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Primary Site of Action of Ketoconazole on *Candida albicans*

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Ketoconazole, an antifungal drug, completely inhibited the growth of *Candida albicans* 7N at concentrations of ≥50 μg/ml (94 μM). However, ketoconazole incompletely inhibited the growth of this opportunistic yeast at concentrations of 25 to 0.2 μg/ml (47 to 0.4 μM). At these lower concentrations, 2,3,5-triphenyl tetrazolium chloride, an electron acceptor, was reduced by several strains of *C. albicans*. This effect resulted in red coloration of colonies. Concomitantly, this phenomenon was not antagonized in the presence of ergosterol. Furthermore, neither ketoconazole nor antimycin A inhibited the growth of *C. albicans* under anaerobic conditions, as revealed by a paper disk method. Ketoconazole at the concentrations stated above inhibited endogenous and exogenous respiration immediately after it was added to a system containing log phase *C. albicans* cells, as determined polarographically. At the same time, ketoconazole inhibited the activity of NADH oxidase at the mitochondrial level. In contrast, higher concentrations of ketoconazole (>100 μM) were required to inhibit the activity of succinate oxidase from rat liver mitochondria. In addition, concentrations of ketoconazole greater than 100 μM were required to impair the uptake of labeled leucine and adenine and, subsequently, the incorporation of the former into protein and the latter into DNA and RNA in intact cells. On the other hand, ketoconazole at concentrations of 10, 1.0, and 0.4 μM had no effect on either membrane permeability or macromolecular synthesis.

Ketoconazole (R41,400) is a new antifungal imidazole compound which has been found to be very active against both superficial and systemic fungal infections when it is administered orally, and its efficacy has been proved in clinical trials. It is known that ketoconazole is much more water soluble and readily absorbed from the gastrointestinal tract than any other chlorinated imidazole derivative synthesized to date. Nevertheless, the excellent in vivo activity of ketoconazole against *Candida albicans* cannot be explained by its fair blood level after oral administration alone, because the blood level attained is far below the minimal (complete) inhibitory concentration (MIC) in vitro (10).

A number of studies have been undertaken to elucidate the mode(s) of action of antifungal imidazole derivatives. The fact that these compounds interact with cell membranes seems to have been well established by the results of Iwata et al. (4, 5) and Yamaguchi and Iwata (21, 22) with clotrimazole, of Swamy et al. (12–14) with miconazole, and of Yamaguchi and Iwata (23–25) with econazole. More recently, Van den Bossche et al. reported the inhibition of ergosterol biosynthesis by miconazole (17) and ketoconazole (16); ergosterol is the characteristic constituent of yeast cell membranes. The same effect was also observed by Henry and Sisler with miconazole and dodecylimidazole (3) and by Marriot with some imidazole-containing antifungal agents (9). These interesting findings may provide some clue concerning the primary site of action of these compounds. However, these results do not differentiate the biochemical actions of miconazole and ketoconazole, and the function of the cell membrane components substituted by the precursors of ergosterol in *C. albicans* is not yet known. Similarly, the majority of these results were obtained after long exposures to the drugs.

In this study our aim was to clarify the principal action of ketoconazole on *C. albicans* in vitro. The growth of test *C. albicans* cells was retarded at a ketoconazole concentration as low as 0.2 μg/ml. Thus, the ratio of MIC to maximum growth allowance concentration (MAC) was much greater than the ratios for clotrimazole and miconazole. Moreover, *C. albicans* cells exposed to low concentrations of ketoconazole reduced 2,3,5-triphenyl tetrazolium chloride (TTC), an electron acceptor, more rapidly and more strongly than untreated cells.

In this paper we describe the inhibition of *C. albicans* respiration as a possible primary site of ketoconazole action.

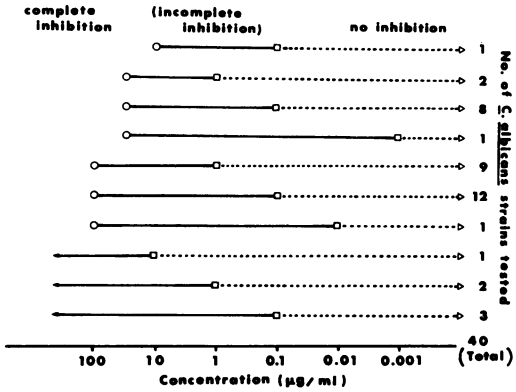

FIG. 1. Comparison of MICs and MACs of ketoconazole for various *C. albicans* strains. MICs (complete inhibition) and MACs (incomplete inhibition) were determined by the agar dilution method in YMA after incubation for 24 h at 37°C.

