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BIOCHEMICAL EFFECTS OF MICONAZOLE ON FUNGI—I

EFFECTS ON THE UPTAKE AND/OR UTILIZATION OF PURINES, PYRIMIDINES, NUCLEOSIDES, AMINO ACIDS AND GLUCOSE BY *CANDIDA ALBICANS*

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Abstract—The antifungal and antibacterial drug miconazole has been shown to inhibit, at concentrations lower than those affecting growth, the transport of adenine, guanine and hypoxanthine by *Candida albicans* in suspension culture. The decrease in the incorporation of purines into nucleic acids seems to be the consequence of an inhibitory effect on their uptake into the cells. When the purines were replaced by adenosine, deoxyadenosine and guanosine, miconazole increased the uptake and incorporation of the radioactivity derived from the nucleosides into macromolecules. The data suggest that the drug-induced increase of nucleoside incorporation into nucleic acids is secondary to enhanced nucleoside transport. Miconazole also slightly affected the uptake of orotic acid. The transport of glucose, glycine and leucine was not affected by miconazole whereas in some way the drug affected glutamine uptake. Studies on the distribution of miconazole and/or its metabolites in the *Candida* cell indicate that in log-phase cells most of the radioactivity was found in the fraction containing cell walls and plasmalemma. In stationary-phase cells the highest radioactivity was found in the fraction which contained the microsomes. Although more information will be needed, the data presented indicate that at low concentrations, miconazole acts primarily on the yeast cell membranes (cell wall and plasmalemma) resulting in a selective inhibition of the uptake of precursors of RNA and DNA (purines) and mucopolysaccharide (glutamine). Higher doses and longer incubation periods also alter the activities of microsomal membranes.

MICONAZOLE (R 14889) is the generic name for 1[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazole nitrate. It is a white, microcrystalline or amorphous powder, very slightly soluble in water and moderately soluble in most organic solvents.

This β-substituted 1-phenethylimidazole has broad-spectrum activity against most pathogenic fungi, such as dermatophytes and *Candida albicans*, against numerous saprophytic fungi as well as against Gram-positive bacteria.[1–5]

The present paper describes effects of miconazole on the uptake and/or utilization of purines, pyrimidines, purine nucleosides, amino acids and glucose by *Candida albicans*. The distribution of miconazole in the *Candida* cell was also determined. The purpose of this study was to formulate a hypothesis on the mode of action of this antifungal agent.

MATERIALS AND METHODS

Materials. Reagents used in the present work were obtained from the following sources: neopeptone, yeast nitrogen base and glucose from Difco (Detroit, U.S.A.);

casein hydrolysate and glycine from Merck (Darmstadt, Germany); L-glutamine from Serva (Heidelberg, Germany); adenine, adenosine, 2′-deoxyadenosine, hypoxanthine and guanosine-5′-phosphate from Boehringer (Mannheim, Germany); orotic acid from Aldrich-Europe (Beerse, Belgium); L-leucine, guanine and guanosine from Calbiochem (Lucerne, Switzerland); DNA from calf thymus, Type V, RNA from yeast, Type XI, 2′-, 3′- and 5′-adenosine-5′-phosphate from Sigma (St. Louis, U.S.A.); adenine-8^{3}H, hypoxanthine-8^{14}C, 2′-deoxyadenosine-^{14}C (U), guanosine-8^{3}H, orotic acid-5^{3}H, L-glutamine-^{14}C (U), glycine-^{14}C (U) and D-glucose-^{14}C (U) were obtained from the Radiochemical Centre (Amersham, U.K.); guanine-8^{14}C from SCK (Mol, Belgium); adenosine-8^{3}H from Schwarz-Mann (Orangeburg, N.Y., U.S.A.).

Media. The following media were used: Sabouraud broth composed of 10 g of neopeptone and 20 g of glucose per litre; Sabouraud agar is similar to Sabouraud broth with the exception that 25 g of agar was added per litre. CYG-medium containing casein hydrolysate (0·5%), yeast extract (0·5%) and glucose (0·5%); Neopeptone medium (NG) composed of 10 g of neopeptone and 5 g of glucose per litre; YNBG-medium containing 6·7 g of yeast nitrogen base and 40 g of glucose per litre.

