JOURNAL OF BACTERIOLOGY, Oct. 1969, p. 310-318

Vol. 100, No. 1
Printed in U.S.A.

Mechanism of Action of the Antifungal Antibiotic Pyrrolnitrin

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Received for publication 22 July 1969

Pyrrolnitrin at 10 μg/ml inhibited the growth of *Saccharomyces cerevisiae*, *Penicillium atrovenetum*, and *P. oxalicum*. The primary site of action of pyrrolnitrin on *S. cerevisiae* was the terminal electron transport system between succinate or reduced nicotinamide adenine dinucleotide (NADH) and coenzyme Q. At growth inhibitory concentrations, pyrrolnitrin inhibited endogenous and exogenous respiration immediately after its addition to the system. In mitochondrial preparations, the antibiotic inhibited succinate oxidase, NADH oxidase, succinate-cytochrome *c* reductase, NADH-cytochrome *c* reductase, and succinate-coenzyme Q_6 reductase. In addition, pyrrolnitrin inhibited the antimycin-insensitive reduction of dichlorophenolindophenol and of the tetrazolium dye 2,2'-di-*p*-nitrophenyl-(3,3'-dimethoxy-4,4'-bi-phenylene)5,5'-diphenylditetrazolium. The reduction of another tetrazolium dye, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, that was antimycin-sensitive, was also inhibited by pyrrolnitrin. The antibiotic had no effect on the activity of cytochrome oxidase, and it did not appear to bind with flavine adenine dinucleotide, the coenzyme of succinic dehydrogenase. In whole cells of *S. cerevisiae*, pyrrolnitrin inhibited the incorporation of ^{14}C-glucose into nucleic acids and proteins. It also inhibited the incorporation of ^{14}C-uracil, ^{3}H-thymidine, and ^{14}C-amino acids into ribonucleic acid, deoxyribonucleic acid, and protein, respectively. The in vitro protein synthesis in *Rhizoctonia solani* and *Escherichia coli* was not affected by pyrrolnitrin. Pyrrolnitrin also inhibited the uptake of radioactive tracers, but there was no general damage to the cell membranes that would result in an increased leakage of cell metabolites. Apparently, pyrrolnitrin inhibits fungal growth by inhibiting the respiratory electron transport system.

Pyrrolnitrin has been isolated from *Pseudomonas pyrrocinia* (1, 11) and *P. aureofaciens* (14). The antibiotic is 3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrole (Fig. 1). Its physical and chemical properties have been described by Arima et al. (2). Pyrrolnitrin is an antifungal antibiotic and is most active against dermatophytic fungi, especially the species of *Trichophyton* (2). It is synthesized from tryptophan, and the synthesis is probably initiated by a chloroperoxidase enzyme system acting on tryptophan (14). The present report deals with the mechanism of action of pyrrolnitrin.

MATERIALS AND METHODS

Pyrrolnitrin was obtained from Eli Lilly & Co., Indianapolis, Ind. The solutions of the antibiotic were prepared in 95% ethyl alcohol. Inorganic and organic chemicals were purchased from either Mallinckrodt Chemical Works, St. Louis, Mo., or Fisher Scientific Co., Pittsburgh, Pa., and biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. The chemicals for determination of radioactivity were obtained from Packard Instrument Co., Inc., Downers Grove, Ill., and radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.

S. cerevisiae, a strain of brewing yeast, was kindly supplied by F. M. Clark, Department of Microbiology, University of Illinois. *Penicillium atrovenetum* and *P. oxalicum* were obtained from Commonwealth Mycological Institute, Kew, England. The yeast culture was maintained in Difco Sabouraud Dextrose Agar slants. Liquid cultures were grown in flasks containing glucose-yeast extract medium (GYE) consisting of 2% glucose and 1% yeast extract in glass-distilled water. The inhibition of growth of *S. cerevisiae* by pyrrolnitrin was studied by growing the yeast in tubes containing glucose-yeast nitrogen base medium (0.2% glucose and 6.7% yeast nitrogen base in glass-distilled water) with or without pyrrolnitrin. Growth was measured turbidimetrically at 650 nm in a Coleman Junior Spectrophotometer. The yield of *P. atrovenetum* and *P. oxalicum* was measured on a dry weight basis after growing the culture for 48 hr on a reciprocal shaker. The mycelium was collected

by filtration on Whatman no. 1 filter paper and dried in an oven at 80 C to a constant dry weight.

Respiratory studies. Yeast cells were grown in GYE medium to a density equivalent to 6.0 mg (dry weight) per ml. They were collected by centrifugation at 12,000 × *g* and washed twice with deionized distilled water and once with 0.2 M sodium phosphate buffer (*p*H 7.4). The washed cells were then suspended in the same buffer and either were used as whole cells or were disintegrated in a French pressure cell at 15,000 psi to make the cell-free extracts. Standard Warburg respirometry was used (25).

