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Changes in Fungi with Age II. Respiration and Respiratory Enzymes of *Rhizoctonia solani* and *Sclerotium bataticola*

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Changes in Fungi with Age

II. Respiration and Respiratory Enzymes of *Rhizoctonia solani* and *Sclerotium bataticola*[1]

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ABSTRACT

VAN ETTEN, JAMES L. (University of Illinois, Urbana), H. PETER MOLITORIS, AND DAVID GOTTLIEB. Changes in fungi with age. II. Respiration and respiratory enzymes of *Rhizoctonia solani* and *Sclerotium bataticola*. J. Bacteriol. 91:169–175. 1966.—The rate of respiration of *Rhizoctonia solani* and *Sclerotium bataticola* decreased with age. This decrease in respiratory rate might be produced by a decrease in the specific activity of one or more enzymes involved in carbohydrate metabolism. Specific activities in cell-free extracts were measured for most of the enzymes in the hexose monophosphate shunt, Embden-Meyerhof-Parnas pathway, tricarboxylic acid cycle, and terminal electron-transport system. In addition, glucose oxidase, isocitritase, and malic enzyme were measured. In *R. solani*, increases in activity with age occurred for hexokinase, α-glycerolphosphate dehydrogenase, malic dehydrogenase, and cytochrome oxidase. Decreases occurred for phosphohexokinase, aconitase, nicotinamide adenine dinucleotide-specific isocitric dehydrogenase, reduced nicotinamide adenine dinucleotide oxidase, and at least one of the enzymes between 3-phosphoglycerate and pyruvate. In *S. bataticola*, increases in activity with age were observed for phosphohexokinase, pyruvic dehydrogenase, fumarase, malic dehydrogenase, and malic enzyme, whereas none of the enzymes decreased. The specific activities of the remaining enzymes did not change with age in either fungus.

The previous paper in this series dealt with changes in chemical composition of fungal cells as they aged (10). An extension of these studies revealed a decrease in the rate of respiration of *Rhizoctonia solani* and *Sclerotium bataticola* with age. That such changes in respiratory rate might be involved in cellular aging is also indicated by reports on a number of other fungi; the Q_{O_2} was greatest during rapid growth but later decreased (*see also* 3, 6, 19). One explanation for such a decrease in the rate of respiration might be a reduced specific activity of one or more of the respiratory enzymes as the cells aged. This report examines the relationship of respiration and enzyme activity to cell age in *R. solani* and *S. bataticola*.

[1] Part of the dissertation of James L. Van Etten, presented to the Graduate Faculty of the University of Illinois in partial fulfillment of requirements for the Ph.D. degree.

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MATERIALS AND METHODS

The two fungi, *R. solani* and *S. bataticola*, were obtained, grown, and harvested as previously described (10). Whole-cell suspensions were prepared for the respiration studies by briefly homogenizing 1 g of mycelium (wet weight) in 10 ml of 0.05 M potassium phosphate buffer, *p*H 7.0 (*R. solani*), or 0.9% sodium chloride (*S. bataticola*), with a metal "micro" Waring Blendor. Because of the high endogenous oxygen consumption by both fungi, the cell suspensions were starved for 2 hr in Warburg flasks prior to the addition of glucose. Dry-weight determinations were made on separate portions of the mycelium from those used for the respiration studies. Respiratory measurements were made by use of standard Warburg techniques at 29.5 C (28). The assay systems always contained the following: cell suspension, 0.5 or 1.0 ml; potassium phosphate buffer (*p*H 7.0), 50 μmoles; magnesium chloride, 10 μmoles; glucose, 50 μmoles (when added); and distilled water to 3.0 ml. When oxygen uptake was measured, 0.2 ml of a 20% solution of potassium hydroxide was placed in the center well. For anaerobic conditions, the Warburg vessels were flushed with nitrogen prior to the initiation of the experiment.

Cell-free extracts were prepared at 4 C by placing fresh mycelium and acid-washed sand (in a ratio of 1:2) in a precooled mortar. Tris(hydroxymethyl)-aminomethane (Tris) buffer (0.05 M, *p*H 7.0) in 0.8 M sucrose was slowly added, and the mixture was ground with a pestle for approximately 5 min until a final ratio of approximately 1 g of mycelium to 2 ml of buffer was attained. The slurry was then centrifuged at 600 × *g* for 20 min, and the pellet was discarded. Microscopic examination of the supernatant fluid and the pellet indicated complete breakage of the cells. The amount of protein in the cell-free extracts was determined by the method of Lowry et al. (17), with bovine serum albumin as a standard.