MATERIALS AND METHODS

Chemicals. Ketoconazole and copiamycin were supplied by Kyowa Fermentation Co., Ltd., Tokyo, Japan. Clotrimazole and miconazole nitrate were supplied by Bayer Yakuhin Co., Ltd., Osaka, Japan, and Eisai Co., Ltd., Tokyo, Japan, respectively. Antimycin A was obtained from Boehringer Mannheim GmbH, Mannheim, Germany, and amphotericin B was obtained from Sigma Chemical Co., St. Louis, Mo.

Ketoconazole was dissolved in 0.1 N HCl and sterilized by filtration. Other antifungal agents were dissolved in ethanol or dimethyl sulfoxide and kept at −20°C. [*U*-^{14}C]leucine (330 mCi/mmol) and [^{3}H]adenine (16.6 mCi/mmol) prepared by New England Nuclear Corp., Boston, Mass., were obtained from Daiichi Pure Chemical Co., Ltd., Tokyo, Japan.

Determination of antifungal activity. A total of 40 clinical isolates of *C. albicans* were used for this study. MICs and MACs were determined by the twofold agar dilution method using yeast morphology agar (YMA). The final concentration of organisms was 2×10^5 cells per ml. MIC and MAC were defined as the lowest drug concentration which resulted in no visible growth and the highest drug concentration which allowed a growth rate equivalent to that of a control culture, respectively. The incomplete growth range between the MIC and the MAC was confirmed by the reduction of TTC.

Effect of ketoconazole on growth rate. *C. albicans* 7N isolated from a patient with pulmonary candidiasis was grown by shaking for 18 h at 37°C in peptone yeast extract glucose broth containing 1% peptone, 0.5% yeast extract, and 1% glucose. The cells were harvested by centrifugation, washed with sterile saline, and suspended in 0.05 M phosphate buffer. After shaking for 20 min, the cells were centrifuged and resuspended in fresh medium (2×10^5 cells per ml) containing different concentrations of ketoconazole. After incubation, samples were withdrawn at 3-h intervals, and the turbidity was measured by optical density at 550 nm with a Hitachi digital spectrophotometer. Viability was determined by a colony count technique, using Sabouraud agar containing 2% glucose.

Anaerobic culture. Anaerobic cultures were grown in GasPak holding jars (BBL Microbiology Systems, Cockeysville, Md.). Antifungal activity under anaerobic conditions was determined by a paper disk method on YMA plates and on YMA plates supplemented with 0.002% ergosterol; the plates were seeded with *C. albicans* 7N as the indicator organism.

Respiration of intact cells. The respiration of *C. albicans* 7N was measured by oxygen consumption, as determined with a Clark type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The vessel of the electrode received (in a final volume of 3 ml) 50 mM potassium phosphate, 20 mM glucose (omitted for endogenous respiration), and log phase *Candida* cells equivalent to 0.3 to 0.5 mg of dry weight. Incubation was carried out at 30°C with stirring. The percent inhibition of oxygen consumption was calculated from the slopes on the chart before and after the addition of ketoconazole.

Preparation of *C. albicans* and rat liver mitochondria. Protoplasts of *C. albicans* 7N were prepared by the method of Wakabayashi (18), using zymolyase (Kirin Brewery Co., Ltd., Tokyo, Japan). Briefly, *C. albicans* cells from a 16-h culture were treated with 0.1 M EDTA and 0.3 ml of 2-mercaptoethanol at 30°C for 40 min. After washing, the cells were suspended in a reaction mixture containing 1.3 M sorbitol, 0.1 mM EDTA, and 10 mM Tris buffer (pH 7.4) (SET) supplemented with 10 mg of zymolyase per g. Protoplasts were harvested by centrifugation at 2,500 × *g* for 10 min at 4°C and, after washing with SET, were disintegrated by sonication. The cell debris and whole cells were removed by centrifugation at 2,500 × *g* for 20 min at 4°C. Mitochondria were harvested from the supernatant by centrifugation at 26,000 × *g* for 30 min at 4°C; the preparation was centrifuged three times, and the resulting pellets were suspended in SET for storage at −80°C. The mitochondrial preparation was used within 3 days.