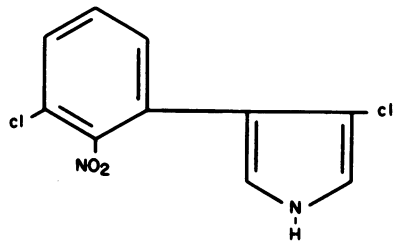
Mitochondria from the yeast cell-free extracts were isolated by the method of Utter et al. (26). Beef heart mitochondria were isolated from a fresh beef heart by the method of Crane et al. (6). The protein content in these mitochondrial preparations was determined by the biuret method of Gornall et al. (7).

The effect of pyrrolnitrin on the production of $^{14}CO_2$ from ^{14}C-glucose was studied in the intra-scintillation vial reaction tubes of Slater et al. (24).

Enzyme assays. Succinate oxidase and cytochrome oxidase were assayed manometrically by measuring the oxygen uptake with sodium succinate and ascorbic acid and cytochrome *c* as substrates, respectively. Activities of other enzymes were assayed on a Beckman DU spectrophotometer attached to a Gilford multiple sample absorbance recorder (model 2000). Absorption cells (1 ml) with a 1-cm light path were used. Reduced nicotinamide adenine dinucleotide (NADH) oxidase was assayed by measuring the decrease in absorbancy at 340 nm with NADH as a substrate. Succinate and NADH-cytochrome *c* reductases were assayed by measuring the increase in the absorbancy at 550 nm resulting from the reduction of cytochrome *c*. An extinction coefficient of 19.2×10^3 liters × mol^{-1} × cm^{-1} was used to calculate the amount of reduced cytochrome *c* (12). Succinate-dichlorophenolindophenol (DPIP) reductase was assayed by the decrease in absorbancy at 600 nm resulting from the formation of the leuco dye (reduced DPIP). The reduction of tetrazolium dyes (formazan formation) was measured by an increase in absorbancy at 530 nm for 2,2′-di-*p*-nitrophenyl-(3,3′-dimethoxy-4,4′-bi-phenylene) 5,5′-diphenylditetrazolium (NBT) and at 570 nm for 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT). Succinate-coenzyme Q_6 reductase activity was measured by the method of Ramasarma and Lester (21). The reaction mixture for each of the enzyme systems is described below.

The possible binding of pyrrolnitrin with flavine adenine dinucleotide (FAD) was measured by the changes in the absorption spectra of FAD and pyrrolnitrin in the mixture of the two compounds.

The effect of pyrrolnitrin on the permeability of yeast cells was determined by two methods, i.e., the uptake of radioactive tracers and the leakage of metabolites from the cells. For leakage experiments, 1 g (wet weight) of late exponential-phase cells was suspended in 25 ml of 0.1 M sodium acetate buffer (*p*H 6.5) in 125-ml Erlenmeyer flasks. After adding appropriate volumes of pyrrolnitrin solutions or



Pyrrolnitrin:

[3-chloro-4-(2'nitro-3'-chlorophenyl)-Pyrrole]

FIG. 1. *Structure of pyrrolnitrin.*

alcohol, the flasks were incubated on a reciprocal shaker for a total of 8 hr at 26 C. Portions (3 ml) were removed after 30 min and after 1, 3, 6, and 8 hr, and the cells were separated by centrifugation. From the supernatant solutions, 0.5-ml samples were used to determine the contents of ninhydrin-positive materials (17), inorganic phosphate (4), reducing sugars (18), and nucleotides. The nucleotides were measured by the absorption at 260 nm.

The effect of pyrrolnitrin on the general cell metabolism was studied with ^{14}C-glucose (UL). A 1-μc amount of ^{14}C-glucose (specific activity, 21 mc/mmole) was added to 25 ml of GYE medium in each of fifteen 125-ml Erlenmeyer flasks. Pyrrolnitrin or alcohol was added to appropriate flasks, and the flasks were inoculated with mid-exponential-phase yeast cells equivalent to 15 mg (dry weight). The flasks were incubated at 26 C for 1 hr on a reciprocal shaker. The $^{14}CO_2$ given off by the cells was absorbed in hyamine hydroxide as described by Gottlieb and Tripathi (10). This absorbed $^{14}CO_2$ was counted in toluene scintillation fluid [2,5-diphenyloxazole (PPO), 5 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 0.3 g; toluene, 1 liter]. Water-miscible samples were counted in dioxane scintillation fluid (PPO, 15 g; POPOP, 0.75 g; ethyl cellosolve, 250 ml; naphthalene, 75 g; dioxane, 1,250 ml). All measurements of radioactivity were done in a Packard Tri-Carb liquid scintillation spectrometer. Quench corrections were made by using ^{14}C-toluene as an internal standard.

