000 *g* for 20 min. to give a supernatant (SN_1) and a particle fraction (P). The fraction (W_0) was re-extracted and allowed to stand 10 min. and the suspension then recentrifuged gently to give a cell-wall fraction (W_2) and a supernatant (SN_2). Further re-extractions of (W_0) were designated (W_3), (W_4) and (SN_3), (SN_4). The fractions were dialysed against 0.01 M-phosphate buffer (pH 7.0) overnight at 2° and adjusted to pH 7.0 before testing.

Measurement of enzyme activity of extracts. Cytochrome *c* oxidase activity was measured as the rate of oxygen uptake in Warburg manometers at 25° . The flasks contained the enzyme extract; cytochrome *c* (Sigma Chemical Co., U.S.A.); 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in the side arm and 0.15 ml. 15% (w/v) KOH on filter-paper

in the centre well. After tipping the side arm, readings were taken at 5 min. intervals for 20 min. and the activity/hr. calculated from the initial rate of O_2 uptake. The O_2 uptake due to autooxidation of the hydroquinone was subtracted and was estimated by extrapolating to zero enzyme concentration at constant cytochrome *c* concentration (Umbreit, Burris & Stauffer, 1949). Autooxidation of hydroquinone occurred even when Versene was present in the assay and grinding media. In order to find the maximum oxidase activity at a constant enzyme concentration, the O_2 uptake with a constant enzyme concentration was measured with different concentrations of cytochrome *c*. The reciprocal of the O_2 uptake values was then plotted against the reciprocal of the cytochrome *c* concentration and the maximum O_2 uptake at this enzyme concentration was calculated by extrapolating to infinite cytochrome *c* concentration (Lineweaver & Burk, 1934). This maximum cytochrome *c* oxidase activity was expressed as $\mu l.$ O_2 uptake/hr./mg. N (Q_{O_2} max. (N)).

Nitrogen determinations of the enzyme preparations were made by precipitating nitrogen compounds with 10% (w/v) trichloroacetic acid, digesting the washed precipitate with concentrated H_2SO_4 + one drop of H_2O_2 and determining the nitrogen by Nessler's method.

Dopa oxidase activity was measured at a wavelength of 480 m μ . at room temperature, using a Unicam S.P. 500 spectrophotometer. Glass cells of light path 1 cm. were used containing in a total volume of 3.0 ml.: enzyme; DL-3:4-dihydroxyphenylalanine (Dopa) and 0.01M-phosphate buffer (pH 7.0). The enzyme was added to the assay system and readings at 10 sec. intervals were commenced 15 sec. after the addition. During the period of the first few readings, the oxidase activity remained approximately constant and the activity/hr. was calculated on this initial rate.

The Dopa oxidase activities of highly turbid suspensions were measured in an EEL colorimeter with a blue filter. Cells contained in a total of 6.0 ml.: enzyme extract; 0.005M-Dopa and 0.05M-phosphate buffer (pH 7.0). The substrate was temperature equilibrated (25°) separately, added to the rest of the system and readings taken at 2 min. intervals. The cells were returned to the water bath in between readings and continuously shaken gently. Autooxidation of substrate and maximum activity were calculated as for cytochrome *c* oxidase. The activity was measured as the change in optical density/min./mg. N in the Unicam and as the change in EEL units/hr./mg. N in the EEL colorimeter. Manometric checks with larger volumes of enzyme were made to ensure that reducing substances were not grossly distorting the assays.

Respiration measurements on intact mycelium. The respiration was measured at 25°, in the presence and absence of inhibitors, by determining the oxygen uptake in Warburg manometers by the direct method (Dixon, 1943). O_2 uptake was referred to mg. dry wt. mycelium, obtained by washing the mycelium from the Warburg flasks on to weighed sintered-glass crucibles, and drying these overnight at 98°.

Application of inhibitors. Inhibitors of the oxidases were used at pH values and concentrations that ensured maximum effectiveness and selectivity (James, 1953b). Inhibition of cytochrome *c* oxidase, tyrosinase and the O_2

uptake of the intact mycelium by various gas mixtures, was measured in Warburg manometers; gas mixtures were introduced into the flasks by an evacuation technique (Umbreit *et al.* 1949). When required, flasks were darkened during temperature equilibration and during dark periods by a dark cloth thrown over the top of the bath. The light source used in the carbon monoxide experiments was a 250 W. mercury vapour lamp and when in use it was necessary to cool the system with a powerful fan. The inhibition was calculated as the percentage decrease of the O_2 uptake in a particular gas mixture, as compared with the O_2 uptake of control samples in air.

