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In Vitro and In Vivo Effects of the Antimycotic Drug Ketoconazole on Sterol Synthesis

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Ketoconazole, an orally active antimycotic drug, is a potent inhibitor of ergosterol biosynthesis in *Candida albicans* when added to culture media which support yeast or mycelial growth or to cultures containing outgrown mycelium. This inhibition coincides with accumulation of sterols with a methyl group at C-14 and can thus be attributed to an interference with one of the reactions involved in the removal of the 14α-methyl group of lanosterol. When administered to rats infected with *C. albicans*, ketoconazole also inhibits fungal synthesis of ergosterol. A six-times-higher dose is required to effect cholesterol synthesis by rat liver.

Ketoconazole (7) (*cis*-1-acetyl-4-{4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1, 3-dioxalan-4-yl]methoxy]phenyl} piperazine) orally active agent with broad-spectrum activity against a variety of yeasts, dermatophytes, and dimorphous fungi (1, 4, 11, 13, 14).

Chemically, ketoconazole is related to miconazole, an antimycotic agent whose activity may be due in part to alteration of membrane permeability originating from interference with the biosynthesis of ergosterol and the accumulation of sterols with a methyl group at C-14 in the fungal cell (8, 15, 16).

This study was undertaken in an attempt to define the action of ketoconazole on sterol synthesis in yeast-phase and mycelial-phase *Candida albicans* cells. The oral effectiveness of ketoconazole makes it possible to study the inhibitory effect of oral ketoconazole on ergosterol synthesis in *C. albicans* present in the vagina of artificially infected rats.

MATERIALS AND METHODS

In vitro studies: strains, inocula, and media. Three different strains of *C. albicans* were used. Their selection was based on the fact that they were growing best as yeast (ATCC 28516 = RV 4688) or mycelium (B 12377) or were highly infective to rats (B 2630).

Inocula of *C. albicans* B 12377 and ATCC 28516 were prepared as previously described (16, 17). Cells were grown aerobically in a reciprocating shaker at 37°C for 64 h, and 0.2-ml portions were used to inoculate either casein hydrolysate-yeast extract-glucose medium (CYG medium) or keratin medium. Cells were grown for 24 or 72 h at 37°C with shaking, and 0.2-ml portions containing 3×10^7 and 2×10^7 cells were used to inoculate 100 ml of CYG or keratin medium, respectively.

Cells were grown in CYG medium for 24 h, and portions (7.5×10^6 cells) were used to inoculate tissue culture dishes or tissue flasks containing 10 or 50 ml of Eagle minimum essential medium (EMEM).

Sabouraud agar, broth, and CYG medium were prepared as previously described (17).

For keratin-medium (A) 20 g of keratin (Merck, Darmstadt, West Germany) and 1 g of NaOH were dissolved in 300 ml of water in an Erlenmeyer flask which contained 10 g of glass beads (diameter, 5 mm) and was placed on a magnetic stirrer for 20 min.; (B) 23.4 g of yeast carbon base (Difco) was dissolved in 1,700 ml of water. A and B were combined with stirring. The pH was corrected at 7. The suspension was then passed through two Endocott sieves of 45 and 400 mesh and filtered through a Büchner filter (S & S no. 1450). The protein content was determined (12) and corrected to 8.4 mg/ml by dilution with B. The medium was dispensed in 100-ml portions and autoclaved.

EMEM was prepared by the method of Aerts and De Brabander (Mykosen, in press). A 500-ml amount of EMEM (Flow Laboratories, 5023 M) was supplemented with 11.4 ml of sodium bicarbonate (7.5%, Flow, 7-041 D), 6 ml of glutamine (200 mM, GIBCO Bio-Cult, 503), and, where mentioned, 50 ml of fetal calf serum (GIBCO Bio-Cult, 629). The serum was made sterile by filtering through a filter (Millex; Millipore Corp.; GS sterile filter unit; pore size, 0.22 μm).

Growth studies. Cells were grown at 37°C in either Erlenmeyer flasks containing CYG or keratin medium with constant agitation (17) or tissue culture dishes containing 10 ml of EMEM in the humidified 5% CO_2 atmosphere of a Heraeus incubator (B 5060 EK CO_2).

Ketoconazole was dissolved in water acidified with 0.1 N HCl (final pH, 2.4) and passed through a 0.22-μm Millipore filter. Dilution was made in sterile water. Fifty microliters of the dilutions was added per 100 ml of the media. Controls were exposed to an equivalent quantity of acidified water.