The amount of ^{14}C-glucose taken up by the cells during incubation was calculated by determining the radioactivity of a portion of the incubation mixture before and after incubation. The cells were removed from the medium by centrifugation and were washed three times with distilled water. The cells were extracted twice with 5 ml of 80% ethyl alcohol to remove small soluble molecules, e.g., sugars. The suspension was centrifuged at 1,200 × *g*, and the supernatant solutions from ethyl alcohol extractions were combined. In this solution, the presence of amino acids and reducing sugars was detected by the ninhydrin test (17) and the anthrone test (18), respectively. The residue from the ethyl alcohol treatment was extracted first with 5 ml of acetone-ether (1:1) for 3 hr and then with 5 ml of ether for 1 hr to remove the lipids. Residual ether from the cell residue was re-

moved by passing nitrogen gas over it. This residue was extracted with 5 ml of ice-cold trichloroacetic acid for 2 hr at 4 C to remove nucleotides, small polysaccharides, etc. The residue obtained after centrifuging this suspension was then extracted with 10 ml of 10% trichloroacetic acid for 1 hr in an oven at 90 C to remove the nucleic acids. Samples from the supernatant solution obtained were used to measure ribonucleic acid (RNA; 3), deoxyribonucleic acid (DNA; 23), and absorbancy at 260 nm. After removing the nucleic acids from the cells, protein was extracted with 5 ml of 0.2 N NaOH at 90 C for 1 hr (10). Samples were removed for ninhydrin (17) and biuret assays (7) to estimate the protein content in this fraction. Samples (1 ml) from each of the above fractions were used to determine the radioactivity. The inhibition of incorporation of ^{14}C-glucose into various fractions was then calculated.

The effect of pyrrolnitrin on the incorporation of ^{14}C-amino acids into protein in yeast cells was studied by using algal protein hydrolysate, uniformly labeled with a specific activity of 15 mc/mmole. Erlenmeyer flasks (500 ml) containing 100 ml of GYE medium were inoculated with yeast equivalent to 2.5 mg (dry weight). After incubating these flasks for 12 hr, alcohol or pyrrolnitrin solutions were aseptically added. After 15 min of incubation to expose the cells to the antibiotic, 4 μc of ^{14}C-amino acid mixture per ml was added to each flask, and the contents were mixed by manual shaking. Samples were removed to determine the radioactivity in the medium. After an additional 2 hr of incubation, the cells were harvested and washed with distilled water and centrifugation. Portions of the supernatant solution were removed to determine the radioactivity taken up by the cells. The cells were then extracted in given order with 10% ice-cold trichloroacetic acid, ethyl alcohol-ether (3:1), ether, and 10% hot trichloroacetic acid (90 C for 1 hr). The protein was then extracted in 0.2 N NaOH by the method of Gottlieb et al. (9). The protein content in the supernatant solution was determined by the method of Lowry et al. (15) by using bovine serum albumin as a standard.

The synthesis of RNA was studied by incorporation of ^{14}C-uracil (30 mc/mmole) into RNA fraction. The same general methods were followed as for protein synthesis except that 125-ml flasks with 25 ml of medium were inoculated with cells equivalent to 0.5 g (dry weight), and the 10% hot trichloroacetic acid and protein extractions were omitted. RNA was extracted by a slightly modified method of Schmidt and Thanhauser (22) as given below. After the cold trichloroacetic acid extraction, the cell residue was hydrolyzed with 0.5 N NaOH at 37 C for 12 hr. The RNA content in the supernatant solution was measured by the orcinol method (3), and the incorporation of ^{14}C-uracil into RNA was calculated as counts per minute per milligram of RNA.

The DNA synthesis in yeast cells was studied by the incorporation of ^{3}H-thymidine (methyl labeled, 2 c/mmole) into 10% hot trichloroacetic acid extract (nucleic acids). A 2-μc amount of ^{3}H-thymidine was added in the medium, and the same general methods were followed as for protein synthesis up to hot trichloroacetic acid extraction. The DNA content in the trichloroacetic acid extract was measured by diphenylamine test (23).

Polyuridylic acid (poly U)-directed in vitro protein synthesis in *Rhizoctonia solani* was studied by the method of Obrig, Cerna, and Gottlieb (19). The in vitro protein synthesis with *Escherichia coli* ribosomes was studied by the method of Clark et al. (5).

RESULTS

Pyrrolnitrin inhibition of the total growth of *S. cerevisiae* increased with increasing concentrations of antibiotic until, at 5 μg/ml, the inhibition was 95% (Fig. 2). Cultures of the yeast treated with 10 μg of pyrrolnitrin per ml did not grow even after 1 week of incubation. The growth of *P. atrovenetum* and *P. oxalicum* was completely inhibited at 10 and 40 μg/ml, respectively. At 10 μg/ml, the yield of *P. oxalicum* was reduced to 50%.