Rat liver mitochondria were isolated by the method of Johnson and Lardy (6).

Protein concentrations were determined by the modified Folin phenol method of Lowry et al. (8).

Leakage of cellular constituents. *C. albicans* 7N cells were suspended in phosphate buffer (pH 7.4) at a final concentration of 2×10^8 cells per ml, and different concentrations of ketoconazole were added. After incubation at 30°C, samples were withdrawn at different times, and the leakage of 260-nm-absorbing materi-

TABLE 1. MICs and MACs of some imidazole drugs for *C. albicans* 7N[a]

Drug	MIC		MAC		Ratio of MIC to MAC
	μg/ml	μM	μg/ml	μM	
Ketoconazole	50.0	94.1	0.10	0.19	500
Clotrimazole	6.25	18.1	0.10	0.29	63
Miconazole nitrate	12.5	26.1	0.20	0.42	63

[a] MICs and MACs were determined by the agar dilution method after 24 h of incubation at 37°C in YMA.

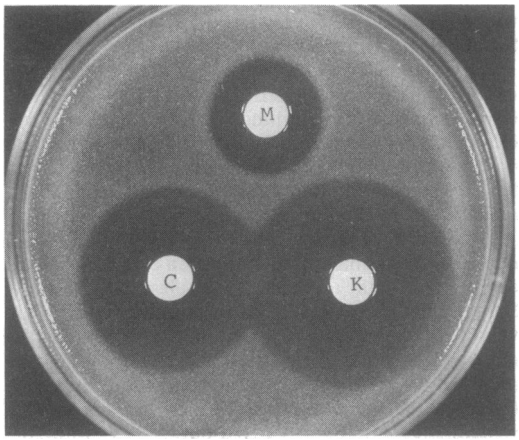

FIG. 2. Complete and incomplete inhibition zones for ketoconazole (K), clotrimazole (C), and miconazole (M) on YMA seeded with *C. albicans* 7N, clearly revealed by TTC reduction. The concentration of each imidazole was 1.0 mg/ml.

al was determined in the supernatant after centrifugation at 3,000 × *g* for 20 min. Similarly, protein leakage was detected by the modified Folin phenol method (8).

Incorporation of labeled compounds into protein, RNA, and DNA of *C. albicans*. *C. albicans* 7N cells grown in peptone yeast extract glucose broth at 30°C for 18 h were collected by centrifugation and washed with saline. The washed cells were suspended in yeast nitrogen base supplemented with 0.5% glucose at a density of 5×10^7 cells per ml. The cell suspensions were incubated at 30°C for 20 min before the addition of ketoconazole at varying concentrations and [^{3}H]adenine (1 μCi/ml) or [^{14}C]leucine (1 μCi/ml). At different times 1-ml samples were withdrawn, and an equal volume of 10% ice-cold trichloroacetic acid was added. The uptake and incorporation of [^{14}C]leucine into protein were assayed by the modified Bollum method (1), and the uptake and incorporation of [^{3}H]adenine into total nucleic acid (RNA and DNA) were assayed by the Yu-Frigelson method (26) and the modified Watson-Yamazaki method (20). Radioactivity was counted in a toluene scintillation fluid with a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Susceptibility of *C. albicans* to ketoconazole. Figure 1 shows a comparison of the MICs and MACs of ketoconazole for 40 strains of *C. albicans*. As this figure shows, the MAC of ketoconazole for 24 strains was 0.1 μg/ml (0.19 μM). The MAC was as low as 0.001 μg/ml (0.0019 μM) for only one strain.

The MICs and MACs of imidazole compounds for *C. albicans* 7N are compared in Table 1. The reason that we selected this strain for further experiments was its high virulence in experimental animals. It was evident from our results that the MACs were approximately the same for ketoconazole, clotrimazole, and miconazole, whereas the ratio of MIC to MAC was much greater for ketoconazole than for the other two compounds.

The complete and incomplete inhibition zones (clearly revealed by TTC reduction) of ketoconazole, clotrimazole, and miconazole on a *C. albicans* 7N plate are shown in Fig. 2. The zone of complete inhibition for ketoconazole was much smaller than the zones for clotrimazole and miconazole; this difference corresponded to the difference in the MICs of these drugs. However, ketoconazole produced a large zone of incomplete inhibition which was stained intensely by the reduction of TTC.