Cell counting method. The number of cells per unit volume of CYG and keratin medium were determined with a Coulter Counter (16).

Determination of dry weight. Cells from one or more tissue culture dishes were collected on pre-

weighed Millipore filters (HAWP 02500; pore size, 0.45 μm) and were dried to a constant weight at 70°C under reduced pressure.

Acetate incorporation into lipids. (i) Growth, disruption of cells and extraction of lipids. For CYG and keratin medium, growth conditions were as described above. Procedures for cell disruption and extraction of lipids were as described previously (16).

For EMEM, *C. albicans* B 12377 was inoculated in 200-ml tissue flasks (Nunc, Roskilder, Denmark) containing 50 ml of EMEM, supplemented with or without serum, to which 1 μmol of sodium acetate, 2.5 μCi of sodium [*U*-^{14}C]acetate, and drug or solvent or both were added immediately before inoculation or after 24 h of growth. Growth, cell collection, and processing were as described above.

(ii) Thin-layer chromatography. The extracted lipids were separated by thin-layer chromatography on precoated silica gel 60F254 plates (Merck no. 5715) using heptane-isopropyl ether-glacial acetic acid, 60:40:4 (vol/vol/vol), as a solvent system (16).

(iii) Determination of radioactivity. Bands on the thin-layer chromatographic plate were located by spraying with 50% aqueous sulfuric acid and heating at 150°C until spots corresponding to free sterols, free fatty acids, and glycerides appeared. The R_f's of the different lipids were compared with those of standards separated with the same solvent system (16). After detection, the bands were scraped out into plastic counting vials and mixed with 1 ml of water and 10 ml of scintillator solution (Lumagel, Lumac). The radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

(iv) Identification of sterols. The chemical structures of the sterols present in ketoconazole-treated (10^{-7} M) or untreated *C. albicans* (strain ATCC 28516) were determined by gas chromatographic-mass spectrometric analysis (16).

In vivo studies: animals. Female Wistar rats, weighing 100 g, were ovariectomized and hysterectomized under Thalamonal anaesthesia. Three and four weeks later the rats were treated subcutaneously weekly with 0.1 mg of estradiol in 1 ml of sesame oil. Rats in pseudo-estrus were infected intravaginally with *C. albicans* (strain B 2630, 8×10^5 cells) on the day of the second estradiol injection.

Treatment. Treatment of the *C. albicans*-infected rats always started 3 days after infection. At the time of treatment, the rats weighed about 250 g.

Mevalonate incorporation into sterols. One gram of DL-mevalonic acid lactone (Sigma) was dissolved in 8 ml of 0.2 N KOH, and the solution was stored at 37°C. After the solution was cooled to room temperature, its pH was adjusted to 7.3 with 0.1 N KOH and 250 μCi of DL-[2-^{14}C]mevalonic acid dibenzylethylenediamine salt (The Radiochemical Centre, Amersham; specific activity, 51 mCi/mmol) was added. The volume was adjusted to 50 ml with water.

Two hours after treatment with ketoconazole or the excipient or both, rats were injected intraperitoneally with 5 μCi of the mevalonate solution. The rats were sacrificed and dissected 2 h later.

The contaminating epithelial cells were disrupted in 0.1 M sodium phosphate buffer at pH 7.4 with a Potter-Elvehjem homogenizer with a Teflon pestle. *C. albicans* withstands such a homogenization procedure and can be collected by centrifugation for 10 min at $310 \times g$.

Livers pooled from 14 rats were cut in small pieces, washed twice in 0.1 M sodium phosphate buffer (pH 7.4), resuspended in the same medium, and homogenized with a Potter-Elvehjem homogenizer with Teflon pestle.

The collected *C. albicans* cells were handled as already described. The lipid content of the liver homogenates was determined by the phosphoric acid-vanillin method of Zöllner and Kirsch (19). The homogenates were diluted to obtain a lipid content of 8.5 mg/5 ml. Extraction and separation of the lipids and determination of radioactivity was essentially the same as that described for *C. albicans* grown in vitro.

RESULTS

Effects on growth. Inhibition of growth of *C. albicans* ATCC 28516 occurred within 3 to 5 h after the addition of 10^{-8} to 10^{-4} M ketoconazole (Fig. 1). At 10^{-7} M ketoconazole, 70% inhibition of cell growth was observed after 24 h of contact.