Pyrrolnitrin inhibited both endogenous and exogenous respiration in *S. cerevisiae* at very low concentrations. The inhibition of respiration increased with increasing concentrations of pyrrolnitrin and was almost complete at 15 μg/ml (Table 1). Pyrrolnitrin also inhibited the production of $^{14}CO_2$ from ^{14}C-glucose, and the CO_2 production decreased with increasing pyrrolnitrin concentrations (Fig. 3). Respiration of cell-free extracts of the yeast was also inhibited by pyrrolnitrin. In these extracts the succinate oxidase activity more than doubled by exogenous cytochrome *c* (Table 2). The oxidation of succinate, with or without exogenous cytochrome *c*, was progressively inhibited with

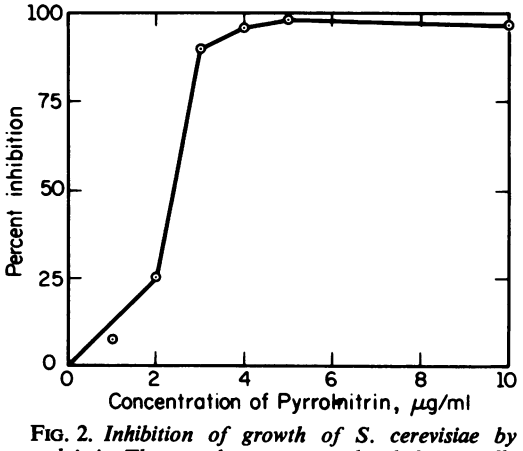


FIG. 2. *Inhibition of growth of S. cerevisiae by pyrrolnitrin. The growth was measured turbidimetrically at 650 nm after 24 hr of incubation.*

TABLE 1. *Inhibition of respiration of S. cerevisiae by pyrrolnitrin*[a]

Amt of pyrrolnitrin	Exogenous respiration		Endogenous respiration	
	O_2 uptake per mg (dry weight) per hr	Inhibition	O_2 uptake per mg (dry weight per hr	Inhibition
μg/ml	*μliters*	%	*μliters*	%
0	100	0	63	0
2	79	21	56	11
5	39	61	36	43
10	18	82	23	64
15	5	95	5	93
20	5	95	1	100
25	4	96	0	100
50	4	96	0	100

[a] Warburg flasks contained sodium phosphate buffer (*p*H 7.4), 50 μmoles; glucose, 200 μmoles (omitted from flasks used for endogenous respiration); magnesium chloride, 35 μmoles; alcohol or alcoholic solutions of pyrrolnitrin, 0.2 ml; and distilled water to make the volume 2.6 ml. A cell suspension of 0.4 ml (1.5 mg, dry weight) was added in the side arm of each flask.

increasing pyrrolnitrin concentrations until about 90% of the succinate oxidation was inhibited at 25 μg/ml.

Respiration of mitochondria was also prevented by pyrrolnitrin. In yeast mitochondria, the antibiotic inhibited the activities of succinate and NADH oxidase 90% at 25 and 10 μg/ml, respectively. This inhibition took place during 10 min of incubation. The activities of these oxidases were inhibited by antimycin A and sodium azide, indicating a normal terminal electron transport pathway (Table 3). The NADH oxidase was also inhibited 50% by 9 mM amytal.

The NADH- and succinate-cytochrome *c* reductases were inhibited by pyrrolnitrin both in beef heart (Fig. 4) and yeast mitochondria (Table 4) immediately after adding the antibiotic. In yeast mitochondria, pyrrolnitrin almost completely inhibited the activities of these enzymes at 10 μg/ml and inhibited them about 60% at 5 μg/ml. The immediate inhibition of cytochrome *c* reductase at low concentrations of pyrrolnitrin again suggested that electron transport is the primary site of action and that the site of action is before cytochrome *c*.

In our studies, yeast mitochondria lost most of their activity within 6 hr of isolation. Therefore, to further delineate the site of action of pyrrolnitrin, beef heart mitochondria, which retain the activity for months, were used. In these mitochondria, cytochrome oxidase activity was not inhibited, even at 100 μg of antibiotic per ml, whereas sodium azide (10 mM) completely inhibited oxygen consumption. Artificial electron acceptors and antimycin A were

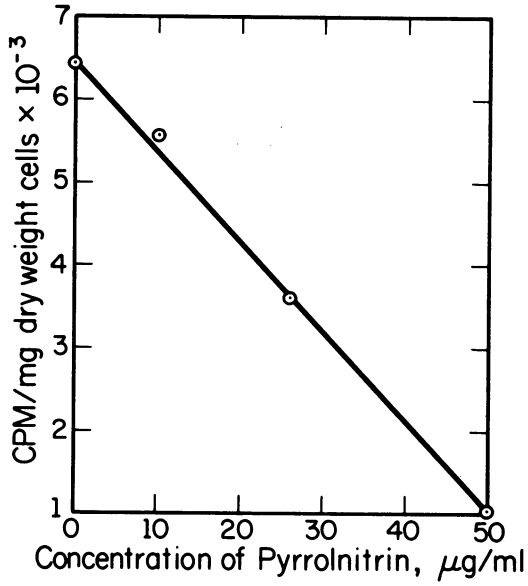


FIG. 3. *Inhibition by pyrrolnitrin of CO_2 production from ^{14}C-glucose by S. cerevisiae. The reaction mixture contained sodium phosphate buffer (pH 7.4), 50 μmoles; magnesium chloride, 20 μmoles; ^{14}C-UL-glucose, 20 μmoles; and alcohol or pyrrolnitrin solution. The final volume of the mixture was 1.5 ml.*