Effect of ketoconazole on the growth rate of *C. albicans* 7N. Figure 3 shows that cells exposed to ketoconazole concentrations of 10, 1.0, and 0.4 μM proliferated at much slower rates than control cells, as determined by viable cell counts and optical density at 550 nm. Both the viability of the cells and the turbidity were affected by

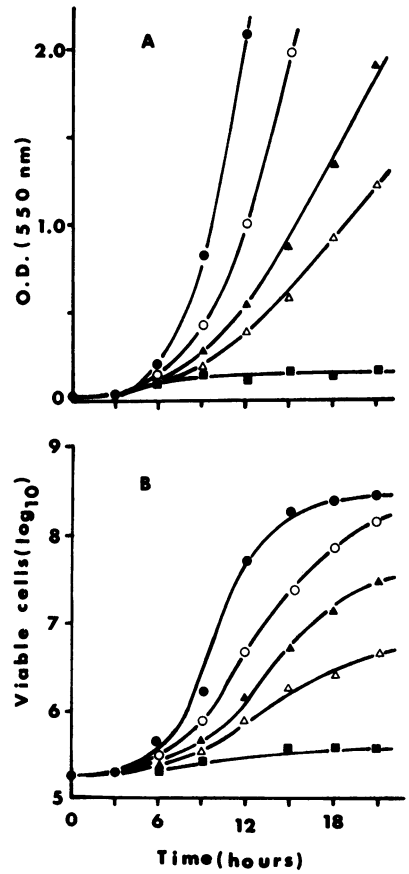

FIG. 3. Effect of ketoconazole on the growth of *C. albicans* 7N. (A) Optical density (O.D.). (B) Viable cells. Symbols: ●, control; ○, 0.4 μM (0.212 μg/ml); ▲, 1.0 μM (0.53 μg/ml); △, 10 μM (5.3 μg/ml); ■, 100 μM (53.1 μg/ml).

TABLE 2. Effects of some antifungal agents on aerobic and anaerobic cultures of *C. albicans* 7N[a]

Antifungal agent	Concn		Diam of inhibition zone (mm)[a]			
	μg/ml	mM	Aerobic cultures		Anaerobic cultures	
			YMA	YMA-E	YMA	YMA-E
Ketoconazole	100	0.19	(24.3)	(27.6)	0	0
	500	0.94	(32.4)	(36.6)	0	0
Antimycin A	500	0.91	(24.6)	(25.3)	0	0
Amphotericin B	500	0.54	16.5	11.4	16.2	11.3
Copiamycin	500	0.47	14.8	16.1	15.1	16.4

[a] Antifungal activity was measured by a paper disk method in YMA and YMA supplemented with 0.002% ergosterol (YMA-E). Parentheses indicate incomplete inhibition.

increasing drug concentrations. Subculture studies revealed that the action was fungistatic rather than fungicidal in nature.

Comparison of the effects of aerobic and anaerobic growth conditions on susceptibility to ketoconazole. The zones of inhibition obtained with ketoconazole, as well as those obtained with antimycin A on plates seeded with *C. albicans* 7N, disappeared completely under anaerobic conditions, whereas the zones of inhibition obtained with amphotericin B (whose target is membrane sterol) and copiamycin (whose target is membrane phospholipids) were not affected by oxygen tension (Table 2). A remarkable feature of this experiment was that the addition of ergosterol to the medium did not antagonize the effect of ketoconazole.

Effect of ketoconazole on the respiration of *C. albicans* 7N intact cells. Ketoconazole inhibited oxygen consumption by *Candida* cells both when glucose was used as a substrate (exogenous respiration) and when glucose was not present (endogenous respiration). As Table 3 shows, the inhibition of respiration was dose dependent and was observed immediately after ketoconazole was added.

Effect of ketoconazole on the respiration of *C. albicans* and rat liver mitochondria. Respiration of isolated mitochondria from *C. albicans* 7N was also inhibited by ketoconazole. This drug inhibited the activity of NADH oxidase when it was measured either spectrophotometrically or polarographically (Table 4). This inhibition was also observed immediately after ketoconazole was added.

Higher concentrations (100 and 10 μM) of ketoconazole were required to inhibit the succinate oxidase of rat liver mitochondria (Fig. 4). No uncoupling effect by ketoconazole was observed, even at a concentration of 100 μM.