The effects of phenylthiourea and KCN on cytochrome *c* oxidase activity and on the O_2 uptake of the mycelium were also measured in Warburg manometers. After a steady rate of O_2 uptake had been established, the inhibitors were added to the contents of the Warburg flasks, phenylthiourea from the side-arm and KCN (at 25°) directly from the outside. In the flasks to which KCN had been added, 3 min. were allowed for temperature equilibration before re-reading the manometers. Inhibitions were calculated as the percentage decrease in O_2 uptake of the sample on addition of the inhibitor. KCN inhibition was calculated from the initial rate of O_2 uptake on adding the inhibitor, as the inhibition quickly diminished due to KCN distilling into the KOH of the centre well.

The effects of KCN, phenylthiourea and Na diethyldithiocarbamate on Dopa oxidase activity were measured spectrophotometrically and calculated as the percentage decrease in activity of samples in the presence of the inhibitors, compared with control samples in their absence.

RESULTS

Factors affecting cytochrome c oxidase activity

Preliminary experiments, performed to ascertain the best conditions for obtaining the maximum extraction and preservation of oxidase activity and the optimum conditions for assay, are not recorded in detail, but they accorded in general with the experience of other workers using cytochrome *c* oxidase prepared from different sources (Hartree, 1955).

Grinding the mycelium with quartz by hand in a mortar gave higher oxidase activities than homogenization with a glass homogenizer, the use of the Atomix blender or hand grinding in a mortar with other abrasives. Maximum activity was found when the temperature was kept as near 0° as possible and when the pH value did not drop below pH 7.0. When cell-free homogenates were kept at room temperature, inactivation of cytochrome *c* oxidase took place. This inactivation was less rapid at 2°; but for maximum activity it was necessary to remove the oxidase from the homogenate as soon as possible. This was done by centrifuging at 5000 g for 20 min. and then washing the particles which sedimented by resuspending them in the grinding media and re-centrifuging. The results (Table 1) showed that 77% of the oxidase activity was in the particle fraction. This value was only approximate however, as some inactivation took place in the homogenate as evidenced by the lower activity

of the homogenate when compared with the combined activities of the other two fractions.

Table 1. *Localization of cytochrome c oxidase activity within the cells*

Cell-free preparations from 36 hr. mycelium as described in text. Warburg manometer flasks contained in a total vol. of 2.0 ml.: 0.5 ml. extract; 10–50 μ M-cytochrome *c*; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in side arm and 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: air; temp. 25°. Recorded O₂ uptake as the calculated maximum corrected for autoxidation (see Methods).

Cell fraction	μ l. O ₂ uptake/hr.	μ l. O ₂ uptake/hr./mg. N
Cell-free homogenate (H)	11,000	1,160
Supernatant (SN)	2,750	550
Particles (P ₁)	9,250	2,000

The endogenous O₂ uptake of washed particle preparations, without added cytochrome *c* and hydroquinone, or with either alone, was negligible. Substrate concentrations greater than 0.08 M-hydroquinone did not give increased O₂ uptake at constant cytochrome *c* concentration; 0.1 M-hydroquinone was therefore used throughout. The concentration of enzyme preparations varied.

Fig. 1. The effect of cytochrome *c* concentration, on the activity at constant cytochrome oxidase concentration. Extract prepared from 48 hr. mycelium, as described in text, except the washed particles were resuspended in the original volume of buffer used for grinding. Warburg manometer flasks contained in a total vol. 2.5 ml.: 1.0 ml. extract; cytochrome *c* as indicated; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in side arm and 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: air; temp. 25°. Activity expressed as μ l. O₂ uptake/hr./ml. extract.

Normally, with constant enzyme concentration, enzyme activity increased with increasing cytochrome *c* concentration. However, with less concentrated enzyme preparations, maximum activity was found with cytochrome *c* concentrations of the order 50 μ M; higher cytochrome *c* concentrations gave lower activities (Fig. 1). In those instances, Q_{O_2} max. was calculated from the straight line part of the curve and the observed optimum Q_{O_2} with saturating cytochrome *c* concentration was not used (Lineweaver & Burk, 1934). The optimum pH value of the enzyme with 0.01 M-phosphate was pH 7.2.

Factors affecting Dopa oxidase activity

Unlike cytochrome *c* oxidase, Dopa oxidase activity was found mainly in the supernatant fraction (Table 2). The enzyme was therefore exposed continuously, from breaking the cells until completion of assay, to any inhibitors or denaturing compounds which might have been present in the crude homogenate.

Table 2. *Localization of Dopa oxidase activity within the cells*

Details of cell-free preparations in text. EEL colorimeter cells contained in total of 6.0 ml.: 1 ml. extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). Fraction W₄ was centrifuged down in assay cell.