Strain. Stock cultures of *Candida albicans* (strain R. V. 4688) were maintained at 25° on Sabouraud agar slants.

Yeast cell inocula. To prepare inocula for experimental studies, cells were grown statically at 37° for 24 hr on Sabouraud agar. A loopful of this culture was used to inoculate 4·5 ml of Sabouraud broth. Cells were grown at 37° for 24 hr and 1·0 or 0·5 ml aliquots (about 40 × 10^{6} cells/ml) of this culture were used to inoculate 100 ml of the above mentioned media.

Growth studies. For all growth experiments cells were grown at 37° aerobically by shaking. Miconazole was dissolved in 50% ethanol and added to the medium. Controls were similarly set up with equivalent quantities of ethanol (final concentration 0·01%). The growth rate was followed turbidimetrically at 546 nm. These readings were converted to mg of dry weight/ml by means of a standard curve.

Uptake and Incorporation studies. Media inoculated with *C. albicans* were supplemented with either purines, pyrimidines, nucleosides, amino acids or glucose as shown in Table 1. These cultures were incubated in the presence of miconazole or ethanol in a shaking water bath at 37°.

(a) Separation of the cold acid-soluble and -insoluble fractions: 10-ml aliquots of the cultures were removed at intervals. The cells were harvested by centrifugation at 3860 *g* for 10 min and the packed cells washed with cold 0·2 M of sodium phosphate buffer (pH 7·4). The cells were suspended in 5 ml of cold 5% (w/v) trichloroacetic acid (TCA). Ten g of glass beads (dia: 0·45 mm) were added and the cells broken in a Braun cell homogenizer (Model MSK) for 3 × 1 min. The glass beads were separated and washed with 5 ml of 5% TCA. The homogenate was centrifuged at 8590 *g* for 10 min. The precipitated material was washed with cold TCA and centrifuged. The supernatants were combined to yield the cold acid-soluble fraction (Pool). The residual material (DNA, RNA and protein) was mixed well with 2 ml of TCA on a Vortex stirrer. One ml of both the pool and the acid-insoluble fraction were mixed with 10 ml of scintillator solution (Insta Gel, Packard) and the radioactivity determined using a Packard Tri-Carb Liquid Scintillation Spectrometer. Correction for quenching was applied by internal standardization.

TABLE 1. PURINES, PYRIMIDINES, NUCLEOSIDES, AMINO ACIDS AND GLUCOSE ADDED TO CULTURES OF *C. albicans*

Compound	Concn in medium ($\times 10^{-6}$ M)	Sp. act. (mCi/mM)
[8^{3}H]Adenine	7·40	130·21
[8^{14}C]Guanine	4·27	11·67
[8^{14}C]Hypoxanthine	7·34	3·21
[5^{3}H]Orotic acid	5·74	36·06
[8^{3}H]Adenosine	3·40	72·48
[^{14}C]Deoxyadenosine (U)	41·80	27·68
[8^{3}H]Guanosine	1·60	312·00
[^{14}C]L-Glutamine (U)	68·44	0·723
[^{14}C]Glycine (U)	133·31	114·00
[^{14}C]L-Leucine (U)	76·27	0·314
[^{14}C]D-Glucose (U)	27,740·00	0·901

(b) Separation of nucleic acids from pool and protein: cells were harvested, washed with cold 0·2 M of sodium phosphate buffer (pH 7·4), homogenized in 10 ml of water as described under (a). Pool-, RNA-, DNA- and protein fractions were separated by the Schmidt and Thannhauser[6] method as modified by Dabrowa *et al.*[7]

Thin-layer chromatography. The alkaline hydrolysates of RNA and the purines and nucleoside 5′-monophosphates present in the Pool were separated, one—dimensionally on poly (ethyleneimine)—cellulose mounted on aluminium sheets (PEI-cellulose sheets were obtained from Merck, Darmstadt, Germany), by stepwise elution with acetic acid (1 M) and LiCl (0·3 M).[8] Prior to application to the sheets the mixtures were neutralized. Standards were run at the same time and were located on the chromatograms by examining the layers under incident short-wave u.v. light. Migration rates of the compounds decrease as follows: adenine > hypoxanthine > 5′-AMP = 2′-AMP ⩾ 3′-AMP > 5′-GMP > ADP.