Effect of ketoconazole on the leakage of cellular constituents. The leakage of cellular constituents (260-nm-absorbing material and protein) as a result of membrane damage by ketoconazole is shown in Fig. 5. When cells were exposed to 1 and 10 μM ketoconazole, no effect was observed, even after 120 min. However, concentrations above 100 μM gave rise to leakage of cellular constituents. Leakage continued to occur as the time of exposure to the drug was prolonged.

Effects of ketoconazole on protein and nucleic acid syntheses. Figure 6 shows the effect of ketoconazole on the uptake and incorporation of [^{14}C]leucine into the cellular protein fraction of *C. albicans* 7N, and Fig. 7 shows the effect of

TABLE 3. Effect of ketoconazole on respiration of *C. albicans* 7N[a]

Inhibitor	Concn (mM)	Endogenous respiration		Exogenous respiration	
		O_2 uptake[b]	Inhibition (%)	O_2 uptake[b]	Inhibition (%)
None	0	2.79×10^{-2}	0	2.14×10^{-2}	0
Ketoconazole	0.001	1.85×10^{-2}	33.7	1.69×10^{-2}	21.1
	0.01	1.36×10^{-2}	51.3	1.21×10^{-2}	43.6
	0.1	0.76×10^{-2}	72.6	0.59×10^{-2}	72.4
Antimycin A	0.01	1.67×10^{-2}	40.0	1.02×10^{-2}	52.2
Potassium cyanide	10.0	1.41×10^{-2}	49.5	0.85×10^{-2}	60.1

[a] Oxygen consumption was measured at 30°C in a medium containing 50 mM phosphate buffer, 20 mM glucose (omitted from the reaction vessel for endogenous respiration), and 0.1 ml of a cell suspension in a total volume of 3 ml. Inhibitors were added to the reaction mixture, and values were determined after 10 min of incubation.

[b] Expressed as micromoles of O_2 per milligram (dry weight) of cells per minute.

TABLE 4. Effect of ketoconazole on NADH oxidation in *C. albicans* 7N mitochondria[a]

Inhibitor	Concn (mM)	Absorbance at 340 nm		O_2 uptake	
		Sp act[b]	Inhibition (%)[c]	Sp act[b]	Inhibition (%)[c]
None		3.31×10^{-3}	0	2.24×10^{-3}	0
Ketoconazole	0.001	2.87×10^{-3}	12.8	1.80×10^{-3}	19.7
	0.01	2.29×10^{-3}	30.7	1.46×10^{-3}	35.0
	0.1	0.97×10^{-3}	70.6	0.64×10^{-3}	71.5
Potassium cyanide	10.0	0.13×10^{-3}	96.0	0.14×10^{-3}	93.7

[a] The reaction mixture contained SET and *C. albicans* mitochondria (1.0 to 1.5 mg/ml) in a total volume of 3 ml (pH 7.4).
[b] Specific activity of NADH oxidase (in micromoles per milligram of protein per minute).
[c] Inhibition was calculated immediately after the chemical was added.

the drug on the uptake and incorporation of [^{3}H]adenine into cellular nucleic acids. The uptake and incorporation of the two precursors were not affected at ketoconazole concentrations of 1 and 10 μM, whereas some effect was observed at a concentration of 100 μM.

DISCUSSION

The experimental evidence presented above indicated that high concentrations of ketoconazole inhibit several cellular functions of *C. albicans*. Since the growth of all organisms is the result of complex, integrated, interdependent processes, antimicrobial agents that affect any cellular process ultimately impair most of the other cellular functions as well. Hence, the important question in the study of the mechanism of action of an antimicrobial agent is the primary site of action. The reaction which is inhibited at the lowest concentration and within the shortest time is usually accepted as the primary site of action. As to the effect of ketoconazole on *C. albicans*, these criteria could be satisfied by the inhibition of respiration because at very low concentrations and short exposures to ketoconazole, the respiration of whole cells, as well as the respiration of mitochrondrial preparations was inhibited to a greater extent than any other cellular function tested so far. Our experiments also demonstrated that ketoconazole affects *C. albicans* only under aerobic conditions. A similar phenomenon was described recently by Sud and Feingold (11) in *Saccharomyces cerevisiae*.