TABLE 2. *Inhibition of succinate oxidation of cell-free extracts of S. cerevisiae by pyrrolnitrin*[a]

Amt of pyrrolnitrin	Without added cytochrome *c*		With cytochrome *c*	
	Amt of O_2 per mg of protein per hr	Inhibition	Amt of O_2 per mg of protein per hr	Inhibition
μg/ml	*μliters*	%	*μliters*	%
0	31	0	71	0
2	28	11	56	21
5	19	39	38	47
10	12	61	25	65
15	9	71	13	81
20	6	81	8	89
25	2	92	5	93
50	2	92	5	93

[a] Contents in the Warburg flasks were the same as described for Table 2, except that 0.4 ml of cell-free extract was added in the side arm and 2 mg of cytochrome *c* was added when desired.

TABLE 3. *Inhibition of succinate oxidase and NADH oxidase activities of yeast mitochondria by pyrrolnitrin*[a]

Inhibitor	Succinate oxidase		NADH oxidase	
	Amt of O_2 per mg of protein per hr	Inhibition	ΔA/min[b]	Inhibition
	μliters	%		%
Pyrrolnitrin (μg/ml)				
0	99	0	0.92	0
5	73	38	0.56	40
10	38	60	0.12	87
15	27	72	0.02	98
25	9	92	0.01	99
50	1	100	0.02	98
Antimycin A (20 μg/ml)	0	100	0	100
Sodium azide (8 mM)	2	99	0	100
Amytal (8 mM)	Not used	Not used	0.47	49

[a] For succinate oxidase, the Warburg flasks contained sodium phosphate buffer (*p*H 7.4), 200 μmoles; sodium succinate, 100 μmoles; cytochrome *c*, 2 mg; and mitochondrial preparation equivalent to 3.0 mg of protein. The control flasks contained 95% alcohol corresponding to the volume of the pyrrolnitrin solution.

[b] For NADH oxidase, the reaction mixture contained sodium phosphate buffer, 50 μmoles; mitochondrial preparation equivalent to 300 μg of protein; NADH, 0.1 μmole; and water to make the volume 1 ml. The reaction was started by adding NADH solution. The oxidation of NADH was measured by the decrease in absorbancy at 340 nm.

used to determine whether pyrrolnitrin inhibits the electron transport prior to the antimycin-sensitive site. The reduction of DPIP was insensitive to antimycin A. In contrast, this reduction was progressively inhibited by increasing pyrrolnitrin concentrations (Fig. 4). About 50% of DPIP reduction was inhibited at 5 μg of pyrrolnitrin per ml, and about 80% was inhibited at 20 μg of pyrrolnitrin per ml. Beef heart mitochondria reduced the tetrazolium dyes NBT and INT. Antimycin A, at 25 μg/ml, had no effect on this succinate-NBT reductase (Fig. 4) but inhibited succinate-INT reductase by 75%, indicating that NBT was reduced before and INT after the antimycin-sensitive site. Pyrrolnitrin inhibited the reduction of NBT in both the presence or absence of antimycin A. The NBT-reductase activity was inhibited 60, 80, and 90% at 25, 50, and 100 μg of pyrrolnitrin per ml, respectively. At the same concentrations, pyrrolnitrin inhibited the reduction of INT 27, 38, and 61%, respectively.

The results with artificial electron acceptors suggested that the site of pyrrolnitrin action was before the antimycin-sensitive site of the mitochondrial respiratory chain. To determine whether this site was before coenzyme Q, the effect of the antibiotic on succinate-coenzyme Q_6 reductase was studied. This coenzyme Q reductase activity was inhibited 50% by 10 mM thenoyltrifluoroacetone, the inhibitor of succinic dehydrogenase, and 90% by 50 μg of pyrrolnitrin per ml. The inhibition of coenzyme Q_6 reductase increased with increasing pyrrolnitrin concentrations (Table 5). The inhibition of the electron