In keratin medium (Fig. 2) the onset of the exponential phase was reached after 10 h of incubation. During the first 4 h, no growth was observed. No inhibition of growth occurred before 24 h or 8 h after addition of 10^{-8} or 10^{-7} M ketoconazole. However, 24 h after the addition of 10^{-7} and 10^{-5} M ketoconazole, the rate of the cell number increase was severely inhibited.

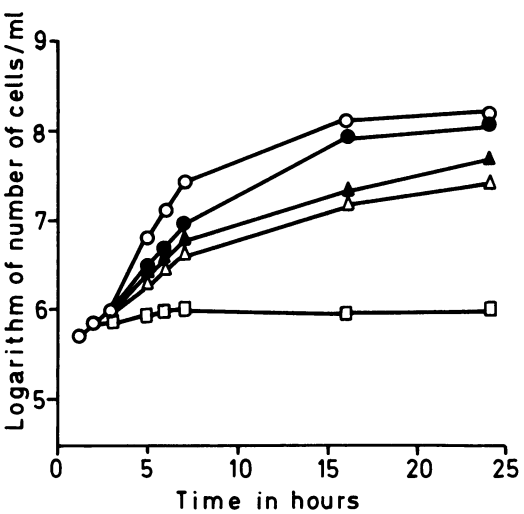

FIG. 1. *Effect of ketoconazole on growth of C. albicans ATCC 28516. Candida were grown at 37°C in CYG medium in the presence of solvent* (○) *and* 10^{-8} (●), 10^{-7} (▲), 10^{-5} (△), *or* 10^{-4} (□) *M ketoconazole. Solvent and drug were added immediately after inoculation. The number of cells per unit volume of CYG medium was determined by the total count method.*

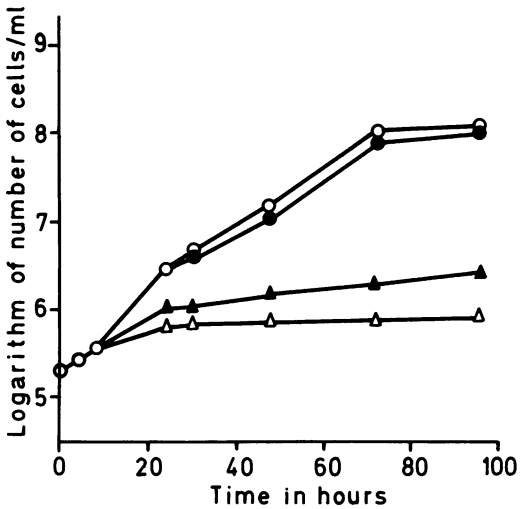

FIG. 2. *Effect of ketoconazole on growth of C. albicans ATCC 28516. Candida were grown at 37°C in keratin medium in the presence of solvent* (○) *and* 10^{-8} (●), 10^{-7} (▲) *or* 10^{-5} (△) *M. Further details are given in the legend to Fig. 1.*

In EMEM the inoculated *C. albicans* B 12377 rapidly transformed into mycelial cells (3). When dry weight of the harvested cells was used as a measure of cell growth, the lag period and exponential growth phase approximated 4 and 20 h (Fig. 3).

When 10^{-8} M ketoconazole was added to *C. albicans* cultures, little inhibition of growth occurred before 16 h after addition of the antifungal agent. However, 16 or 9 h after the addition of 10^{-5} or 10^{-4} M ketoconazole, the rate of dry-weight increase was significantly inhibited (Fig. 3).

The results presented in Table 1 indicate that the presence of serum in the medium did not significantly influence the growth inhibitory effects of ketoconazole.

Effect on ergosterol synthesis. (i) *C. albicans* grown in CYG medium. Incubation of *C. albicans* for 4 and 16 h in the presence of [^{14}C]acetate, followed by thin-layer chromatographic separation of the lipid fractions, indicated that almost 16 and 19% of the total radioactivity present in the lipid fraction was incorporated into sterol esters and free sterols.

Although 4 h of incubation of *C. albicans* in the presence of ketoconazole had no appreciable effect on growth and [^{14}C]acetate uptake, remarkable differences were observed in the radioactivities incorporated in the 4-desmethyl-, 4,14-dimethyl-, and 4,4',14-trimethylsterols between control and treated cells (Fig. 4). A 50% decrease in acetate incorporation into 4-desmethylsterols was already obtained at 5×10^{-9} M. Concomitantly, a significantly increased incorporation was noticed in the fractions containing 4,14-dimethyl- and 4,4',14-trimethylsterols.