A number of attempts have been made to elucidate the mode(s) of action of the imidazole antifungal agents. However, no experiment has fully explained the primary site of action of ketoconazole. Van den Bossche et al. (16) reported potent inhibition of ergosterol biosynthesis in *C. albicans* by ketoconazole at low concentrations. However, a relatively long ex-

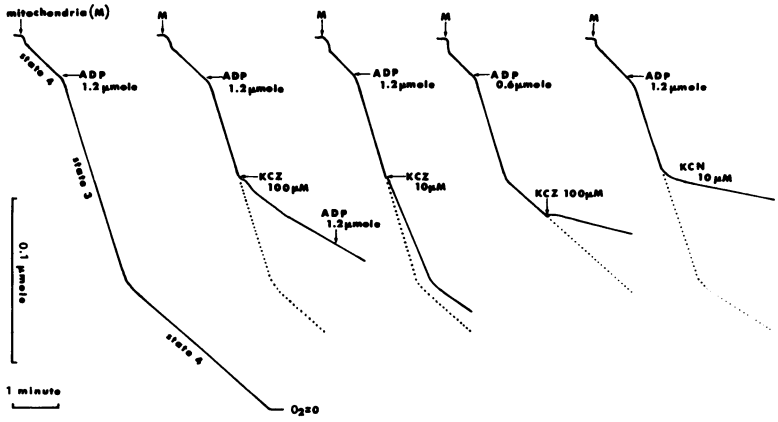

FIG. 4. Effect of ketoconazole (KCZ) on the oxidation of succinate by rat liver mitochondria. Respiration was measured in a reaction mixture containing 225 mM sucrose, 5 mM potassium phosphate, 10 mM Tris-hydrochloride, 5 mM magnesium chloride, 0.5 mM EDTA, 15 mM succinate, and 1.5 mg of rat liver mitochondria at 30°C in a final volume of 3 ml (pH 7.4).

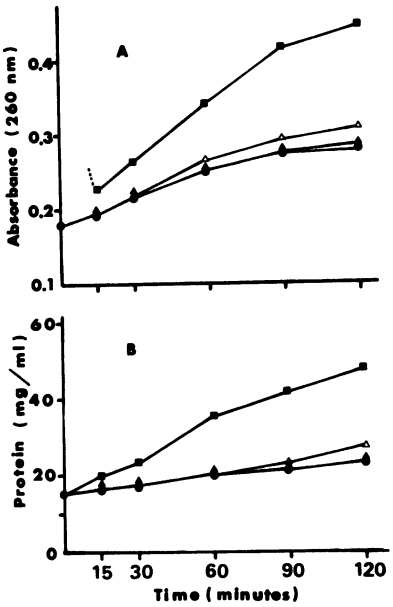

FIG. 5. Effect of ketoconazole on leakage of nucleic acids (A) and proteins (B) from *C. albicans* 7N. Symbols: ●, control; ▲, 1.0 μM; △, 10 μM; ■, 100 μM.

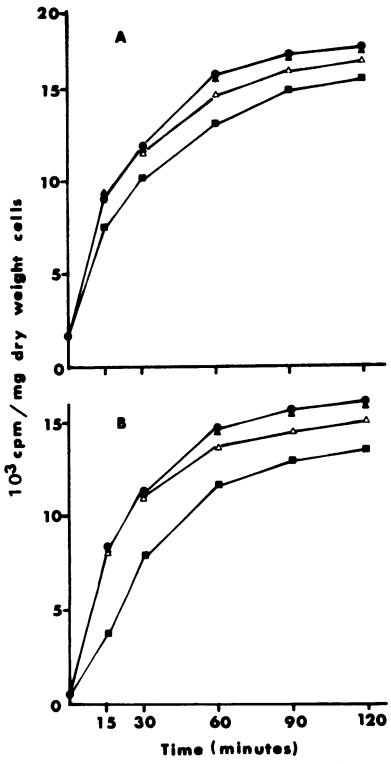

FIG. 6. Effect of ketoconazole on the uptake of [^{14}C]leucine (A) and its incorporation into protein (B) of *C. albicans* 7N. Symbols: ●, control; ▲, 1.0 μM; △, 10 μM; ■, 100 μM.

