THE MODE OF ACTION OF ANTIFUNGAL AGENTS: EFFECT OF PYRROLNITRIN ON MITOCHONDRIAL ELECTRON TRANSPORT

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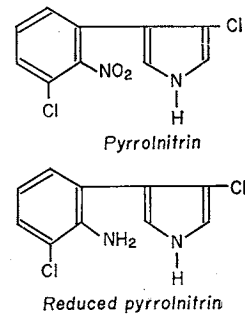
Pyrrolnitrin preferentially inhibited the oxidation of NADH-linked substrates in monkey kidney cells, and in rat liver mitochondria (RLMw) and the oxidation of NADH by submitochondrial particles (SMP) of beef heart. The antibiotic inhibited the reduction of 2, 6-dichlorophenolindophenol and cytochrome *c* by NADH and by succinate, but it did not affect the flavins of NADH dehydrogenase and succinate dehydrogenase. Pyrrolnitrin probably blocked electron transfer between the dehydrogenases and the cytochrome components of the respiratory chain. The effect on the phosphorylation of ADP (RLMw) and energy transduction (SMP) as indicated by the fluorescence of 8-anilino-naphthalene-1-sulfonic acid were secondary. Reduced pyrrolnitrin had similar effects on respiration, but it was a less potent inhibitor.

The biological activity of pyrrolnitrin, 3-(2'-nitro-3'-chlorophenol)-4-chloropyrrol, isolated from *Pseudomonas* species has been studied in some detail[1,2,3]. Pyrrolnitrin strongly inhibited the growth of fungi and, less effectively, the growth of yeast and bacteria. Studies on the mode of action of pyrrolnitrin showed that it bursts the cell membrane by reacting with phospholipid components[4]. Although pyrrolnitrin inhibited the respiration of intact cells, the oxidative phosphorylation of mitochondria isolated from *Candida utilis* was not inhibited[4].

In this communication we will present the effect of pyrrolnitrin on the respiration of intact monkey kidney cells, isolated rat liver mitochondria and beef heart submitochondrial particles. These studies showed that pyrrolnitrin affected electron transfer to a greater extent than the energy transfer pathway of the respiratory chain. Parallel studies on effect of reduced pyrrolnitrin and of some known metabolic inhibitors were made.

Fig. 1. Structures of pyrrolnitrin and reduced pyrrolnitrin.



Materials and Methods

Cultures of monkey kidney cells were kindly supplied by Dr. R. HULL of the Lilly Research Laboratories. Rat liver mitochondria were isolated and twice washed with 0.25 M sucrose[5]. Submitochondrial particles were prepared, in the presence of EDTA[6], from frozen beef heart mitochondrial preparations[7]. Protein was determined by a modified biuret method[8].

A Clark type electrode was used to measure respiration polarographically at 30°C. The index of oxidative phosphoryla-

tion, ADP/O ratio, was obtained as described by CHANCE and WILLIAMS[9]. Spectrophotometric methods were used to monitor the reduction of cytochrome *c*, 2, 6-dichlorophenolindophenol (DCIP) and ferricyanide[10]. Redox states of flavoproteins were measured with an Aminco-Chance dual wavelength spectrophotometer. The fluorescence of 8-anilino-naphthalene-1-sulfonic acid (ANS) was detected by an Aminco-SPF-125 spectrofluorometer. Pyrrolnitrin and reduced pyrrolnitrin (structures shown in Fig. 1) were provided by Dr. R. HAMILL of our laboratories. NADH was purchased from Boehringer, and other chemicals were obtained from Sigma.

Results

According to polarographic measurement, intact monkey kidney cells respired on endogenous substrates at 9.4 (11) ± 1.3* mμatom oxygen/min/mg protein. Pyrrolnitrin and reduced pyrrolnitrin at 200 μM and 500 μM, respectively, suppressed 90 % of the respiratory rate (Fig. 2). At these concentrations of the antibiotics, the addition of succinate restored respiration to 3.3 (2) ± 0.23 mμatom/min/mg, a rate similar to that of cells treated with 2 μM rotenone, 2.5 (2) ± 0.45 mμatom/min/mg. Thus, it was suggested that there might be similarity in actions of these antibiotics and rotenone.

In the presence of ADP, intact rat liver

Fig. 2. Effect of pyrrolnitrin and reduced pyrrolnitrin on endogenous respiration of monkey kidney cells.