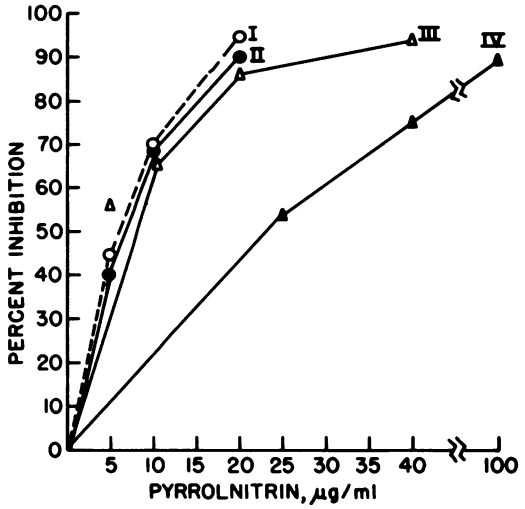


FIG. 4. *Inhibition of NADH-cytochrome c reductase (I), succinate-cytochrome c reductase (II), succinate-DPIP reductase (III), and succinate-NBT reductase (IV) of beef heart mitochondria by pyrrolnitrin. The following reaction mixtures were used for these enzyme assays. (I) NADH-cytochrome c reductase: phosphate buffer (pH 7.4), 50 μmoles; sodium azide, 5 μmoles; cytochrome c, 0.7 mg; mitochondria equivalent to 100 μg of protein; NADH, 0.2 μmole; and water to make the volume 1.0 ml. (II) Succinate-cytochrome c reductase: same as in NADH-cytochrome c reductase except that 20 μmoles of sodium succinate was used instead of NADH, and 300 μg of mitochondrial protein. (III) Succinate-DPIP reductase: phosphate buffer (pH 7.4), 50 μmoles; ethylenediaminetetraacetic acid, 1 μmole; sodium succinate, 30 μmoles; DPIP, 0.05 μmole; mitochondria equivalent to 300 μg of protein; and water to make the volume 1.0 ml. (IV) Succinate-NBT reductase: phosphate buffer (pH 7.4), 50 μmoles; sucrose, 25 μmoles; sodium succinate, 30 μmoles; antimycin A, when added, 10 μg; mitochondria equivalent to 600 μg of protein; NBT, 1.0 μmole; and water to make the volume 1.0 ml. The reduction of NBT was measured spectrophotometrically at 530 nm.*

TABLE 4. *Inhibition of NADH- and succinate-cytochrome c reductase of yeast mitochondria by pyrrolnitrin*

Amt of pyrrolnitrin	NADH-cytochrome *c* reductase[a]		Succinate-cytochrome *c* reductase[b]	
	ΔA/min[c]	Inhibition	ΔA/min.	Inhibition
μg/ml		%		%
0	0.50	0	0.48	0
2	0.41	18	0.32	33
5	0.21	58	0.18	60
7	0.10	80	0.12	75
10	0.01	98	0.01	98
15	0.0	100	0.01	98
20	0.0	100	0.0	100

[a] The reaction mixture for succinate-cytochrome *c* reductase contained sodium phosphate buffer (*p*H 7.4), 50 μmoles; sodium azide, 5 μmoles; cytochrome *c*, 0.7 mg; mitochondrial preparation equivalent to 300 μg of protein; and water to make the volume 0.95 ml. The reaction was initiated by adding 0.05 ml of sodium succinate (20 μmoles).

[b] The conditions for the assay of NADH-cytochrome *c* reductase were the same except that 0.2 μmole of NADH was added in place of succinate, and the amount of mitochondrial preparation was reduced to correspond to 100 μg of protein.

[c] Absorbancy was measured at 550 nm.

transfer from succinate to coenzyme Q could be due to the interference of pyrrolnitrin with succinic dehydrogenase which has FAD as its coenzyme. The ability of pyrrolnitrin to bind FAD was therefore studied by measuring the absorption spectrum of both FAD and pyrrolnitrin singly and in a mixture of the two compounds. The criteria for binding of pyrrolnitrin to FAD, or vice-versa, were any changes in the absorption spectrum of either FAD or pyrrolnitrin. FAD had peaks at 375 and 445 nm. Pyrrolnitrin had a single absorption peak at 250 nm. In the mixture of two compounds, these absorption spectra were not altered.

Pyrrolnitrin also inhibited the synthesis of various macromolecules. The antibiotic had no inhibitory effect on the incorporation of ^{14}C-glucose into the 80% ethyl alcohol extract (amino acids, soluble sugars, etc.) or the 5% cold trichloroacetic acid extract (nucleotides, small polysaccharides, etc.). The synthesis of nucleic acids and protein from ^{14}C-glucose were inhibited by pyrrolnitrin (Table 6). Studies with ^{3}H-thymidine, ^{14}C-uracil, and ^{14}C-amino acids confirmed that the antibiotic inhibited the syntheses of DNA, RNA, and protein. For example, at 50 μg/ml, pyrrolnitrin inhibited the incorporation of ^{3}H-thymidine into DNA by 76%; the incorporation of ^{14}C-uracil into RNA and that of ^{14}C-amino acids into protein were both inhibited by 100% (Table 7).

Despite inhibition by pyrrolnitrin of protein synthesis in whole cells, the antibiotic had no effect on in vitro protein synthesis in *R. solani* or in *E. coli*. In *R. solani*, the poly U-directed incorporation of phenylalanine was inhibited by ribonuclease and puromycin, a characteristic of the typical in vitro protein synthesis system.