All spectrophotometric enzyme assays were conducted on a Bausch and Lomb Spectronic-505 spectrophotometer coupled with a Bausch and Lomb V.O.M. recorder. Endogenous activity (assay system without substrate) served as the blank, and boiled cell-free extract was used as a control. An extinction coefficient of 6.22 (11) was used to calculate the enzymatic activity in all of the reactions dependent on the reduction or oxidation of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP).

The specific activities of the following enzymes were measured by methods similar to those described by Caltrider and Gottlieb (5): hexokinase, phosphoglucoisomerase, 6-phosphofructokinase, aldolase, triose phosphate isomerase, α-glycerolphosphate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, isocitric dehydrogenase, malic dehydrogenase, and the reaction sequence involving phosphoglyceromutase, enolase, and pyruvate kinase. Glyceraldehyde-3-phosphate dehydrogenase was assayed according to the method of Krebs (15). Pyruvic dehydrogenase was assayed by the methods described by Dowler, Shaw, and Gottlieb (8) and by Schindler (24). Alcohol and lactic dehydrogenases were measured by determining the oxidation or reduction of pyridine nucleotide in the presence of appropriate substrates (14, 23). Aconitase and fumarase were assayed according to the method of Racker (22). α-Ketoglutaric dehydrogenase was determined by the method of Kaufman (12). Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase, succinic dehydrogenase, and $NADH_2$-cytochrome *c* reductase were assayed according to Dowler et al. (8). Isocitritase was assayed by two methods, forming either the glyoxylate phenylhydrazone (7) or the glyoxylate semicarbazone (21). Malic enzyme was assayed by the method of Ochoa (20) and glucose oxidase by the method of Bentley (2).

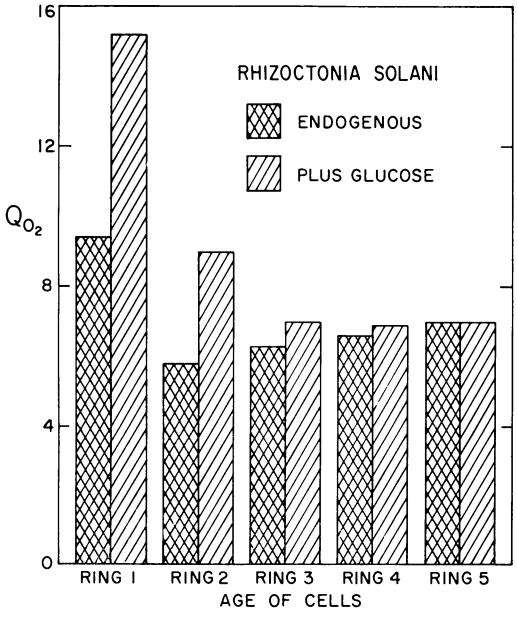

FIG. 1. *Relationship between fungal age of Rhizoctonia solani and* Q_{O_2} *in the presence and absence of glucose. The cells were starved for 2 hr prior to the addition of glucose. Ring 1 contains cells 0 to 16 hr old; ring 2, 16 to 31 hr; ring 3, 31 to 43 hr; ring 4, 43 to 56 hr; ring 5, 56 to 80 hr.*

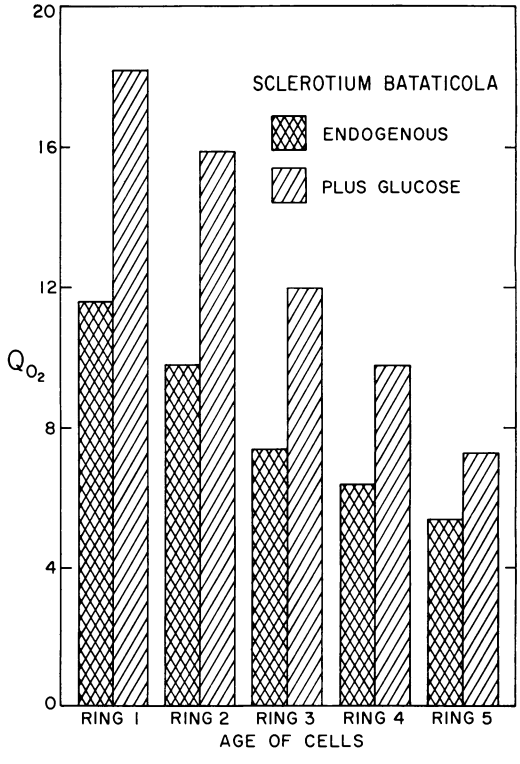

FIG. 2. *Relationship between fungal age of Sclerotium bataticola and* Q_{O_2} *in the presence and absence of glucose. The cells were starved for 2 hr prior to the addition of glucose. Ring 1 contains cells 0 to 28 hr old; ring 2, 28 to 57 hr; ring 3, 57 to 78 hr; ring 4, 78 to 102 hr; ring 5, 102 to 144 hr.*