Oxygen consumption was measured with a Clark electrode in a medium containing NaCl, 140 mM; KCl, 48 mM; $MgCl_2$, 1 mM; KH_2PO_4, 8 mM, pH 7.4 and cell culture, 7.6 mg protein/ml.

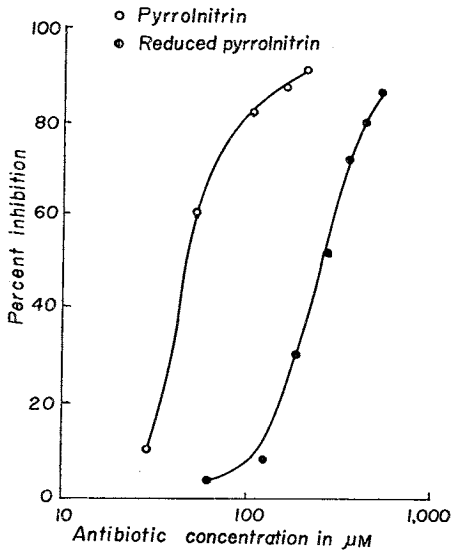
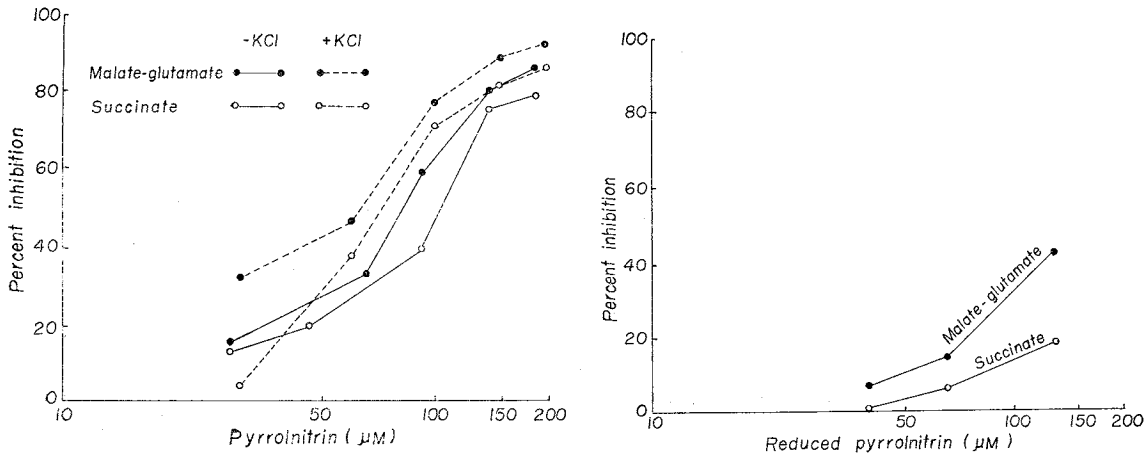


Fig. 3A and 3B. Effect of pyrrolnitrin (A) and reduced pyrrolnitrin (B) on respiration of isolated rat liver mitochondria.

Mitochondrial respiration was measured with a Clark type oxygen electrode in a medium containing sucrose, 0.25 M; KH_2PO_4, 20 mM and pH 7.4; mitochondrial protein, 1.5~2 mg/ml; KCl, 10 mM if it is added; malate-glutamate (Tris), 3 mM each or succinate (Tris), 5 mM and a sufficient amount of ADP to consume 30~40 μM of molecular oxygen.



* Values within parenthesis denote number of observations and values after ± signs signify standard deviation.

Table 1. Effect of pyrrolnitrin and reduced pyrrolnitrin on oxidative phosphorylation of isolated rat liver mitochondria

	Malate-Glutamate		Succinate	
	ADP/O	O_2-Uptake mμatom o/min/mg	ADP/O	O_2-Uptake mμatom o/min/mg
Control	2.64(10)±0.28	47.55(22)±8.5	1.75(3)±0.09	62.88(11)±13.1
Pyrrolnitrin, 4.7×10^{-5} M	2.40(4) ±0.20	31.34(7) ±2.73	1.33(2)±0.13	51.30(4) ± 8.0
% Inhibition	9 %	34 %	24 %	18.4 %
Reduced pyrrolnitrin, 1.3×10^{-4} M	2.52(2) ±0.06	27.45	1.53(2)±0.0	57.5 (2) ± 0.0
% Inhibition	6.5 %	42 %	17.5 %	8.5 %

Experimental conditions were the same as described in the legend for Fig. 3. Index of oxidative phosphorylation, ADP/O ratio was calculated according to CHANCE and WILLIAMS[9].