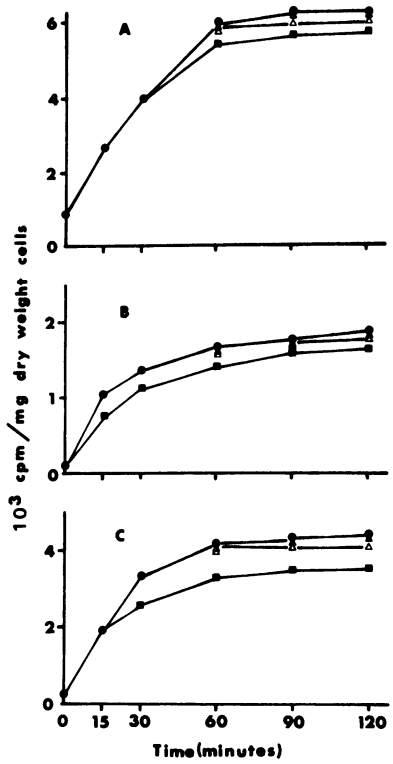

FIG. 7. Effect of ketoconazole on the uptake of [^{3}H]adenine (A) and its incorporation into DNA (B) and RNA (C) of *C. albicans* 7N. Symbols: ●, control; ▲, 1.0 μM; △, 10 μM; ■, 100 μM.

posure was required to detect this effect. Our strain of *C. albicans* 7N was rather resistant to ketoconazole compared with other *C. albicans* strains. The reason why this strain was chosen is its high virulence in experimental animals. With other test strains, inhibition of respiration was attained at ketoconazole concentrations similar to those used by Van den Bossche et al. (16).

On the other hand, our results showed that the activity of ketoconazole was not antagonized by the addition of ergosterol to the medium under aerobic conditions. It is well known that ergosterol is not synthesized by yeast plasma membranes and mitochondrial membranes under anaerobic conditions (7, 15, 19). Strong arguments based on these studies suggested the possibility that the inhibition of ergosterol biosynthesis might be a consequence of respiratory impairment of mitochondria caused by ketoconazole.

The specific site of ketoconazole action on the respiratory system of *C. albicans* 7N has been analyzed further by difference spectrophotometry and will be described elsewhere.

With respect to mammalian mitochondria, Dickinson (2) reported that miconazole caused an uncoupling effect on isolated rat liver mito-

chondria, with concurrent membrane damage. In our studies, an uncoupling effect was not observed with ketoconazole at concentrations ranging from 100 to 0.4 μM. Ketoconazole inhibited respiration in rat liver mitochondria only at concentrations as high as 100 μM. This requirement for a high concentration of ketoconazole for inhibition of mammalian mitochondrial respiration might be reflected in the low toxicity of this drug to mammalian cells.

We concluded that three major imidazoles (miconazole, clotrimazole, and ketoconazole) basically act in the same manner. However, miconazole and clotrimazole cause direct membrane damage to *C. albicans* cells at lower concentrations than ketoconazole. This fact might correlate well with the higher toxicities of miconazole and clotrimazole to mammalian cells compared with the toxicity of ketoconazole. On the other hand, the inhibitory effect of ketoconazole on *C. albicans*, as determined by incomplete respiration or impairment of respiratory function, occurred at the lowest concentration observed among the imidazole compounds developed to date.