Cytochrome oxidase was measured potentiometrically in a G. M. E. Oxygraph, model K (Gilson Medical Electronics, Middleton, Wis.). The assay system contained: potassium phosphate buffer (*p*H 7.0), 100 μmoles; cytochrome *c*, 2 mg; ascorbic acid, 11.4 μmoles; cell-free extract; and water to a volume of 2.0 ml.

Each value reported in the respiration and enzyme studies was the average value of at least two experiments, and each experiment contained at least two replicates.

RESULTS

The endogenous and total Q_{O_2} values of both *R. solani* (Fig. 1) and *S. bataticola* (Fig. 2) declined with age. Starvation of the young cells for 2 hr resulted in the oxidation of exogenous substrate by both fungi, but older cells did not respond to this treatment. When glucose was added after the starvation period, the Q_{O_2} values of the younger cells were restored to their original levels prior to the starvation period. When the microliters of oxygen consumed per hour were calculated on a protein basis instead of a dry-weight basis, the decrease in total oxygen uptake with age was less pronounced for both fungi (Table 1). However, there was still about a 40% decrease in respiration with age. Anaerobic carbon dioxide production in *S. bataticola* decreased from 4.5 μliters per hr per mg of dry weight in the youngest cells to 1.8 in the oldest cells. A decrease in $Q_{CO_2}^{N_2}$ from 2.5 to 1.8 with age also occurred in *R. solani*, but, since the carbon dioxide production was so low, the values were probably insignificant.

Neither endogenous respiratory quotients (RQ) nor the RQ with glucose as substrate changed for either fungus with age (Table 2). The endogenous RQ values of *S. bataticola* were 0.6 to 0.7 in cells of all ages, but, when glucose was added as a substrate, the RQ values increased to about 1.0. No such shift was observed with *R. solani*, which had a RQ between 0.8 and 0.9 at all ages in the presence and absence of glucose.

Since the rate of respiration decreased with age in both fungi, the activities of the respiratory enzymes were investigated. The decrease in respiratory rate with age could be explained if there were a decrease in the specific activity of one or several enzymes involved in carbohydrate metabolism.

The specific activities of the respiratory enzymes in *R. solani* and *S. bataticola* which did not change with age are listed in Table 3. The enzymes in which the specific activity changed with the age of the cell are listed in Table 4 for *R. solani* and Table 5 for *S. bataticola*. In both fungi the three enzymes from 3-phosphoglyceric acid to pyruvic acid were measured as a unit, and, since the overall reaction in both fungi was sensitive to fluoride, the normal enolase enzyme was assumed to be present. In both fungi, pyruvic dehydrogenase was only detected by the method of Schindler (24).

In *R. solani*, the specific activities of phosphohexokinase, aconitase, NAD-specific isocitric dehydrogenase, $NADH_2$ oxidase, and at least one of the enzymes between 3-phosphoglyceric acid and pyruvic acid decreased with age. Hexokinase, α-glycerolphosphate dehydrogenase, malic dehydrogenase, and cytochrome oxidase increased with cell age (Table 4). No change with age in

TABLE 1. *Relationship between fungal age of Rhizoctonia solani and Sclerotium bataticola and the amount of oxygen consumed per milligram of protein, with glucose as a substrate*

Organism	Ring	Age	Amt of oxygen taken up per hr per mg of protein
		hr	*μliters*
*R. solani**	1	0–16	125
	2	16–31	91
	3	31–43	71
	4	43–56	72
	5	56–80	72
*S. bataticola**	1	0–28	124
	2	28–57	133
	3	57–78	111
	4	78–102	106
	5	102–144	87

* The first hour after starving the cells for 2 hr.