Table 2. Effect of pyrrolnitrin and reduced pyrrolnitrin on electron transfer of submitochondrial particles from beef heart

	μ equivalent/min/mg protein and % of control							
Substrate	NADH				Succinate			
Electron acceptors	O_2	cyto *c*	DCIP	$Fe(CN)_6^{=}$	O_2	cyto *c*	(PMS) DCIP	$Fe(CN)_6^{=}$
Control	1.04(7) ±0.11*	0.05(6) ±0.00	94.3(4) ±10.5	3.03(4) ±0.19	0.84(6) ±0.05	0.04(9) ±0.05	0.16(4) ±0.01	0.07(4) ±0.00
Pyrrolnitrin, 16 μM	58%	22.2%	59.4%	93.3%	—	—	—	—
78 μM	4.0%	10.8%	33.3%	111.5%	44%	69.6%	51.0%	66.7%
391 μM	—	—	—	—	6.0%	16.0%	9.6%	60%
Reduced pyrrolnitrin, 117 μM	50%	30%	45%	86%	—	—	—	—
291 μM	3.0%	9.4%	31%	96%	—	—	—	—
335 μM	—	—	—	—	50%	60%	48%	56%
1,014 μM	—	—	—	—	6%	5.3%	22%	25%
Rotenone, 83 nM	13%	26%	100%	100%	100%	100%	98%	—

Respiration was measured as described in Fig. 4. The reduction of cytochrome *c*, 2,6-dichlorophenolindophenol (DCIP) or DCIP with phenazine methosulfate (PMS) and $K_3Fe(CN)_6$ were measured spectrophotometrically by a Gilford spectrophotometer at 550 nm, 600 nm and 420 nm respectively. Reaction mixture consisted of sucrose 0.25 M, $KHPO_4$ 20 mM and pH 7.4, mitochondrial protein 0.08 mg/ml, KCN 3.3 mM and one of the following electron acceptors: cytochrome *c*, 17 μM, $K_3Fe(CN)_6$ 0.06 mM and DCIP 0.1 mM (or plus PMS 30 μM). Either NADH 0.44 mM or succinate (Tris) 6.7 mM was used as substrate.
* Standard deviation.

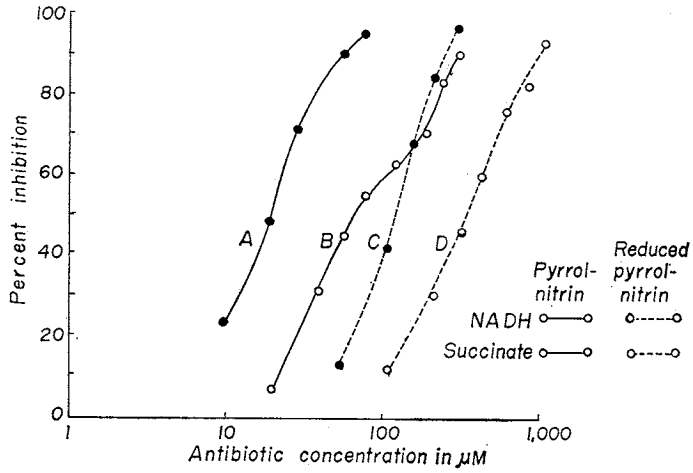
mitochondria consumed oxygen at a rate of 47.5(22)±8.5 mμatom/min/mg with malate-glutamate as substrate and at 62.8(11)±13.1 with succinate. With 10 mM potassium, the respiratory rates rose to 129.7(6)±15.5 mμatom/min/mg and 171.2(7)±12.4 mμatom/min/mg, respectively. In the absence or presence of potassium, pyrrolnitrin at 200 μM caused 80~90 % inhibition of respiration with malate-glutamate or with succinate as substrate (Fig. 3A). At equivalent concentrations, reduced pyrrolnitrin was about half as inhibitory (Fig. 3B). Both antibiotics were more effective inhibitors of malate-glutamate oxidation than of succinate oxidation.

Table 1 shows the effect of pyrrolnitrin and reduced pyrrolnitrin on phosphorylation index, (ADP/O ratio) and respiratory rates. When mitochondria respired at the expense of malate-glutamate, 47 μM pyrrolnitrin or 130 μM reduced pyrrolnitrin caused only about 9 % and 6.5 % of inhibition on phosphorylation of ADP. The

inhibition of oxygen consumption was 34 % and 42 % respectively. On the other hand, when succinate was the substrate, the ADP/O ratio was affected to a greater extent than oxygen consumption.