Fraction	EEL units/hr./ fraction	EEL units/hr./ mg. N
Supernatant (SN ₀)	4080	350
Cell wall (W ₀) (by calculation)	1000	—
Supernatant (SN ₁)	4080	500
Particle (P)	0	—
Supernatant (SN ₂) (2nd extract)	620	460
Supernatant (SN ₃) (3rd extract)	200	210
Supernatant (SN ₄) (4th extract)	80	40
Cell wall (W ₄) (after 4th extract)	100	—

Although enzyme activity was found mainly in the supernatant fluid, some activity was only removed from the cell wall + quartz fraction by repeated re-extractions of this fraction with buffer. However, after a single re-extraction of the debris fraction, 81 % of the total activity was in the soluble fractions and with further re-extraction of the debris all but 2 % was found in the soluble fraction. No Dopa oxidase activity was associated with the particle fraction. During these experiments the enzyme, when stored in the cold, increased in activity; this increase depended on the pH value during extraction and storage.

Table 3. *The effect of pH of extraction and dialysis on the Dopa oxidase activity of extracts*

Extracts made from 3-day mycelium. Activity tested immediately and after dialysis overnight at 2° against 0.01 M-phosphate buffer. Extracts adjusted to pH 7.0 before assay. Spectrophotometer cells contained in a total of 3.0 ml.: 0.2 ml. enzyme extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). Extract preparation and assay details in text.

pH during extraction	pH of dialysis buffer	pH of prepara- tion after dialysis	μg. N/ ml.	Δ o.d./ min./ mg. N
7.0	Assayed at once	—	98	0.85
	7.0	7.0	90	4.7
	6.0	6.2	85	3.6
	5.0	5.4	60	0.98
	Overnight at 2°—no dialysis	—	95	4.0
6.0	Assayed at once	—	96	0.73
	6.0	6.1	78	3.1
	Overnight at 2°—no dialysis	—	90	2.5

Table 3 gives the Dopa oxidase activities of preparations extracted from the mycelium at various pH values at 2°. The greatest activity was found with preparations which had been extracted and dialysed at pH 7.0, although the initial activities of preparations extracted at pH 7.0 and 6.0 were not greatly different. The possibility that the increase in activity of dialysed preparations was due to the removal of a dialysable inhibitor was excluded since the only effect of undialysed preparations on the activity of dialysed ones was to cause a lag period, varying from several seconds to minutes, before oxidation of substrate took place. When the preparations were not dialysed, but left overnight at 2° at the pH value of extraction, an increase in activity occurred which was nearly as great as that in similar preparations which had been dialysed.

Table 4. *The effect of 'ageing' conditions on the Dopa oxidase activity of cell-free extracts*

Extracts made from 4-day mycelium and tested after 'ageing' as indicated. Spectrophotometer cells contained in a total of 3.0 ml.: 0.2 ml. extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). Extract preparation and assay details in text.

Conditions of 'ageing'	Δ O.D./min./ 100 μ g. N
Tested at once	0.09
After 1 hr. at 27°	0.16
After 3 hr. at 27°	0.18
After 8 hr. at 27°	0.32
After 12 hr. at 27°	0.32
After 1 hr. at 2°	0.13
After 8 hr. at 2°	0.35
After dialysis overnight at 2°, pH 7.0	0.39
After dialysis overnight at 2°, pH 7.0, then left for 3 days at 2°	0.39
After 8 hr. at 2° and 12 hr. at 27°	0.33

Maximum activity was found after 8 hr. at 2° and this activity was stable for at least several days in dialysed preparations stored at pH 7.0 and 2°. Dialysed preparations were also stable at 27° over the period tested (12 hr.). When fresh preparations were allowed to stand at room temperature they increased in activity faster than similar preparations left at 2°; but the final activity was less.

At constant enzyme concentrations, the activities of enzyme extracts increased with increasing substrate concentration, to 0.005 M-Dopa (Fig. 2). The optimum pH value for enzyme activity in 0.01 M-phosphate buffer was pH 7.0.

Substrate specificity of Dopa oxidase

The Dopa oxidase was tested for action on various possible substrates. It oxidized L-tyrosine and *p*-cresol with lag periods, Dopa and pyrocatecho rapidly and *p*-phenylenediamine very slowly; it did not oxidize hydroquinone.