TABLE 2. *Respiratory quotients for Rhizoctonia solani and Sclerotium bataticola at various ages, with and without glucose*

Organism	Ring	Age	RQ	
			Endogenous	Plus glucose
		hr		
*R. solani**	1	0–16	0.9	0.9
	2	16–31	0.8	0.8
	3	31–43	0.9	0.8
	4	43–56	0.9	0.8
	5	56–80	0.8	0.8
*S. bataticola**	1	0–28	0.7	0.9
	2	28–57	0.7	1.0
	3	57–78	0.7	1.0
	4	78–102	0.6	1.0
	5	102–144	0.6	0.9

* Cells were starved for 2 hr prior to the addition of the substrate.

TABLE 3. *Specific activities in cell-free extracts of the enzymes involved in carbohydrate metabolism of Rhizoctonia solani and Sclerotium bataticola which did not change with age*[a]

Enzyme	Amt of substrate transformed per min per mg of protein	
	R. solani	*S. bataticola*
	μmoles	μmoles
Phosphoglucomutase	0.010	0.019
Hexokinase	I	T
Phosphoglucoisomerase	0.053	0.12
Phosphohexokinase	D	I
Aldolase	0.05	0.12
Triose phosphate isomerase	1.7	14.6
α-Glycerolphosphate dehydrogenase	I	0
Glyceraldehyde-3-phosphate dehydrogenase	0	+[b]
2,3-Phosphoglyceric mutase[c]	D	27.4
Enolase[c]	D	27.4
Pyruvic kinase[c]	D	27.4
Pyruvic dehydrogenase	0.008	I
Lactic dehydrogenase	0	0
Alcohol dehydrogenase	0.003	0
Glucose-6-phosphate dehydrogenase	0.18	0.11
6-Phosphogluconate dehydrogenase	0.050	0.054
Glucose oxidase	0	0
Aconitase[d]	D	149
Isocitric dehydrogenase (NAD)	D	0
Isocitric dehydrogenase (NADP)	0.011	0.003
α-Ketoglutarate dehydrogenase	0	0
Succinic dehydrogenase	0.019	0.020
Fumarase[d]	342	I
Malic dehydrogenase	I	I
Malic enzyme	0	I
Isocitritase	T	T
$NADH_2$ oxidase	D	0.071
$NADH_2$-cytochrome *c* reductase	NM	0.058
Cytochrome oxidase	I	NM

[a] Where changes in specific activity occurred, I denotes increase and D denotes decrease in activity with age. The quantitative values for the changes are given in Tables 4 and 5. Symbols: T, trace; NM, not measured.

[b] Activity was detected, but the specific activity was not calculated because the reaction was not linear with time.

[c] Micromoles of pyruvate produced per hour per milligram of protein based on a 10-min incubation period.

[d] Specific activity is expressed as units per minute per milligram of protein. A unit is defined as that amount of enzyme which gives an initial increase in optical density of 0.001 per min at 240 mμ.

the specific activities of the remaining enzymes measured was observed (Table 3). Glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, glucose oxidase, α-ketoglutarate dehydrogenase, and malic enzyme were not detected in *R. solani*.

In *S. bataticola*, the specific activities of phosphohexokinase, pyruvic dehydrogenase, fumarase, malic dehydrogenase, and malic enzyme increased with age, but none of them showed decreased activity (Table 5). The specific activity of glyceraldehyde-3-phosphate dehydrogenase in *S. bataticola* was not calculated, since a linear relationship between activity and time was not obtained. In addition, the enzyme was only active for about 2 min. No differences in the specific activities of the remaining enzymes were observed with age. Activities for α-glycerolphosphate dehydrogenase, lactic dehydrogenase, alcohol dehydrogenase, glucose oxidase, NAD-specific isocitric dehydrogenase, and α-ketoglutarate dehydrogenase were not detected in the cell-free extracts from *S. bataticola*.

The specific activity for $NADH_2$ oxidase in *R. solani* decreased markedly with age from a specific activity of 0.046 in the young cells to 0.012 in the old cells. However, when cytochrome *c* was omitted from the assay system for $NADH_2$ oxidase, no difference in the specific activity with age was observed. Therefore, the young cells of *R. solani* apparently had a greater potential for oxidizing $NADH_2$ than did the old cells when cytochrome *c*, or perhaps some other electron acceptor, was added. $NADH_2$-cytochrome *c* reductase could not be measured in *R. solani*, because none of the common inhibitors of cytochrome oxidase would completely block cytochrome *c* oxidation. As soon as reduced cytochrome *c* was formed, it was reoxidized, even in the presence of azide or cyanide. This reoxidation of cytochrome *c* was more rapid in the old cells than in the young ones, even though the percentage of inhibition of $NADH_2$ oxidase by azide and cyanide was approximately the same for cells of all ages. Reduced cytochrome *c* from *R. solani* was apparently oxidized by at least two pathways. When cytochrome oxidase was assayed on the oxygen electrode, it was completely inhibited by both azide and cyanide. But if $NADH_2$-cytochrome *c* reductase was measured spectrophotometrically at 550 mμ, azide or cyanide was able to inhibit only partially the