Examination of the sterol fractions from *C. albicans* after 16 h of incubation also revealed a shift from 4-desmethylsterols to sterols with methyl groups at C-4 and C-14 (Fig. 5). At 10^{-8} M, a ketoconazole concentration that had only a minor effect on growth severely inhibited [^{14}C]acetate incorporation into 4-desmethylsterols.

Identification of the three free sterols in cells collected after 16 h of growth in the presence of

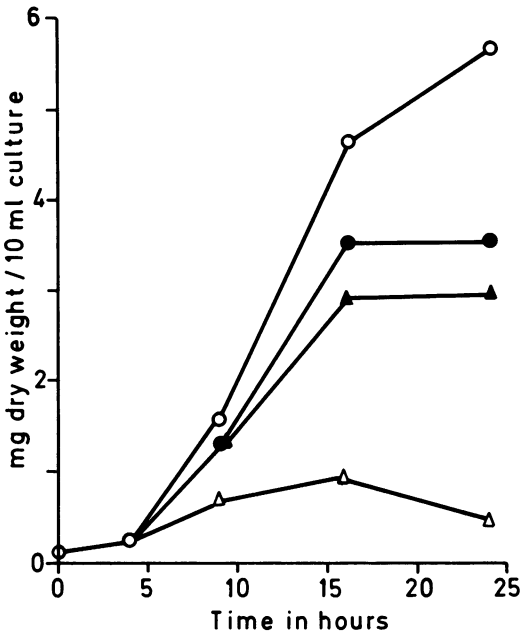

FIG. 3. *Effect of ketoconazole on growth (dry weight) of C. albicans B 12377 in EMEM (without serum). Cells were grown at 37°C in the presence of solvent and* 10^{-8} (●), 10^{-5} (▲), *or* 10^{-4} (△) *M.*

TABLE 1. *Effect of serum on the inhibitory effects of ketoconazole on the growth of C. albicans in EMEM*[a]

Ketoconazole (M)	% of control	
	EMEM + serum	EMEM − serum
10^{-8}	69.1 ± 15.4 (8)	63.0 ± 5.5 (8)
10^{-5}	56.9 ± 3.4 (7)	50.4 ± 10.0 (10)
10^{-4}	23.2 ± 2.3 (4)	16.8 ± 6.0 (6)

[a] Solvent and ketoconazole were added to *C. albicans* cultures immediately after inoculation. The number of cells was counted after a 24-h growth period. The results presented are mean values ± standard deviation followed by the number of experiments in parentheses.

ketoconazole or solvent or both was performed by thin-layer chromatography and by a gas chromatographic-mass spectrometric analysis (Table 2). The 4-desmethyl fraction collected from control cells consisted of ergosterol, and the small amount of 4,4',14-trimethylsterol was lanosterol.

In cells treated for 16 h with 10^{-7} M, the 4-desmethylsterol fraction contained 14-methylfecosterol instead of ergosterol; in the 4,14-dimethylsterol fraction, obtusifoliol or 4,14-dimethyl-ergosta-8,24 (28)-dien-3β-ol was found. The 4,4',14-trimethylsterol fraction contained lanosterol and a sterol whose mass spectrum was identical with that published for 24-methylenedihydrolanosterol (16).

The sterol esters isolated from a control culture or from a culture incubated for 16 h in the presence of 10^{-7} M ketoconazole were saponified, and the sterols were separated by gas-liquid chromatography and identified by mass spectrometry. The total ion chromatogram of the control culture showed two major sterol fractions. The mass spectrum of the first eluting sterol corresponded to that of ergosta-7,24 (28)-dien-3β-ol (episterol). The spectrum of the second major sterol corresponded to that of 4,4',14-trimethylcholesta-8, 24-dien-3β-ol (lanosterol). Two minor sterols with a 24-methylene side chain were detected. Complete elucidation of these structures was not possible. Analysis of the sterol fraction obtained from the treated culture showed no evidence of the sterols mentioned above. Three sterols were found. The first

