Respiration in Organic Acid-Forming Molds

Part III. Alteration of the Electron Transport System during Lactate Fermentation in *Rhizopus oryzae*

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Mycelia of *Rhizopus oryzae*, a lactate forming fungus, were used to study respiration and glucose degradation in this organism.

The respiration of the mycelia obtained during an early stage of shake culture growth, when only a little lactate was produced, was completely inhibited by KCN, while the respiration of the mycelia obtained from older shake cultures, or from surface cultures, regardless of the age, was not inhibited by KCN. These results suggest that energy for growth of *Rhizopus oryzae* is predominantly obtained by a respiratory system involving cytochromes and that a loss of this system occurs in older shake cultures which then begin to accumulate lactate. This observation was confirmed by the enzymatic studies on cell-free extracts.

INTRODUCTION

In the previous paper,¹⁾ we reported the contents of cytochrome c, flavins and coenzyme Q₉ in the mycelia of lactate forming *Rhizopus oryzae*. The results showed that the mycelia of shake cultures were rich in cytochrome c and FAD, and lower in FMN than surface culture mycelia. Moreover, the contents of cytochrome c and FAD decrease with the progress of the fermentation.

In the present study an attempt was made to determine the respiration of *Rhizopus oryzae* in order to clarify the reason for the large amount of lactic acid accumulated by the organism under certain aerobic condition.

METHODS

The microorganism and its cultural conditions were described in the preceding paper.¹⁾

Respiratory quotient and lactate formation by mycelium. Warburg experiments were carried out with glucose as the substrate in order to determine CO₂ and O₂ balance. The residual glucose and lactate

formed were determined by the Somogyi method modified by Kobayashi and Tabuchi²⁾ and the Barker-Summerson method,³⁾ respectively. The rate of oxygen uptake (QO₂) was expressed as the volume (μl) of oxygen absorbed per mg dried mycelia per hour.

Enzyme activities of cell-free extracts. Washed mycelia were ground with an equal weight of quartz sand in a porcelain mortar, and then suspended in 20 ml of 0.1 M phosphate buffer (pH 6.8) per 1 g mycelia (dried weight). The suspension was stirred for 20 minutes in an ice bath and the cell-free extract was obtained by centrifugation at 20,000×g for 20 minutes at 0°C.

To compare the various enzymatic activities of the cell-free extract, the following assay methods were adopted; namely, the method of Racker⁴⁾ for alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1), which was described previously⁵⁾ for L-lactic dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27); the method of Mahler⁶⁾ for NADH₂-cyto-

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chrome c reductase (NADH₂-cytochrome c oxidoreductase, EC 1.6.2.1); the method of Smith⁷ for cytochrome c oxidase (cytochrome c: O₂ oxidoreductase, EC 1.9.3.1); the method of Fukui⁸ and Mizushima⁹ for NADH₂ peroxidase (NADH₂: H₂O₂ oxidoreductase, EC 1.11.1.2) and the NADH₂ oxidase system. For the dehydrogenation of NADH₂ or NADPH₂, Kikuchi's method¹⁰ of NADPH₂ diaphorase (NADPH₂: (acceptor) oxidoreductase, EC 1.6.99.1) was adopted, using 2,6-dichlorophenol indophenol, vitamin K₃ and coenzyme Q₀ as hydrogen acceptors. As for vitamin K₃ and coenzyme Q₀, 2 μ moles and 1 μ mole, respectively, were separately dissolved in 0.5 ml methanol, and each 0.05 ml of the methanol solution was added to 3 ml of reaction mixtures.

The enzyme activities were expressed as the oxidation or reduction velocity of substrates (mole/mg protein/hour), from the change of optical density, using a Hitachi EPU-2A spectrophotometer (d=1 cm).

Protein was determined by the colorimetric method of Lowry et al.,¹¹ for which bovine albumin was used as the standard.