TABLE 4. *Specific activities of the enzymes involved in carbohydrate metabolism in cell-free extracts of Rhizoctonia solani which change with age*

Enzyme	Micromoles of substrate transformed per min per mg of protein				
	Ring 1 (0–16 hr)	Ring 2 (16–31 hr)	Ring 3 (31–43 hr)	Ring 5 (56–80 hr)	Per cent change[a]
Hexokinase	Trace	Trace	0.002	0.008	+
Phosphohexokinase	0.09	0.07	0.06	0.06	−35
α-Glycerolphosphate dehydrogenase	0.012	0.015	0.015	0.018	+50
2,3-Phosphoglyceric mutase[b]	18.4			10.4	−43
Enolase[b]	18.4			10.4	−43
Pyruvic kinase[b]	18.4			10.4	−43
Aconitase[c]	218	194	167	153	−30
Isocitric dehydrogenase (NAD)	0.002	0.003	0	0	−100
Malic dehydrogenase	2.42	2.90	3.74	4.36	+80
$NADH_2$ oxidase	0.046	0.016	0.012	0.012	−74
Cytochrome oxidase[d]	15	13	39	59	+293

[a] Symbols: +, per cent increase; −, per cent decrease.

[b] Micromoles of pyruvate produced per hour per milligram of protein based on a 10-min incubation period.

[c] Specific activity is expressed as units per minute per milligram of protein. A unit is defined as that amount of enzyme which gives an initial increase in optical density of 0.001 per min at 240 mμ.

[d] An amount of oxygen taken up per minute per milligram of protein.

TABLE 5. *Specific activities of the enzymes involved in carbohydrate metabolism in cell-free extracts of Sclerotium bataticola which change with age*

Enzyme	Micromoles of substrate transformed per min per mg of protein				
	Ring 1 (0–28 hr)	Ring 2 (28–57 hr)	Ring 3 (57–78 hr)	Ring 5 (102–144 hr)	Per cent increase
Phosphohexokinase	0.10	0.13	0.14	0.15	50
Pyruvic dehydrogenase	0.003	0.003	0.006	0.008	170
Fumarase*	490	470	500	600	22
Malic dehydrogenase	2.58	2.56	3.00	3.30	28
Malic enzyme	0.022	0.053	0.063	0.070	220

* Specific activity is expressed as units per minute per milligram of protein. A unit is defined as that amount of enzyme which gives an initial increase in optical density of 0.001 per min at 240 mμ.

reoxidation of cytochrome *c*. A similar reoxidation of cytochrome *c* in the presence of azide or cyanide was obtained when succinate-cytochrome *c* reductase was assayed.

The effect of cyanide, azide, and antimycin A on $NADH_2$ oxidase from the youngest and oldest cells of *R. solani* and *S. bataticola* was determined. $NADH_2$ oxidase from both the youngest and oldest cells of *S. bataticola* was inhibited 70% by 20 μmoles of sodium azide per ml and 100% by 5 μg of antimycin A or 15 μmoles of potassium cyanide per ml. *R. solani* $NADH_2$ oxidase was relatively insensitive to the same concentrations of these inhibitors. $NADH_2$ oxidase was inhibited about 80% by potassium cyanide in the youngest and oldest cells, and sodium azide and antimycin A inhibited 60 and 25%, respectively.

The specific activities of $NADH_2$ oxidase from the young cells of both fungi were stimulated approximately two- to threefold by the addition of 0.7 mg of cytochrome *c*. The same enzyme in the old cells of *S. bataticola* was stimulated about twofold by exogenous cytochrome *c*, but exogenous cytochrome *c* had no effect on $NADH_2$ oxidase activity in the old cells of *R. solani*.