TABLE 5. *Inhibition of succinate-coenzyme Q_6 reductase of beef heart mitochondria by pyrrolnitrin*[a]

Inhibitor	Absorbancy	Inhibition
μg/ml		%
Pyrrolnitrin		
0	0.401	0
5	0.369	8
10	0.320	20
15	0.312	22
20	0.244	39
25	0.196	51
50	0.040	90
75	0.039	90
100	0.039	90
Thenoyltrifluoroacetone (10 mM)	0.195	49

[a] The assay mixture contained the following, in glass-stoppered tubes: sodium phosphate buffer (*p*H 7.4), 100 μmoles; sodium azide, 5 μmoles; sodium succinate, 50 μmoles; coenzyme Q, 0.4 mg as an alcoholic solution; ethyl alcohol or pyrrolnitrin solution; 0.25 M sucrose to a total of 3.0 ml. Absorbancy was measured at 519 nm.

TABLE 6. *Effect of pyrrolnitrin on the metabolism of ^{14}C-glucose by S. cerevisiae*[a]

Amt of pyrrolnitrin	Per cent inhibition of			
	Uptake of ^{14}C-glucose	Production of $^{14}CO_2$	Hot trichloroacetic acid-soluble fraction[b]	Protein
μg/ml				
0	0	0	0	0
10	7	12	13	2
25	14	25	25	5
50	27	56	75	55
100	75	86	95	96

[a] Exponential-phase cells were incubated in GYE medium containing ^{14}C-glucose. The disappearance of the radioactivity from the medium was a measure of uptake of ^{14}C-glucose. The macromolecules were obtained as described.

[b] Nucleic acid.

But pyrrolnitrin, even at 40 μg/ml (20 μg per assay), did not inhibit the phenylalanine incorporation. Instead, the antibiotic stimulated this incorporation about 80% (Table 8). In vitro protein synthesis in an *E. coli* system directed by turnip yellow mosaic virus genome was also insensitive to pyrrolnitrin. Even at 125 μg/ml (the minimal growth inhibitory concentration for *E. coli* was 100 μg/ml), pyrrolnitrin had no effect on this protein synthesis (Table 9). However, no stimulation was observed in the *E. coli* system.

TABLE 7. *Effect of pyrrolnitrin on the synthesis of RNA, DNA, and protein by S. cerevisiae*[a]

Amt of pyrrolnitrin	Per cent inhibition of					
	DNA		RNA		Protein	
	Uptake of ^{3}H-thymidine	Incorporation into DNA	Uptake of ^{14}C-uracil	Incorporation into RNA	Uptake of ^{14}C-amino acids	Incorporation into protein
μg/ml						
0	0	0	0	0	0	0
5	28	29	17	10	23	31
10	37	43	21	23	41	56
20	44	57	48	62	67	91
50	64	76	89	100	92	100

[a] Exponential-phase, washed cells were incubated for 2 hr in GYE medium containing the appropriate tracer. The uptake of the tracer was measured by its disappearance from the medium. The cells were fractionated as described.

TABLE 8. *In vitro protein synthesis in Rhizoctonia solani in the presence of pyrrolnitrin*

Assay condition	Counts per min per assay[a]
Complete system	
0 min	68
60 min	2,590
plus pyrrolnitrin (20 μg)	4,417
plus puromycin (50 μg)	558
plus ribonuclease (30 μg)	478
minus ribosomes	389
minus supernatant fraction	257
minus poly U	157

[a] All of the data are from the 60-min incubation, unless otherwise indicated. The complete assay system, in 0.5 ml, contained Tris(hydroxymethyl)-aminomethane-hydrochloride buffer (*p*H 7.8), 50 μmoles; magnesium acetate, 10 μmoles; NH_4Cl, 25 μmoles; 2-mercaptoethanol, 7.5 μmoles; reduced glutathione, 0.5 μmole; ATP, 1.5 μmoles; guanosine triphosphate, 0.1 μmole; phosphoenolpyruvate, 2.5 μmoles; pyruvate kinase, 10 μg; yeast transfer RNA, 200 μg; poly U, 60 μg; ^{14}C-L-phenylalanine, 0.3 μc; 19 other ^{12}C-amino acids, 0.005 μmole each; 105,000 × *g* supernatant fraction equivalent to 0.44 mg of protein; ribosomes equivalent to 0.29 mg of RNA; pyrrolnitrin, when added, 20 μg.

The antibiotic inhibited the uptake of glucose, uracil, thymidine, and amino acids (Table 6 and 7) by *S. cereviseae*. This effect was not due to a breakdown of general cell permeability, since there was no leakage of phosphate, ninhydrin-positive materials, reducing sugars, nucleotides, etc.

DISCUSSION

Since growth of all organisms is the result of complex, integrated, and interdependent processes, antibiotics affecting any one cellular process would ultimately impair most of the other cellular functions. The important question in the study of the mode of action of an antibiotic is the primary site of action. The reaction which is inhibited at the lowest concentration and shortest time is usually accepted as the primary site of action. For pyrrolnitrin, these criteria were satisfied by the inhibition of respiration, since very low concentrations completely inhibited the respiration in whole cells, cell-free extracts, and mitochondrial preparations. Whole cell respiration, as well as mitochondrial respiratory enzymes, were rapidly inhibited, within 5 to 10 min. The inhibition of syntheses of RNA, DNA, and proteins, on the other hand, required higher concentrations of pyrrolnitrin and much longer times, more than 1 hr.