Fig. 4. Effect of pyrrolnitrin and reduced pyrrolnitrin on NADH oxidase and succinate oxidase of submitochondrial particles (SMP) from beef heart.

The oxidase activities of SMP were measured polarographically in a medium containing sucrose, 0.25 M; KH_2PO_4, 20 mM and pH 7.4; mitochondrial protein, 0.17 mg/ml and NADH, 1 mM or succinate (Tris) 10 mM.



To examine the effects of the antibiotics on the respiratory enzymes directly, we subsequently used submitochondrial particles (SMP) of beef heart muscles. In agreement with the studies with intact cells and mitochondria, both antibiotics inhibited the oxidation of NADH 3-fold stronger than the oxidation of succinate (Fig. 4). Furthermore, pyrrolnitrin (traces A and B) was about 5-fold more potent an inhibitor than was reduced pyrrolnitrin (traces C and D).

Artificial electron acceptors have been found useful in the elucidation of site specificity of the respiratory inhibitors[11]. Such study of pyrrolnitrin and reduced pyrrolnitrin is shown in Table 2. At 78 μM pyrrolnitrin, the transfer of electrons from NADH to oxygen was reduced to 4.0 %, cytochrome *c* 10.8 %, DCIP 33.3 % and ferricyanide 111.5 %. At that concentration of the inhibitor, the transfer of electrons from succinate toward molecular oxygen was reduced to 44 %, cytochrome *c* 69.6 %, DCIP (PMS) 51 % and ferricyanide 66.7 %. When the concentration of pyrrolnitrin was raised to 391 μM, the reduction of electron acceptors by succinate was further reduced to 6 %, 16 %, 9.6 % and 60 % respectively. It is significant to note that pyrrolnitrin had an intermediate effect on reduction of DCIP by NADH but no inhibitory effect on the reduction of ferricyanide by the same substrate. On the other hand, when succinate was the substrate, the reduction of ferricyanide was decreased to 60 % by the inhibitor. However, rotenone, a well-known inhibitor[12], inhibited only the transfer of electrons from NADH towards molecular oxygen and cytochrome *c* and was without effect on the reduction of DCIP or ferricyanide. Rotenone did not inhibit the transfer of electrons to various acceptors from succinate via the respiratory chain. Although higher concentrations of reduced pyrrolnitrin were needed, its inhibitory effect on the reduction of these electron acceptors by NADH and succinate resembles that of pyrrolnitrin.

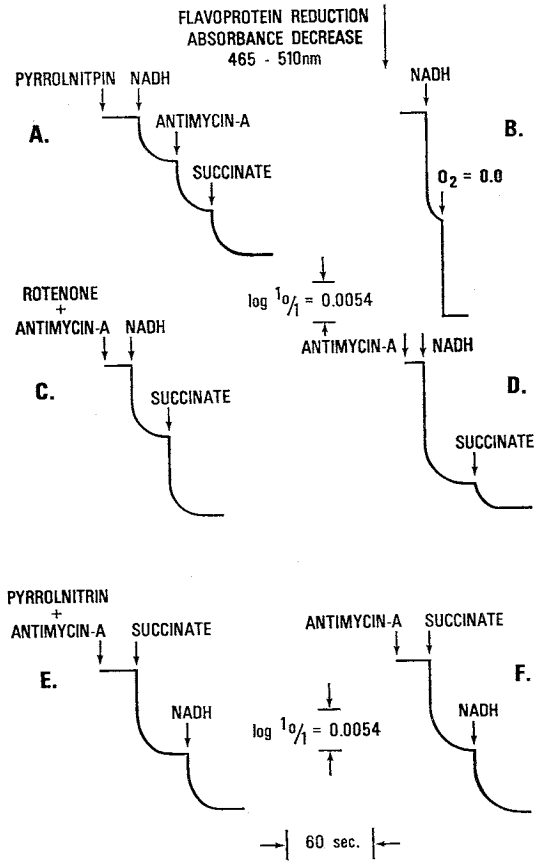
Pyrrolnitrin differs from rotenone in that pyrrolnitrin inhibits the reduction of DCIP and inhibits succinate oxidation, whereas rotenone does neither. Presumably the mechanism of inhibition by pyrrolnitrin is different from that by rotenone. We

do not know the molecular basis of inhibition by either inhibitor. However, artificial electron acceptors like DCIP or PMS are known to accept electrons from non-heme-iron protein moieties of succinate dehydrogenase[11]. We speculate that these non-heme-iron proteins probably are the sites of inhibition by pyrrolnitrin.