Presence of cytochrome and oxidase in mycelia of various ages

The visible absorption bands were at approximately 603, 564, 550 and 518 $m\mu$., corresponding to the cytochrome bands (*a a₃*), *b*, *c* and *d* of yeast at 603, 564, 549 and 519 $m\mu$. respectively (Keilin, 1925). On passing O_2 gas or oxidising suspensions with a drop of H_2O_2 , the bands faded and were replaced by a diffuse and weak band at *c.* 566 $m\mu$.

Fig. 2. The effect of DL-3:4-dihydroxyphenylalanine concentration on the activity at constant Dopa oxidase concentration. Extract prepared as in text. Spectrophotometer cells of 1 cm. light path contained in a total vol. 3.0 ml.; 10 μ g. extract N; Dopa concentration as indicated and 0.01 M-phosphate buffer (pH 7.0). Activity expressed as Δ o.d./10 sec./10 μ g. extract N.

The bands could be seen *in situ* in reduced mycelial felts up to and including 3–4 days old. In older material, owing to brown pigment formation, they were not seen. Particle preparations were therefore made from the older mycelium, by the same method as for cytochrome oxidase determinations, except that 18% (w/v) of sucrose was included in the preparative and washing fluids. The washed particle preparations were resuspended in 70% (w/v) sucrose in water with a little sodium dithionite and examined spectroscopically. In this way, it was possible to establish the presence of the cytochrome system at all mycelial ages up to 7 days.

Table 5. *The cytochrome c oxidase and Dopa oxidase activity of extracts prepared from mycelia of various ages*

Extracts prepared and activity assayed as in text.

Age of mycelium in days	Cyt. <i>c</i> oxidase activity (μ l O_2 uptake/hr./mg. N)	Dopa oxidase activity (Δ o.d./min./mg. N)
1	1500	0
1½	1950	0
2	1900	0.4
3	1760	4.7
4	1600	4.0
5	1200	4.0

Assays for cytochrome *c* oxidase and Dopa oxidase activity of extracts prepared from mycelia of various ages showed that, while cytochrome *c* oxidase activity remained approximately constant with age of mycelium, Dopa oxidase activity was undetected before 48 hr. (Table 5). Maximum specific activity of Dopa oxidase was found with extracts from 3-day mycelium and the activity of extracts from 4- and 5-day mycelia was approximately constant. Undialysed extracts of young mycelia showed no Dopa oxidase activity.

Effects of some respiratory inhibitors on the activity of the extracted enzymes

Cytochrome *c* oxidase was inhibited by CO in the dark and by KCN but not by phenylthiourea. Dopa oxidase was inhibited by CO in the light and in the dark, by KCN, phenylthiourea and sodium diethyldithiocarbamate (Table 6).

Table 6. *Effects of some respiratory inhibitors on the cytochrome c oxidase and Dopa oxidase activity of cell-free extracts prepared from the mycelium*

For cytochrome *c* oxidase activities, Warburg manometer flasks contained in a total of 2.5 ml.: 0.5 ml. extract; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 50 μ M-cytochrome *c*; 0.1 M-hydroquinone in side arm and 0.15 ml. (w/v) KOH in centre well. Gas phase: air or as indicated; temp. 25°. Extract from 36 hr. mycelium.

For Dopa oxidase activities, Warburg manometer flasks contained in a total of 2.0 ml.: 0.5 ml. extract; 0.005 M-Dopa; 0.01 M-phosphate buffer (pH 7.0); 0.15 ml. 15% (w/v) KOH in centre well. Gas phase: as indicated; temp. 25°. Spectrophotometer cells of 1 cm. light path contained in a total volume of 3.0 ml.: 0.2 ml. extract; 0.005 M-Dopa and 0.01 M-phosphate buffer (pH 7.0). Inhibitors (as in table) added to comparable samples. Extract from 4-day mycelium.

Details of extracts and assays in text.

Enzyme system	Inhibitor	Inhibition (%)
Cytochrome <i>c</i> oxidase	17:1, N ₂ :O ₂ } Dark	0
	17:1, CO:O ₂ } Dark	70
	17:1, N ₂ :O ₂ } Light	0
	17:1, CO:O ₂ } Light	0
	0.001 M-KCN	95
	Sat. phenylthiourea	0
Dopa oxidase	17:1, N ₂ :O ₂ } Dark	0
	17:1, CO:O ₂ } Dark	60
	17:1, N ₂ :O ₂ } Light	0
	17:1, CO:O ₂ } Light	60
	0.001 M-KCN	75
	Sat. phenylthiourea	100
	0.001 M-dieca	65