The locus of inhibition of cellular respiration was the mitochondrial electron transport system, since pyrrolnitrin inhibited the activities of succinate and NADH oxidase of isolated mitochondria. In this electron transport system, the site of inhibition by pyrrolnitrin was before cytochrome *c* because pyrrolnitrin inhibited the NADH- and succinate-cytochrome *c* reductase without any inhibitory effect on cytochrome *c*

TABLE 9. *In vitro protein synthesis of Escherichia coli system in the presence of pyrrolnitrin*

Assay condition	Counts per min per assay
Complete system	
0 min	697
60 min	140,692
minus viral genome[a]	3,714
plus pyrrolnitrin (25 μg/ml)	145,222
plus pyrrolnitrin (125 μg/ml)	153,214
Background	46

[a] Turnip yellow mosaic virus.

oxidase. Since pyrrolnitrin inhibited the antimycin-insensitive reduction of DPIP and NBT, this inhibition must have been prior to the antimycin-sensitive site in the electron transport system. The inhibition by the antibiotic of INT reduction, which was antimycin-sensitive, would be expected since the INT accepted electrons after the antimycin-sensitive site. Lester and Smith (13) also found that the reduction of NBT in beef heart mitochondria is prior to and INT after the antimycin-sensitive site. According to these authors, the site of NBT reduction is at or before the site of coenzyme Q (13). Since pyrrolnitrin inhibited the NBT reduction, this inhibition should be at or before coenzyme Q in the respiratory chain. The inhibition of succinate-coenzyme Q_6 reductase by pyrrolnitrin suggests that the antibiotic inhibited the electron transfer between succinate and coenzyme Q (Fig. 5).

This inhibition of respiratory electron transport would also block adenosine triphosphate (ATP; energy) formation (16). Since energy is required for the active uptake of various metabolites and for the biosynthesis of macromolecules (16), pyrrolnitrin should ultimately affect these energy-dependent processes. The data fit this concept which explains the fact that pyrrolnitrin inhibits the synthesis of RNA, DNA, and protein and that the antibiotic also reduces the uptake of amino acids, uracil, and thymidine. The effect of pyrrolnitrin on the biosynthesis of macromolecules could be either on the incorporation of the precursor into the macromolecules or on the inhibition of uptake of precursors. Probably it is uptake that is prevented because pyrrolnitrin had no effect on the in vitro protein-synthesizing systems of *R. solani* and *E. coli*. Based on these facts, we conclude that the primary site of action of pyrrolnitrin is on the respiratory electron transport system.

After submission of this paper, an article on the mechanism of action of pyrrolnitrin by Nose and Arima was published (19). Their data generally agree with ours in showing that pyrrolnitrin inhibits growth, synthesis of protein, RNA, DNA, and uptake of metabolites. In our experiments on *S. cerevisiae*, pyrrolnitrin did not stimulate leakage of 260-nm absorbing materials, sugars, and phosphate, whereas, in Nose and Arima's work on *Candida utilis*, the antibiotic caused a leakage of 260-nm absorbing materials. Besides the different organisms used in the two studies, the age of the cells used in these studies also differed. We used late exponential-phase cells, whereas Nose and Arima used early exponential-phase cells. The results on oxygen consumption also differed. Pyrrolnitrin, at 50 μg/ml, almost completely inhibited the oxygen uptake

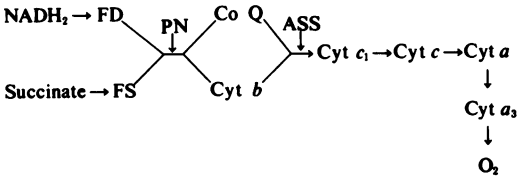


FIG. 5. *Components and their sequence in the respiratory electron transport system of mitochondria (12) and the most likely site of pyrrolnitrin action (PN). Abbreviations: FD, NADH-dehydrogenase flavoprotein; FS, succinate dehydrogenase flavoprotein; Co Q, coenzyme Q; Cyt, cytochrome; ASS, antimycin-sensitive site.*

by *S. cerevisiae* in our experiments, whereas Nose and Arima reported that in *C. utilis* the inhibition was transitory. The oxygen uptake in this yeast was completely inhibited for 10 min and then the yeast began to consume oxygen but never at the rate of the control. At 20 and 30 min, the inhibition was still about 90 and 50%, respectively. Nose and Arima attribute the antibiotic action of pyrrolnitrin to the permeability because of the deleterious effect of the antibiotic on bacterial protoplasts. On the other hand, our data with *S. cerevisiae* whole cells, cell-free extracts, and mitochondria, as well as beef heart mitochondria, all indicate a direct effect on aerobic respiration.

ACKNOWLEDGMENT

We thank Paul D. Shaw and Tom Obrig, Department of Plant Pathology, and John Clark, Jr., Department of Chemistry, University of Illinois, for valuable assistance.

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