The reduced levels of flavoproteins associated with the respiratory chain were measured spectrophotometrically as shown in Fig. 5. In the presence of pyrrolnitrin (Fig. 5A), only 30% of flavoproteins were reduced with NADH. The subsequent addition of antimycin A and succinate gave additive reduction of flavoproteins. In comparison with the steady state level (without inhibitor, as in Fig. 5B) and the totally reduced level (with antimycin A, as in Fig. 5D), pyrrolnitrin could divide the reducible flavoproteins into two portions as was demonstrated with rotenone (Fig. 5C)[13]. On the other hand, the reduced levels with succinate as substrate were not affected by pyrrolnitrin (Fig. 5E & F).

Fig. 5. Effect of pyrrolnitrin, rotenone and antimycin A on respiratory chain-linked flavoproteins.

The redox levels of flavoproteins were measured spectrophotometrically by an Aminco-Chance dual wavelength spectrophotometer at wavelength 465 minus 510 nm. The reaction mixture consisted of sucrose 0.25 M, KH_2PO_4 20 mM and pH 7.4, mitochondrial protein 2.6 mg/ml. Subsequent additions were as follows: A, pyrrolnitrin 520 μM, NADH 0.44 mM, antimycin A 10 μg and succinate 6.7 mM; C, rotenone 5 μM; E, pyrrolnitrin 260 μM and otherwise the amount of reagents added were the same as in A.

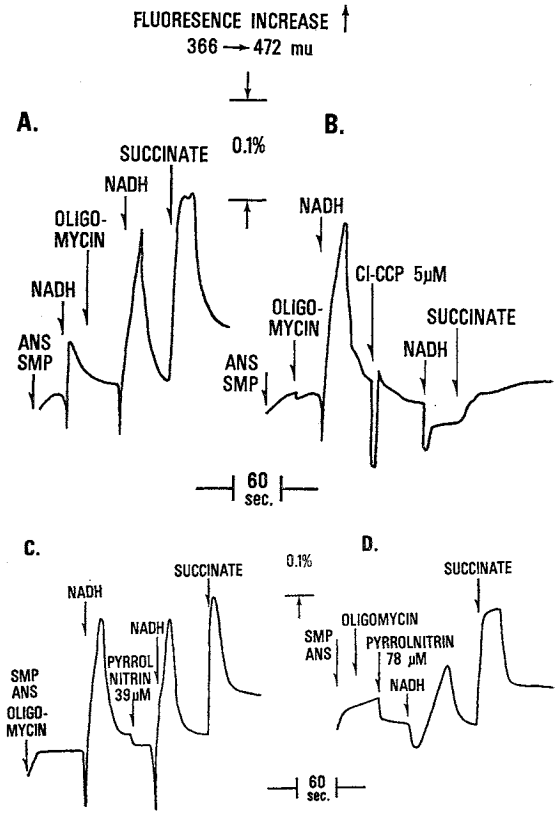


Recently, 8-anilino-naphthalene-1-sulfonic acid (ANS) has been used as a fluorescence probe for the structural transition of mitochondrial membrane as a function of metabolic states[14]. The energized state of mitochondrial membrane can be induced with either ATP or oligomycin[6], as shown in Fig. 6. In the presence of oligomycin, the addition of 0.26 mM NADH caused an increase of ANS fluorescence and then a decrease of fluorescence upon exhaustion of the substrate (Fig. 6A). The subsequent addition of succinate again increased fluorescence until oxygen was exhausted in the medium. However, in the presence of an uncoupling agent, carbonylcyanide *m*-chlorophenylhydrazone (Cl-CCP), the addition of NADH and succinate did not initiate fluorescence changes of ANS (Fig. 6B). Thus, we have confirmed here the association of fluorescence changes of ANS with the energetic state of the mitochondrial membrane[14]. In the presence of pyrrolnitrin, 39 μM, the fluorescence cycle did not show significant difference with the addition of NADH or succinate (Fig. 6C). When the concentration

was increased to 78 μM, pyrrolnitrin reduced the cycle to half and lengthened the period of time to exhaust NADH but the fluorescence cycle created by the oxidation of succinate was only reduced by 10 % (Fig. 6D). In agreement with the previous data on rat liver mitochondria, the oxidation of substrate was first inhibited before the energy transfer reactions were affected as the concentration of antibiotic was increased. Pyrrolnitrin has the same effect whether added before or after the fluorescence probe, ANS.