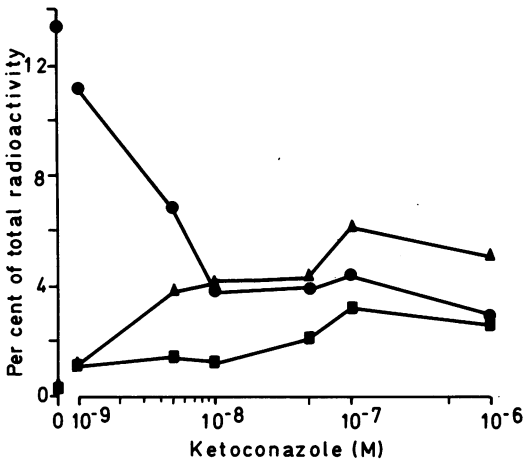

FIG. 4. *Effect of ketoconazole on the [^{14}C]acetate incorporation into sterols by C. albicans grown in CYG medium for 4 h. Solvent and ketoconazole were added to C. albicans cultures immediately after inoculation. The results presented are mean values of at least two experiments. Total radioactivity represents the radioactivity derived from [^{14}C]acetate incorporated into the lipid fraction of the cells. C4-desmethyl- (●), 4,14-dimethyl- (■), and 4,4',14-trimethyl- (▲) sterols.*

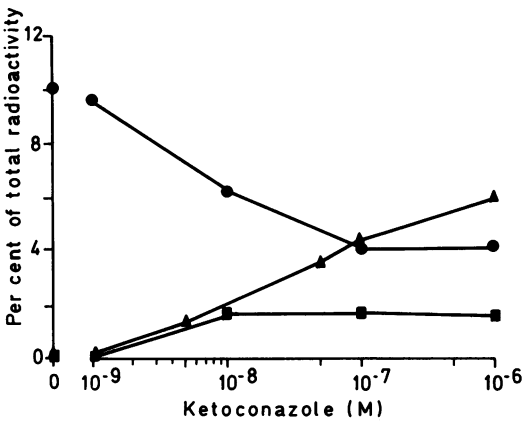

FIG. 5. *Effect of ketoconazole on the [^{14}C]acetate incorporation into sterols by C. albicans grown in CYG medium for 16 h. C4-desmethyl- (●), 4,14-dimethyl- (■) and 4,4',14-trimethyl- (▲) sterols. Further details are given in the legend to Fig. 4.*

TABLE 2. *Sterols present in C. albicans after 16 h of growth in the absence and presence of ketoconazole*[a]

Fractions	Ketoconazole (M)	Sterols		
		C4-desmethyl	4,14-Dimethyl	4,4',14-Trimethyl
Free sterols	0	Ergosterol	—	Lanosterol (tr)
	10^{-7}	14-Methylfecosterol	Obtusifoliol	Lanosterol 24-Methylenedihydrolanosterol
Sterolesters	0	Episterol[b]	—	Lanosterol
	10^{-7}	14-Methylfecosterol	Obtusifoliol	24-Methylenedihydrolanosterol

[a] *C. albicans* was grown in CYG medium for 16 h. Isolation and identification of sterols were by thin-layer chromatography, gas-liquid chromatography and mass spectrometry. —, Absent.

[b] Episterol, Ergosta-7,24 (28)-dien-3β-ol

eluting sterol could be identified as 14-methylfecosterol, the second was obtusifoliol, and the third was 24-methylenedihydrolanosterol. No ergosterol could be found.

(ii) *C. albicans* grown in keratin medium. No marked effect was found on the radioactivity present in lipid extracts of *C. albicans* exposed for the first 4 h of the lag phase to [^{14}C]acetate and to ketoconazole.

However, between control and ketoconazole-treated cells remarkable differences were evident in the radioactivity incorporated into different sterols (Fig. 6). The incorporation of acetate into the 4-desmethylsterols was significantly decreased at ketoconazole concentrations $\geq 10^{-9}$ M. Fifty percent inhibition was achieved at 4×10^{-9} M. Concomitantly, an increased incorporation of [^{14}C]acetate was noticed in the fractions containing 4,14-dimethyl- (obtusifoliol) and 4,4',14-trimethylsterols (lanosterol and 24-methylenedihydrolanosterol).