Distribution of Miconazole in the Candida *cell. Candida* were grown in CYG-medium to which $2{\cdot}08 \times 10^{-7}$ M of miconazole-^{3}H was added. After 7 or 24 hr of incubation the cells were collected on Millipore filters, washed, suspended in H_2O and broken in a Braun cell homogenizer for 25 min. The homogenate was separated into a 16,000 *g* pellet (P1), a 100,000 *g* pellet (P2) and a soluble fraction (S) as described by Gilbert.[9]

Determination of DNA, RNA and protein. DNA was estimated in the TCA-extract by incubation of 1 ml of the extract with 1 ml of 2 M $HClO_4$ and 2 ml of diphenylamine reagent prepared according to Giles and Myers.[10] The mixtures were incubated for 15 hr at 30° and the difference between the absorbance at 595 and 700 nm determined.[10] DNA from calf thymus was heated at 70° for 15 min in the presence of 2 ml $HClO_4$ (2 M). One ml of this extract (containing 0·3 mg/ml) was used as standard. The RNA content was determined by the orcinol procedure as described by Hatcher and Goldstein.[11] Yeast RNA was used as standard. Protein was determined by the Folin-phenol method of Lowry *et al.*[12]

RESULTS

Effect of miconazole on the uptake and incorporation of purines by C. albicans. As can be seen in Fig. 1 concentrations of miconazole, lower than those affecting growth,

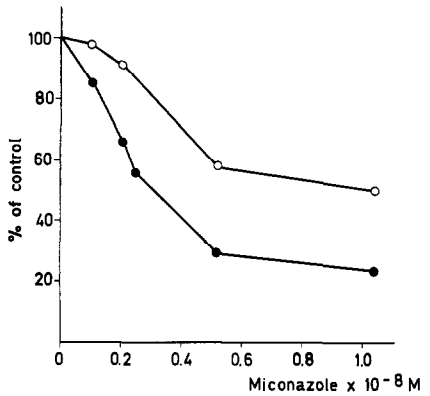

FIG. 1. Effect of miconazole on the growth of *C. albicans* (○) and incorporation of adenine into TCA-insoluble material (●). Cultures were incubated for 7 hr in the presence of miconazole and [8^3H]adenine. The optical density was measured. The cells were then harvested and fractionated as described in methods.

significantly inhibited the incorporation of adenine into trichloroacetic acid-insoluble material (TCA-insoluble). The addition of 5 ng miconazole/ml of incubation mixture ($1{\cdot}04 \times 10^{-8}$ M) to 3- and 4-hr-old cultures did not significantly affect the amount of cells present 3 and 4 hr later (Table 2). However, in both cases the levels of adenine incorporated into TCA-insoluble material were markedly lower than those found in the controls. The results presented in Table 2 also indicate that addition of the drug prior to the purine did not result in a higher inhibition of the incorporation. When miconazole was added to log-phase cells (5-hr-old) no effect was observed either on growth or on the incorporation of adenine. Table 2 also shows that miconazole influenced the size of the Pool of TCA-soluble material derived from adenine.

Miconazole interfered with the incorporation of guanine (Fig. 2c) and hypoxanthine (Fig. 2d). It should be noted that the Pool derived from guanine was much

TABLE 2. EFFECT OF MICONAZOLE ON THE ADENINE UPTAKE AND INCORPORATION INTO COLD TCA-SOLUBLE AND -INSOLUBLE MATERIAL IN *Candida albicans**

Time of addition (hr)		Radioactivity (b) (cpm/ml cells)				Growth† (mg dry wt/ml culture)	
		Pool		TCA-insoluble			
M	Ad.	C	M	C	M	C	M
0	0	2426	649 (26·7)	12,474	2906 (23·3)	0·80	0·30 (37·5)
0	3	2182	642 (29·4)	12,592	3514 (27·9)	0·66	0·29 (43·9)
3	3	2471	1267 (51·3)	8897	3960 (45·1)	0·63	0·50 (79·3)
3	4	2559	767 (29·9)	12,943	4726 (36·5)	0·82	0·61 (74·4)
4	4	1582	601 (38·0)	9465	5936 (62·8)	0·90	0·85 (94·4)
5	5	1137	1237 (108·8)	14,979	14,988 (100·0)	1·01	1·04 (102·9)

*All cells were collected 7 hr after inoculation in CYG-medium.
M = miconazole ($1{\cdot}04 \times 10^{-8}$ M); Ad. = adenine; C = control.
† Figures in parenthesis are percentage of controls.