Fig. 6. Effect of Cl-CCP and pyrrolnitrin on the fluorescence of 8-anilinonaphthalene-sulfonic acid (ANS) in SMP.

Fluorescence was measured with a spectrofluorometer, Aminco SPF 125, at wavelength 366 nm for excitation and 472 nm for emission. Reaction mixture consists of sucrose 0.25 M, KH_2PO_4 20 mM, mitochondrial protein, 2 mg/ml and ANS, 100 μM. Subsequent additions were made as follows: A, NADH 256 μM, oligomycin 1 μg, NADH 256 μM and succinate (Tris) 6.7 mM; B, carbonyl cyamide *m*-chlorophenyl hydrazone 16.6 μM, C, pyrrolnitrin, 36 μM; D, pyrrolnitrin 78 μM, and otherwise the amount of reagents added were the same as in A.



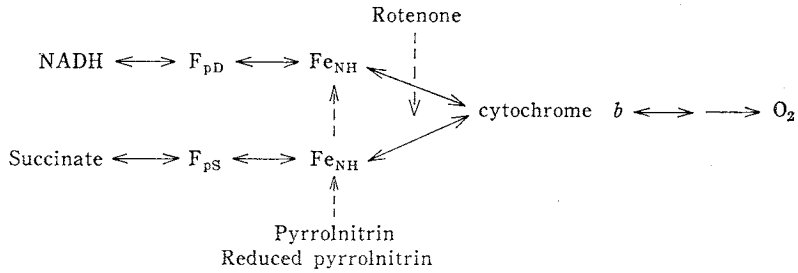
Discussion

The present study clearly shows that pyrrolnitrin and reduced pyrrolnitrin inhibit respiration of mitochondria. This is quite contrary to the previous observation[4]. Perhaps, the failure of NOSE and ARIMA to find an effect of pyrrolnitrin on mitochondrial respiration was due to the choice of *Candida utilis*, whose growth was only mildly inhibited by the antibiotics[2]. With respect to the present study, in all three stages of biological organization—monkey kidney cells, intact rat liver mitochondria and submitochondrial particles of beef heart —consistent inhibitory effects of both pyrrolnitrin and reduced pyrrolnitrin were observed. We did not determine the nature of endogenous substrates in fresh cultures of monkey kidney cells, but the observation that respiration was almost completely abolished with 2 μM rotenone or pyrrolnitrin and one third of the activity was restored with succinate indicates that the majority of the endogenous substrates were NADH-linked. This was confirmed by our observations that endogenous respiration could be abolished by storing cells overnight, but was restored to the original rate with added malate-glutamate.

Pyrrolnitrin did not entirely abolish the fluorescence changes of ANS as an uncoupling agent would, but it did reduce the NADH and succinate induced fluorescence. Perhaps pyrrolnitrin had some unspecific binding on the membrane competing with ANS.

The direct measurement of flavoproteins indicated that pyrrolnitrin as well as rotenone did not alter the reduced level of the primary NADH-dehydrogenase (F_{pD}) and succinate dehydrogenase (F_{pS}). However, the ability of pyrrolnitrin and reduced pyrrolnitrin to inhibit the reduction of cytochrome *c* could account for their effects on oxygen consumption of SMP. Therefore, the segments of the respiratory chain between these two dehydro-

Scheme 1. Sites of inhibition by pyrrolnitrin and reduced pyrrolnitrin on the respiratory chain.



F_{pD}=NADH-dehydrogenese; F_{pS}=succinate dehydrogenase; F_{NH}=non-heme-iron protein; solid arrow : electron transfer pathway; broken arrows : suggestive sites of inhibition.

genases and the cytochrome components are probably the site(s) of inhibition caused by pyrrolnitrin and reduced pyrrolnitrin (Scheme 1).

The above findings have been substantiated by recent studies indicating that pyrrolnitrin did inhibit the respiratory chain-linked dehydrogenases of another fungus, *Microsporum gypseum*[15]. Furthermore, respiration and activities of mitochondrial dehydrogenase from *Saccharomyces cerevisiae* were also inhibited by pyrrolnitrin[16].

Acknowledgement

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