Chemicals. NAD, NADH₂ and NADPH₂ were purchased from Sigma Chemical Company. Vitamin K₃ was purchased from Daiichi-Kogyoseiyaku. Cytochrome c and coenzyme Q₀ were obtained from *Rhizopus oryzae* as described in the preceding paper.⁹

EXPERIMENTAL AND RESULTS

1. Respiratory quotient and lactate formation of the mycelia

Comparative investigations were carried out using the Warburg respirometric technique on the degradation of glucose by the mycelia obtained from surface and shake cultures at various periods of incubation. Typical results are shown in Fig. 1 and Table I.

The mycelia of surface cultures demonstrated oxygen uptake, carbon dioxide evolution and lactate formation in an almost constant ratio, regardless of the cultural age. The

FIG. 1. Change in the Oxygen Uptake (Q_{O_2}) of Mycelia during the Course of Shake Culture Incubation.

Each Warburg vessel contained 5–10 mg of mycelia (dry weight), 30 μ mol. of glucose and 0.5 ml of 0.1 M phosphate buffer (pH 6.5). Total volume was brought to 2.5 ml with distilled water.

Reaction temperature: 35°C.

Broken line: KCN was added to give the final concentration of 3.3×10^{-3} M.

End: endogenous respiration.

respiratory quotient (Q_{O_2} (glucose)) was 25 to 30 in the mycelia obtained in the presence of 4.0 mg $ZnSO_4 \cdot 7H_2O$ per 100 ml medium and 9 to 15 in those obtained without zinc. Moreover, oxygen uptake was activated rather than inhibited by 3.3×10^{-3} M KCN.

On the other hand, younger mycelia from about 14-hour shake cultures produced a small amount of lactate. Both oxygen uptake and glucose degradation of the mycelia were completely inhibited by 3.3×10^{-3} M KCN. The inhibitory effects of KCN decreased with increasing culture age and finally glucose con-

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TABLE I. RESPIRATION AND LACTATE FORMING ACTIVITY OF MYCELIA

Cultural condition and age of mycelia	Shake culture						Surface culture			
	14 hr.			22 hr.			48 hr.		96 hr.	
	— KCN		Acrif	— KCN		Acrif	— KCN		— KCN	
Glucose consumed*	0.55	0	0	1.53	2.46	0	0.47	0.48	0.41	0.39
QO ₂ (glucose)**	40	0	0	55	57	0	10.8	12.3	9.2	9.9
CO ₂ evolution***	3.30	—	—	1.61	1.04	—	1.06	1.11	1.00	1.13
Lactate formed****	0.64	—	—	1.21	1.52	—	1.34	1.46	1.37	1.49

Medium: basal medium added ZnSO₄·7H₂O 0.4 mg/100 ml

KCN: final concentration, 3.3×10^{-8} M.

Acrif: acriflavin, 1.6×10^{-8} M.

Reaction: glucose 25 μ mol., mycelia 5–10 mg (dry wt.), pH 6.5, total volume 2.5 ml.

* Glucose consumed: μ mol./mg dry mycelia /hr.