DISCUSSION

The low RQ values of *S. bataticola* and the somewhat higher RQ values of *R. solani* are rational on the basis of the nature and quantity of their storage products (10). *S. bataticola* had a high lipid content and an endogenous RQ of 0.6 to 0.7 in cells of all ages, indicating a predominant fatty acid metabolism (9). The RQ increased to approximately 1.0 when glucose was

added, indicating a shift to carbohydrate metabolism. The high endogenous RQ of *R. solani*, 0.8 to 0.9, reflects its high carbohydrate content. Thus, adding glucose did not cause a shift in its RQ. Though actual values of RQ are sometimes misleading (18), the shifts in RQ in the presence of glucose correlate with the fungal composition, and probably indicate the nature of the endogenous substrate being metabolized.

Since the rate of respiration decreased with age in both fungi, one might expect that a change in the specific activity of one or more of the respiratory enzymes would be associated with this decrease. Changes in specific activities were found for one or more enzymes in both fungi; however, no single enzyme, common to both fungi, showed a decreased activity. In fact, the only change common to both fungi was an increase in malic dehydrogenase. It is possible that the age-dependent decreased respiratory rate might be caused by an alteration in activity for different enzymes in different fungi. If this were the case, then the diminished respiratory rate in *R. solani* could be explained by the decrease in activity for phosphohexokinase, some of the enzymes involved in the transformation of 3-phosphoglyceric acid to pyruvic acid, aconitase, NAD-specific isocitric dehydrogenase, or $NADH_2$ oxidase. However, the decrease in the rate of respiration in *S. bataticola* could not be explained on this basis, since none of the respiratory enzymes decreased in activity with age. Since, of all the enzymes measured, there were very few changes with age, it is difficult to explain the decrease in the rate of respiration of both fungi as due to a general deficiency of respiratory enzymes. These results are similar to those of Barrows, Falzone, and Shock (1), who studied respiration in rat tissues. These investigators attributed the decreased respiration rate to a reduction in the number of mitochondria per cell. Studies on the number of mitochondria per cell at various stages of development are lacking for mycelial fungi, but changes in their morphology with cell age have been observed (26).

The investigation of enzyme activity cannot be considered as definitive, since it was difficult or impossible to detect some of the enzymes in cell-free extracts. Lactic dehydrogenase, glucose oxidase, and α-ketoglutarate dehydrogenase activities were not found in either fungus. The inability to find α-ketoglutarate dehydrogenase activity in mycelial fungi has also been reported by Dowler et al. (8) and Caltrider and Gottlieb (5). These investigators were also unable to detect pyruvic dehydrogenase activity by the usual techniques. In the present study, pyruvic dehydrogenase activity was detected only by the method of Schindler (24). Glyceraldehyde-3-phosphate dehydrogenase was detected in *S. bataticola* but not in *R. solani*. However, the activity in *S. bataticola* lasted for only about 2 min, and during that time the rate was not linear. Glyceraldehyde-3-phosphate dehydrogenase in *Penicillium chrysogenum* (25) required NADP for activity instead of NAD; however, NADP did not cause activity in *R. solani*. Therefore, some other explanation must be sought for the inactivity of this enzyme in cell-free extracts of *R. solani*, since it is probably present in the whole cells. That α-glycerolphosphate dehydrogenase activity was found in *R. solani* and not in *S. bataticola* was surprising, since the latter is extremely high in fatty acids (10), and this enzyme is probably necessary for triglyceride formation.

The effect of azide and antimycin A to inhibit only partially the electron transport in *R. solani* was not completely surprising. Dowler et al. (8) reported that $NADH_2$ oxidase in a number of fungi, including *Rhizoctonia* sp., was only partially inhibited by antimycin A, and Tolmsoff (27) also reported that *R. solani* mitochondria were resistant to a number of classical respiratory inhibitors other than cyanide.

The reoxidation of reduced cytochrome *c* by extracts of older cells of *R. solani* in the presence of inhibitors of cytochrome *c* oxidase was surprising. This phenomenon could be explained if a cytochrome *c* peroxidase was present in this fungus.

One possible explanation for the decrease in the rate of respiration with age could be that, even though old cells apparently have the same enzyme potential as young cells, old cells are not able to function to maximal capacity because of the accumulation of feed-back inhibitors, lack of substrate, cofactor, or some other controlling agent. Another possibility for the decrease in respiration with age is that cell permeability to oxygen or other nutrients decreases. There is some evidence that cell permeability is altered with age in both higher plants and animals (4, 13, 16). It is possible that the cell membrane of fungi is altered, since the ergosterol content in *R. solani*, *S. bataticola*, and *Penicillium atrovenetum* decreased with age (10, 29). The effects of sterols in changing cell permeability, which have been mentioned in the preceding paper (10), might explain the respiratory decreases reported in this paper.

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