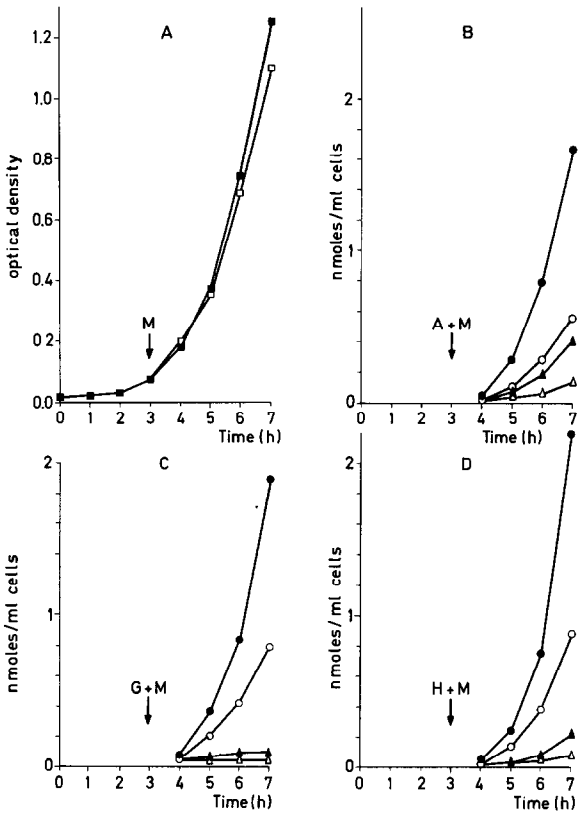

FIG. 2. Time course of effect of miconazole ($1{\cdot}04 \times 10^{-8}$ M) on growth (■, □) of *Candida* (A), on the incorporation of adenine (B), guanine (C) and hypoxanthine (D) (●, ○) and on the pool of TCA-soluble material derived from the purines (▲, △). The solid squares, circles and triangles represent the controls. Arrows indicate the addition of miconazole and purines.

TABLE 3. EFFECT OF MICONAZOLE ON THE UPTAKE OF ADENINE AND ON INCORPORATION OF RADIOACTIVITY FROM [8^3H]ADENINE INTO DNA AND RNA OF *C. albicans**

	Adenine (nmoles/mg protein per 7 hr)		
		Miconazole‡	
Fraction†	Control	A	B
H	28·50	20·32	13·06
P	3·73	2·91	1·89
RNA	24·00	18·57	11·36
DNA	1·04	0·77	0·42

* Miconazole and adenine were added to CYG-media immediately after inoculation; 7 hr later the radioactivity present in the different fractions was measured.

† H = homogenate; P = Pool.

‡ A = $4{\cdot}16 \times 10^{-9}$ M; B = $8{\cdot}32 \times 10^{-9}$ M miconazole.

TABLE 4. CHROMATOGRAPHY OF POOL-AND RNA-FRACTION FROM CELLS LABELLED WITH [8^3H]ADENINE IN THE PRESENCE AND ABSENCE OF MICONAZOLE*

Fraction	Miconazole ($\times 10^{-8}$ M)	Total ^{3}H (cpm/ml cells)	Per cent of total†			
			GMP	AMP	Hypoxanthine	Adenine
Pool	0	3604	3·3	10·1	6·2	64·4
	1·04	1904	4·4	14·5	6·9	55·2
RNA	0	3596	7·4	43·6	9·8	16·8
	1·04	1206	10·3	38·2	10·6	14·6

* *C. albicans* were harvested 7 hr after inoculation in CYG-medium to which 7·4 μM [8^3H]adenine was added. The cold acid-soluble (Pool) and the RNA-fraction were isolated, the radioactivity determined (Total ^{3}H) and analysed chromatographically.