Effects of the inhibitors on the endogenous respiration of intact mycelium

In order to measure the light-reversible inhibition of respiration by CO, the O₂ uptake of samples of washed mycelium were determined in the presence and absence of CO in the light and in the dark. Mycelia of all ages had an endogenous activity which was not greatly affected by the presence of glucose; glucose was therefore omitted from the assay medium in later experiments. The inhibition was determined relative to control samples respiring in air; also included in Table 7 are the results of experiments with samples in N₂/O₂ mixtures. Since the CO inhibition was competitive with respect to O₂, it was

necessary to use a high CO:O₂ ratio; however, very low proportions of O₂ caused inhibition of the respiration and the ratio of 17:1 (v/v) CO or N₂ to O₂ was therefore chosen. Even with 17:1 mixtures, it was necessary (*a*) to have growth conditions such that the mycelium grew as fine strands, and (*b*) not to have more than 2 mg. dry wt. of 24-hr.-old mycelium, increasing to 8 mg. at 4 days old, if the lowered O₂ tension was not to cause inhibition of O₂ uptake when compared with rates in air. Under these conditions, there was no significant difference between the rates in air and the rates in 17:1 (v/v) N₂:O₂ except at 2½ and 3 days, when samples respiring in the N₂/O₂ mixture were slightly inhibited. The CO inhibition was completely reversed by light, except at 2½ days, when about 10% remained (Table 7).

Table 7. *Inhibition of the respiration of intact mycelium of different ages in 17:1 (v/v) CO:O₂ mixtures, in the light and in the dark*

Warburg manometer flasks contained in a total volume of 2.0 ml.: 2–8 mg. dry wt. mycelium; 0.01 M-phosphate buffer (pH 6.0); 0.15 ml. 15% (w/v) KOH in centre well. Gas phase: as indicated; temp. 25°.

Age of mycelium in days	Q _{O₂} μl./hr./mg. dry wt.		Q _{O₂} (air) – Q _{O₂} (CO:O ₂) Q _{O₂} (air) × 100 %	
	Air	N ₂ :O ₂	Light	Dark
1	58	59	0	65
1½	50	49	0	60
2	36	38	0	60
2½	30	26	Variable about 20	65
3	27	23	10	60
4	20	22	0	60
5	19	—	—	—
6	17	18.5	—	—

0.001 M-KCN inhibited the respiration of 3½-day mycelium 80–90% while phenylthiourea was without effect.

DISCUSSION

Darby & Goddard (1950*a*) showed that with standard conditions of inoculation, culture and assay, it was possible to obtain reproducible results in respiration studies with fungal material. This has been confirmed with *Gelasinospora tetrasperma*. Cytochrome oxidase is the major terminal oxidase in the respiration of this organism. This enzyme was extracted from the mycelia of various ages and the light-reversible CO inhibition of its activity was 70%. With similar conditions, the CO inhibition of the respiration was about 60% and, except with 2½-day mycelia, it was completely reversed by light. If therefore the extracted enzyme was inhibited to the same extent when it was within the mycelium, the cytochrome system was responsible for approximately 85% of the O₂ uptake of the mycelium. This was confirmed by using cyanide, which inhibited the extracted enzyme almost completely and the respiration 80–90%. These results are in contrast with those of Darby &

Goddard (1950*b*) who extracted cytochrome oxidase from the mycelium of *Myrothecium verrucaria*; this enzyme was inhibited by CO and KCN yet the respiration was unaffected by these inhibitors.

A Dopa oxidase was extracted from 2- to 5-day mycelia and characterized as tyrosinase by its substrate specificity and inhibition with CO in the light and in the dark. This enzyme was more or less inactive *in situ* since the CO inhibition of the respiration was completely reversed by light, apart from 10 % of that of 2½-day mycelia.

Horowitz & Shen (1952) showed that a dialysable inhibitor caused inactivity of the tyrosinase of *Neurospora crassa* when *in situ*. This is not so, however, with *Gelasinospora tetrasperma* since undialysed extracts when added to dialysed extracts did not inhibit their activity.

Tyrosinase activities of extracts tested immediately on grinding the mycelium were considerably less than when stored for a period under suitable conditions, suggesting that the enzyme was in an inactive form within the mycelium. A similar conclusion has been reached by Mallette & Dawson (1949) and Mason (1956) who found that changes in the enzyme took place on purification which led to an increased diphenolase activity. Singer & Kearney (1954) pointed out that these changes may have occurred during extraction rather than purification.

Lastly, it may be that the inactivity of the enzyme when *in situ* is partly due to conditions within the mycelium such as non-availability of substrate or low O₂ tension (Nelson, 1950).

The small proportion of the respiration unaffected by KCN or CO may be explained by the presence of an enzyme system insensitive to these poisons; this system might only function when the metal-containing enzymes have been inhibited (James, 1953*a*).

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