(iii) *C. albicans* grown in EMEM. Interference of ketoconazole with ergosterol synthesis was also observed when drug was added to media in which the yeast phase of *C. albicans* transformed to the mycelial phase. Table 3 presents the results obtained in EMEM after 24 h of incubation in the presence and absence of ketoconazole. The ketoconazole-induced inhibi-

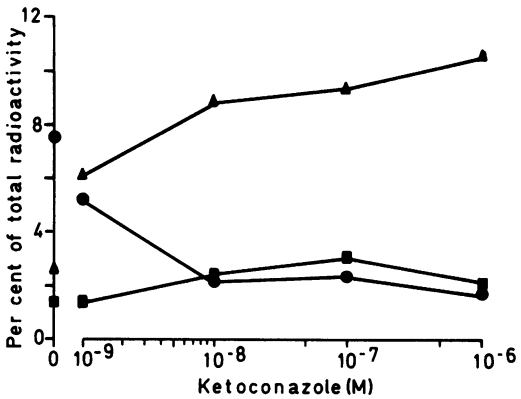

FIG. 6. *Effect of ketoconazole on the [^{14}C]acetate incorporation into sterols by C. albicans grown in keratin medium for 4 h. C4-desmethyl- (●), 4,14-dimethyl- (■), and 4,4',14-trimethyl- (▲) sterols. Further details are given in the legend to Fig. 4.*

TABLE 3. *Effect of ketoconazole on the [^{14}C]acetate incorporation into sterols by C. albicans grown in EMEM*[a]

Serum	Ketoconazole (M)	% of total radioactivity		
		C4-desmethyl	4,14-Dimethyl	4,4',14-Trimethyl
Absent	0	14.4	0.8	0.9
	2×10^{-9}	11.7	1.6	2.7
	4×10^{-9}	6.2	2.8	3.2
	10^{-8}	6.0	3.3	2.8
Present	0	18.4	0.5	0.5
	2×10^{-9}	15.4	0.6	0.8
	4×10^{-9}	10.8	1.7	2.6
	10^{-8}	7.1	3.0	3.0

[a] Results are mean values of at least three experiments. The ketoconazole concentrations given were added immediately after inoculation. Incubation time was 24 h.

TABLE 4. *Effect of ketoconazole on the [^{14}C]acetate incorporation into sterols by grown-out mycelium of C. albicans*[a]

Serum	Ketoconazole (M)	% of total radioactivity		
		C4-desmethyl	4,14-Dimethyl	4,4',14-Trimethyl
Absent	0	16.4	1.3	1.5
	2×10^{-9}	9.0	2.1	4.8
	4×10^{-9}	5.7	2.6	6.5
	10^{-8}	5.0	3.2	7.1
Present	0	19.8	0.6	0.8
	2×10^{-9}	18.5	0.8	1.0
	4×10^{-9}	9.3	1.8	2.8
	10^{-8}	6.2	2.9	4.1

[a] *C. albicans* B 12377 was inoculated in EMEM and grown for 24 h. Drug or solvent or both and [^{14}C]acetate were added, and cells were incubated for another 24 h. Results are mean values of at least three experiments.

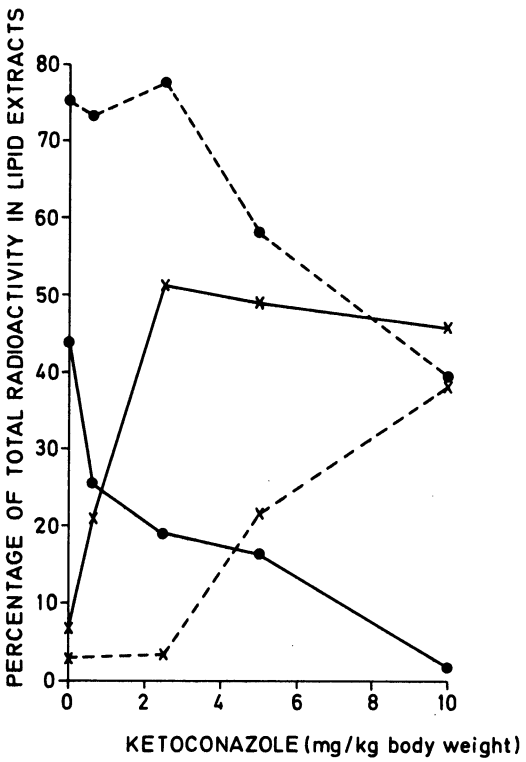

FIG. 7. *Effect of ketoconazole on sterol metabolism in vivo. C. albicans-infected rats were treated orally with ketoconazole or solvent and 2 h later were injected intraperitoneally with [^{14}C]mevalonate. Two hours later the rats were sacrificed and the lipids were extracted from C. albicans* (——) *and liver* (- - - - -). *Radioactivity was determined in the total lipid fraction, in the ergosterol or cholesterol* (●) *fraction, and in the fraction containing the 4,14-dimethyl- and 4,4',14-trimethylsterols* (×).

tion of the ergosterol synthesis was not affected by serum (Table 3).