† The radioactive spots found on the chromatogram were designated GMP, AMP, Hypoxanthine and Adenine since their R_f values corresponded to those of nucleotides and purines run at the same time.

smaller than that obtained from adenine (Fig. 2b) and hypoxanthine. This may indicate that most of the labelled guanine taken up by the cell was incorporated directly into the cold TCA-insoluble material (DNA and RNA). The results shown in this figure clearly indicate that the effects of miconazole on growth (Fig. 2a) were much smaller than those on the incorporation of the three purines studied.

The effects of the drug on the uptake of [8^3H]adenine into the cells (homogenate) and on the amount of radioactivity derived from this purine in the Pool-, DNA- and RNA-fractions over a 7 hr period are shown in Table 3. The data presented here indicate that the incorporation of the [^{3}H]labelled purine into total cell material (H) was reduced to about the same extent as its incorporation into Pool, RNA and DNA. The small amount of adenine found in the DNA-fraction reflected the low DNA-content of *Candida* compared with the RNA-content of these cells. The mean values of five determinations of the DNA-content and RNA-content in 7-hr-old cultures were 8·84 and 112·35 μg/ml cells. Chromatographic analysis of Pool- and RNA-fractions from cells labelled with [8^3H]adenine in the absence or presence of miconazole ($1{\cdot}04 \times 10^{-8}$ M) indicate that, although miconazole decreased the total radioactivity found in Pool- and RNA-fractions, it had no effect on the relative distribution of label among the intra-cellular purines and nucleotides found in both fractions (Table 4). It should be noted that the Pool still contained significant amounts of adenine.

In the experiment illustrated in Fig. 3 two suspensions of *Candida* cells in CYG-medium supplemented with $1{\cdot}04 \times 10^{-8}$ M of miconazole were incubated together with two controls at 37° for 4 hr. One treated and one untreated culture were put on ice whereas the cells of the two other cultures were collected, washed and resuspended in fresh CYG-medium. All cultures were then supplemented with [8^3H]adenine, incubated at 37° and at various times samples of each suspension were analysed. The data presented here indicate that the incorporation of adenine into the TCA-soluble and -insoluble material was inhibited by miconazole to about the same extent whether or not the cells were washed prior to adenine addition.

Effect of miconazole on the uptake and incorporation of purine nucleosides. When adenine and guanine were replaced by their nucleosides, adenosine (Fig. 4a) and guanosine (Fig. 4b), miconazole induced an increased uptake and incorporation. Four hr after the addition of miconazole and [8^3H]adenosine or [8^3H]guanosine to 3-hr-old cultures, the amount of radioactivity in the TCA-insoluble material was