** QO₂ (glucose): O₂ uptake μ l/mg dry mycelia /hr.

*** CO₂ evolution: mole ratio to glucose consumed

**** Lactate formed: mole ratio to glucose consumed

TABLE II. HYDROGEN OR ELECTRON TRANSPORTING ACTIVITIES OF CELL-FREE EXTRACTS

Cultural condition and age of mycelia		(hr.)	Shake		Surface	
			14	22	48	96
Spectrophotometric assay for enzyme reactions						
Reaction		Wavelength measured (m μ)	Activity of extract μ mol./mg protein/hr.			
Cytochrome c oxidase		549 (cyt-c)	1.52	0.21	0.24	0.15
" + KCN			0	0	—	—
NADH ₂ dehydrogenation H-acceptor						
Cytochrome c (NADH ₂ -cyt c reductase)		549	1.16	0.45	0.11	0.25
2,6-DCIP		340 (NADH ₂)	20.25	12.18	8.82	7.94
Oxygen (NADH ₂ oxidase)		"	—	$\pm$	0	—
Vitamin K ₃		"	11.00	4.22	3.44	—
Coenzyme Q ₉		"	0	0	0	0
Pyruvate (Lactic dehydrogenase)		"	*3.80	6.30	5.47	3.50
NADPH ₂ dehydrogenation H-acceptor						
Cytochrome c (NADPH ₂ -cyt c reductase)		549	—	0.30	—	—
2,6-DCIP		340	—	9.63	—	—
Vitamin K ₃		"	—	5.79	—	—
Coenzyme Q ₉		"	—	0	—	—
Pyruvate		"	—	0	—	—
L-Lactate dehydrogenation H-acceptor						
2,6-DCIP (L-lactic dehydrogenase)		600 (2,6-DCIP)	0.33	1.26	2.59	1.39
" + acriflavin		"	*0.70	1.59	2.10	1.26
Ethanol dehydrogenation H-acceptor						
NAD (alcohol dehydrogenase)		340	—	7.31	7.60	—
Mycelial wt. corresponding to 1 mg protein of extract (mg)			3.5	3.5	6.4	4.4
			*3.5	4.2	5.3	6.2

The mycelia used for the preparation of cell-free extract were the same with those of Table I.

For the calculation of the activities (μ mol./mg protein/hr.), the following wavelengths and millimolar extinction coefficients were adopted: 19.2 at 549 m μ for cytochrome c (difference between the optical densities at 549 m μ of reduced and oxidized cytochrome c); 6.22 at 340 m μ for NADH₂ and NADPH₂; 19.1 at 600 m μ for 2,6-dichlorophenol indophenol.

*: Aliquots of another cell-free extract were used for assay of the enzyme activities.

sumption and lactate formation were markedly activated by KCN. It is noteworthy that the respiratory quotient was increased in the presence of KCN, and oxygen uptake per equivalent of glucose consumed was much lower than that without KCN.

As shown in Table I, oxygen uptake and glucose degradation were completely inhibited in shake culture mycelia by 1.6×10^{-3} M acriflavin. Though not shown in the Table, a similar inhibitory effect by vitamin K₃ (1.2×10^{-3} M) and 2,4-dinitrophenol (10^{-3} M) was observed.

Endogenous respiration of mycelia was increased with increase in culture age, and the ratio of carbon dioxide evolution to oxygen uptake was less than 1, namely 0.7 to 0.8, indicating that fatty substances from the mycelia were being oxidized as the substrate of endogenous respiration.

2. Enzymatic activities of the cell-free extract

Various enzyme activities of the cell-free extract are listed in Table II. Although crude cell-free extracts were used for the enzyme assay, it is possible to compare the activities. Remarkable contrast was observed among the activities of cytochrome c oxidase, of NADH₂-cytochrome c reductase and of lactic dehydrogenase. The first two enzymes demonstrated high activity in the young mycelia of 14-hour

shake cultures; while the latter showed high activity in the mycelia of 22-hour shake cultures and in those of surface cultures.

3. Effect of KCN on the lactate fermentation in shake culture

Addition of 2×10^{-4} M KCN to the medium at the beginning of the incubation markedly inhibited the growth of *Rhizopus oryzae* in shake culture. Therefore, the microorganism was cultured on the shaker in the basal medium supplied with 0.4 mg ZnSO₄·7H₂O per 100 ml. Potassium cyanide was added to a broth culture when lactate accumulation began (21 hours) and incubation was continued. These results are shown in Table III. A high yield of lactate was not obtained in this experiment, presumably because of the addition of zinc to the medium. However, the addition of KCN increased the consumption of glucose and the yield of lactate. The highest yield of lactate was obtained at the concentration of 2×10^{-4} M KCN.

DISCUSSION

Aerobic respiration plays an important part in lactate fermentation by *Rhizopus oryzae*. However, little was previously known about the respiration in this fungus.