LITERATURE CITED

1. **Bollum, F. J.** 1968. Filter paper disk techniques for assaying radioactive macromolecules. Methods Enzymol. **12B**: 169–173.
2. **Dickinson, D. P.** 1977. The effect of miconazole on rat liver mitochondria. Biochem. Pharmacol. **26**:541–542.
3. **Henry, H., and H. D. Sisler.** 1979. Effects of miconazole and dodecylimidazole on sterol biosynthesis in *Ustilago maydis*. Antimicrob. Agents Chemother. **11**:158–166.
4. **Iwata, K., and H. Yamaguchi.** 1973. Mechanism of action of clotrimazole. I. Survey of the principal action on *Candida albicans*. Jpn. J. Bacteriol. **23**:513–521.
5. **Iwata, K., H. Yamaguchi, and T. Hiratani.** 1973. Mode of action of clotrimazole. Sabouraudia **11**:205–209.
6. **Johnson, D., and H. Lardy.** 1967. Isolation of liver or kidney mitochondria. Methods Enzymol. **10**:94–96.
7. **Jollow, D., G. M. Killerman, and A. W. Linnane.** 1968. The biogenesis of mitochondria. III. The lipid composition of aerobically and anaerobically grown *Saccharomyces cerevisiae* as related to the membrane systems of the cells. J. Cell Biol. **37**:221–230.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. **Marriott, M. S.** 1980. Inhibition of sterol biosynthesis in *Candida albicans* by imidazole-containing antifungals. J. Gen. Microbiol. **117**:253–255.
10. **Shigematsu, M. L., J. Uno, and T. Arai.** 1981. Correlative studies on in vivo and in vitro effectiveness of ketoconazole (R41400) against *Candida albicans* infection. Jpn. J. Med. Mycol. **22**:195–201.
11. **Sud, I. J., and D. S. Feingold.** 1981. Heterogeneity of action mechanisms among antimycotic imidazoles. Antimicrob. Agents Chemother. **20**:71–74.
12. **Swamy, K. H. S., A. Joshi, and G. R. Rao.** 1976. Mechanism of action of miconazole: labilization of rat liver lysosomes in vitro by miconazole. Antimicrob. Agents Chemother. **9**:903–907.
13. **Swamy, K. H. S., M. Sirsi, and G. R. Rao.** 1974. Studies on the mechanism of action of miconazole on respiration and cell permeability of *Candida albicans*. Antimicrob. Agents Chemother. **5**:420–425.
14. **Swamy, K. H. S., M. Sirsi, and G. R. Rao.** 1976. Studies on mechanisms of action of miconazole. II. Interaction of miconazole with mammalian erythrocytes. Biochem. Pharmacol. **25**:1145–1150.
15. **Taketani, S., T. Osumi, and H. Katuki.** 1978. Characterization of sterol-ester hydrolase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **524**:87–92.
16. **Van den Bossche, H., G. Willemsens, W. Cools, F. Cornelissen, W. F. Lauwers, and J. M. Van Cutsem.** 1980. In vitro and in vivo effects of the antimycotic drug ketoconazole on sterol synthesis. Antimicrob. Agents Chemother. **17**:922–928.
17. **Van den Bossche, H., G. Willemsens, W. Cools, W. F. Lauwers, and L. Le Jeune.** 1978. Biochemical effects of miconazole on fungi. II. Inhibition of the ergosterol biosynthesis in *Candida albicans*. Chem. Biol. Interact. **21**:59–78.
18. **Wakabayashi, K.** 1974. Preparation of mitochondria particles from yeast, p. 349–357. *In* Y. Anraku (ed.), Protein, nucleic acid and enzyme. Kyoritsu Shuppan, Tokyo.
19. **Wallace, P. G., M. Huang, and A. W. Linnane.** 1968. The biogenesis of mitochondria. II. The influence of medium composition on the cytology of anaerobically grown *Saccharomyces cerevisiae*. J. Cell Biol. **37**:207–220.
20. **Watson, R., and H. Yamazaki.** 1973. Alkali hydrolysis of RNA of *Escherichia coli* deposited on filter paper disks. Anal. Biochem. **51**:312–314.
21. **Yamaguchi, H.** 1977. Antagonistic action of lipid components of membranes from *Candida albicans* and various lipids on two imidazole antimycotics, clotrimazole and miconazole. Antimicrob. Agents Chemother. **12**:16–25.
22. **Yamaguchi, H., and K. Iwata.** 1974. Mechanism of action of clotrimazole. II. Effects on the cell membrane of *Candida albicans*. Jpn. J. Bacteriol. **29**:379–385.
23. **Yamaguchi, H., and K. Iwata.** 1979. Studies on the mechanism of action of econazole. I. Differential inhibition of cellular function in *Candida albicans*. Jpn. J. Med. Mycol. **20**:31–39.
24. **Yamaguchi, H., and K. Iwata.** 1979. Studies on the mechanism of action of econazole. II. Effect of econazole on cytoplasmic membranes of fungal, bacterial and mammalian cells. Jpn. J. Med. Mycol. **20**:201–208.
25. **Yamaguchi, H., and K. Iwata.** 1979. Studies on the mechanism of action of econazole. III. Effect of lipid composition on sensitivity of liposomes to econazole. Jpn. J. Med. Mycol. **20**:260–265.
26. **Yu, F. L., and P. Frigelson.** 1971. Paper disc estimation of radioactive RNA. Studies on the presence and elimination of metabolically generated artifacts from labeled purine and pyrimidine precursors. Anal. Biochem. **39**:319–321.