Ketoconazole also is a potent inhibitor of the conversion of lanosterol into ergosterol when added to cultures containing outgrown mycelium (Table 4). However, under these circumstances a slight serum dependence was seen. Inhibition at the 50% level was achieved at a concentration of 2×10^{-9} and 4×10^{-9} M with and without serum (Table 4). The cholesterol of serum did not replace ergosterol. Ergosterol was the main sterol in control cells.

Effects on sterol metabolism in vivo. Rats were treated orally with 0, 0.63, 2.5, 5.0, or 10.0 mg of ketoconazole per kg of body weight 3 days after inoculation with *C. albicans*. Two hours later the rats were injected intraperitoneally with [^{14}C]mevalonate, and 2 h thereafter they were sacrificed. As shown in Fig. 7, there was a dose-dependent decrease in the radioactivity incorporated into the ergosterol of *C. albicans* which coincided with increased incorporation of radioactivity into 4,14-dimethyl- and 4,4',14-trimethylsterols (here fractionated together out of the lipid extract). The shift from ergosterol to C-14 methylated sterols was apparent at 0.63 mg/kg, reached the 50% level at 1.7 mg, and was almost complete at 10 mg/kg. At the latter dose there was a 50% decrease in incorporation of radioactivity into cholesterol into rat liver. Thus, as compared with *C. albicans*, almost six times more ketoconazole is needed to obtain a similar accumulation of radioactivity in C-14 methylated sterols of liver.

DISCUSSION

The experimental evidence presented indicates that the imidazole derivative, ketoconazole, is a potent inhibitor of ergosterol biosynthesis in *C. albicans* grown in culture media which support yeast or mycelial growth. In all circumstances investigated, the inhibition of ergosterol biosynthesis occurred before any measurable effect on growth. Of great interest is the interference of ketoconazole with ergosterol synthesis in fully developed mycelium, a form mostly encountered in vivo. Low doses of ketoconazole administered orally to rats artificially infected with *C. albicans* also affect the ergosterol synthesis in *Candida*. A six-times-higher dose is required to effect cholesterol synthesis by rat liver.

This greater inhibitory effect may be due at least partly to differences in the sterol metabolism of yeast and mammalian cells. Preliminary results indicate that the mevalonate incorporation into ergosterol by a $10{,}000 \times g$ supernatant fraction of yeast cells is much more sensitive to ketoconazole than the incorporation into cholesterol by a similar fraction isolated from rat liver (manuscript in preparation).

Under all circumstances studied, the ketoconazole-induced inhibition of the ergosterol biosynthesis that coincided with an accumulation of sterols with a methyl group at C-14 can thus be attributed to an interference with one of the reactions involved in the removal of the 14α-methyl group of lanosterol.

Studies of the effects of C-14 methyl sterols on properties of artificial membranes (6, 8, 10, 18), on growth of anaerobic yeast and certain mutant animal cell lines (5), and on the developmental process of pupating insects have shown that lanosterol is unable to replace 4-desmethylsterols (e.g., cholesterol) in sterol-requiring eucaryotic cells (2). For example, *Saccharomyces cerevisiae* kept under anaerobic conditions will not grow without a sterol supplement because in the absence of oxygen the cy-

lization of squalene cannot proceed. This requirement for an exogenous sterol is fully met by ergosterol and partly by cholesterol, whereas lanosterol sustained growth of such cells only marginally (2; Van den Bossche, unpublished data).

The presence of a 14α-methyl is the principal structural feature responsible for the disparate membrane effects of lanosterol and cholesterol (2). This was evidenced by studying the properties of artificial phospholipid vesicles as a function of sterol composition. By using glucose permeability as a parameter, it was shown that the incorporation of cholesterol into lecithin vesicles reduced the release of entrapped glucose from 50 to about 5%. However, when lanosterol replaced cholesterol in the vesicles, the exit of trapped glucose from the vesicles was delayed only slightly (10).

Based on all these studies, it is reasonable to speculate that the presence of sterols with a methyl group at C-14 in the ketoconazole-treated *C. albicans* may lead to functional changes in the membranes. Changes in the membranes may be at the origin of permeability changes, leaky membranes, inhibition of growth, and increased susceptibility of the invasive cells to the host defense system.

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