This study has provided evidence that the mycelia of young shake cultures contain more

TABLE III. EFFECT OF KCN ON THE LACTATE FERMENTATION IN SHAKE CULTURE

Concentration of KCN added (after 21 hr. incubation)	—	1×10^{-4} M	2×10^{-4} M	5×10^{-4} M
At 21 hr. incubation				
Glucose consumed (mg)	1,710	1,750	1,740	1,830
Lactate formed (mg)	480	540	540	500
At 45 hr. incubation				
Glucose consumed (mg)	3,480	3,360	4,110	3,960
Mycelia (mg)	330	360	314	300
Products (mg)				
Lactic acid	1,590	1,760	2,520	2,190
Ethanol	330	390	430	430
Fumaric acid	300	260	250	230
Yield of lactic acid (%)	45.6	52.5	61.2	55.3

cytochrome c oxidase and NADH₂-cytochrome c reductase than older cultures, and its respiration is inhibited by KCN. With aging of the culture the activities of these enzymes fell off and its respiration turned into a KCN-resistant form of respiration, characterized by the accumulation of lactate. From these results, it may be concluded that in the earlier period of shake culture growth, the fungus obtains energy through the cytochrome system functioning in respiration. Respiration via the cytochrome system then is changed to a KCN-resistant respiration, and consequently the accumulation of lactate takes place. This conclusion seems to be supported by the high content of cytochrome c in the younger mycelia of shake cultures, as was described in the preceding paper.¹⁾

Although the results from the Warburg respirometric analysis may be open to question, surface mycelia are taken to respire predominantly through a cytochrome-independent system rather than a cytochrome system, since the respiration which proceeded with glucose degradation was KCN-resistant.

This supposition is in agreement with the results of Terui et al.¹²⁾ with *Aspergillus oryzae*, who reported that surface mycelia of the mold react and respire with gaseous oxygen by the flavin system during surface cultivation. On the other hand, the fungus adapts to form the cytochrome system for respiration with oxygen in the submerged condition, and operates predominantly with this system in shake culture.

Rhizopus oryzae seems to behave almost in the same way toward oxygen as *Asp. oryzae*, except for the phenomenon that *Rhizopus oryzae* in older shake culture respire predominantly with the system of flavins and does not respire with the cytochrome system. The alternation of the respiratory systems in shake culture may be looked upon as one of the

reasons why *Rhizopus oryzae* can accumulate large amounts of lactate even in shake culture. In other words, the accumulation of lactate occurs when the fungus respire with the KCN-resistant system; and when the cytochrome system is the predominant form of respiration, the yield of lactate is very low, presumably because glucose consumption decreases and oxygen uptake and carbon dioxide evolution per unit of glucose consumed increase. An oxygen uptake (Q_{O_2}) of from 10 to 25 was found for mycelia of *Rhizopus oryzae* grown without zinc in shake culture under conditions appropriate for lactate formation. The addition of zinc to the medium increased the oxygen uptake of the mycelia to 40~60, which resulted, as shown in Fig. 1, from the KCN-resistant respiration at the later stage of incubation. The increased oxygen uptake for mycelia obtained from zinc containing media corresponds to the increased FMN content in mycelia obtained as described previously¹⁾ from zinc containing media. However, the addition of zinc to the medium retarded lactate formation. The relatively high oxygen uptake, even if it is KCN-resistant respiration, might be cited as a reason for the decreased accumulation of lactate.

While studying of the effect of zinc on *Saccharomyces*, Miwa and Ueyama¹³⁾ observed that the oxygen uptake of the yeast grown in the absence of zinc was 10.2, while that of the yeast grown in its presence was 42.0. The effect of zinc on *Rhizopus oryzae* was very similar to the result of Miwa and Ueyama.

Coenzyme Q₉ obtained from the mycelia of *Rhizopus oryzae* did not act as a hydrogen acceptor for NADH₂ dehydrogenation by cell-free extracts of the mycelia. No recognizable activities of NADH₂-oxidase system and NADH₂-peroxidase were observed in the cell-free extract. Therefore, the enzymes which act in the KCN-resistant respiration system

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are yet to be elucidated.

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