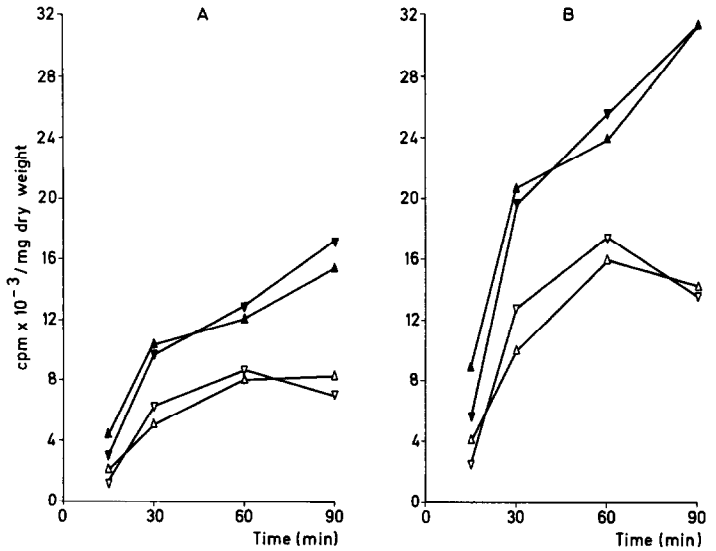

FIG. 3. Effect of miconazole ($1{\cdot}04 \times 10^{-8}$ M) on adenine incorporation into acid-soluble (A) and -insoluble material (B). Two cultures were supplemented with miconazole and incubated with 2 controls at 37° for 4 hr. The cells of one treated and one untreated culture were collected, washed and resuspended in fresh medium. All cultures were then supplemented with [8^3H]adenine ($7{\cdot}4 \times 10^{-6}$ M), incubated at 37° and at various times samples of each culture were collected and monitored for radioactivity in acid-soluble and -insoluble material. (▽) cells washed free of miconazole; (△) without washing. Solid triangles represent the controls.

2·5 and 1·3 times higher than that found in the controls. Further examination of the graphs, presented in Figs. 2 and 4, indicates that the uptake of these nucleosides was only one tenth of that observed with the purines.

The effect of miconazole on the uptake of [8^3H]adenosine and on the incorporation of radioactivity from this nucleoside into DNA and RNA of *C. albicans* is shown in Table 5. Since the results indicate that acid-soluble radioactivity (Pool) and radioactivity in DNA- and RNA-fractions were increased to about the same extent it can be suggested that the drug-induced increase in the nucleoside incorporation into nucleic acids was secondary to enhanced adenosine transport.

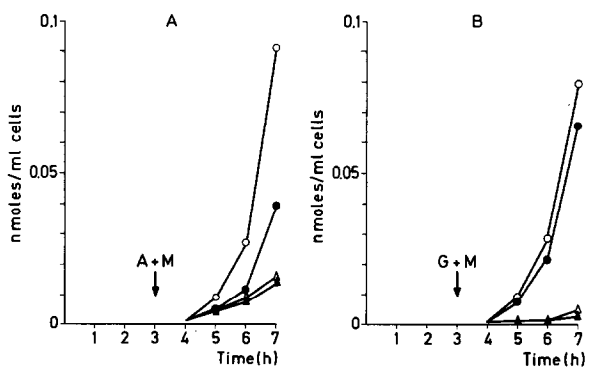

FIG. 4. Time course of effect of miconazole ($1{\cdot}04 \times 10^{-8}$ M) on adenosine (A) and guanosine (B) incorporation into acid-soluble (△) and -insoluble material (○). Solid triangles and circles represent the controls. Arrows indicate the addition of miconazole and nucleosides.

TABLE 5. EFFECT OF MICONAZOLE ON THE UPTAKE OF [8^3H]ADENOSINE AND ON INCORPORATION OF RADIOACTIVITY FROM THIS NUCLEOSIDE INTO DNA AND RNA OF *C. albicans**

	Adenosine (nmoles/mg protein per 4 hr)		
Fraction†	Control	A	B
H	0·994	1·581	2·490
P	0·137	0·261	0·450
RNA	0·793	1·163	2·220
DNA	0·017	0·031	0·030

* Miconazole (A = 0·521 and B = $1·04 \times 10^{-8}$ M) and adenosine ($3·40 \times 10^{-6}$ M) were added to 3 hr-old cultures. Four hr later the radioactivity in the different fractions was measured. Results are expressed as nmoles of adenosine incorporated or absorbed.

† H = homogenate; P = Pool.

When *Candida* was grown in Neopeptone–glucose medium to which miconazole and 2'-deoxyadenosine-8^3H were added, the acid-soluble and acid-insoluble radioactivity were also increased compared with the control values (Fig. 5a) although the growth was inhibited by about 50 per cent (Fig. 5b). However, the results obtained with this deoxynucleoside differ from those obtained with adenosine in that the amount of radioactivity recovered in the pool was higher than that found in the acid-insoluble fraction.

Effect of miconazole on the uptake and incorporation of pyrimidines. The results shown in Table 6 indicate that extremely low amounts of orotic acid were absorbed by *C. albicans*. After exposure to $1·04 \times 10^{-8}$ M of miconazole for periods as long as 4 hr, the amount of radioactivity, derived from [5^3H]orotic acid, in the acid-soluble (Pool) and in the RNA- and DNA-fraction was still about 70 per cent of that

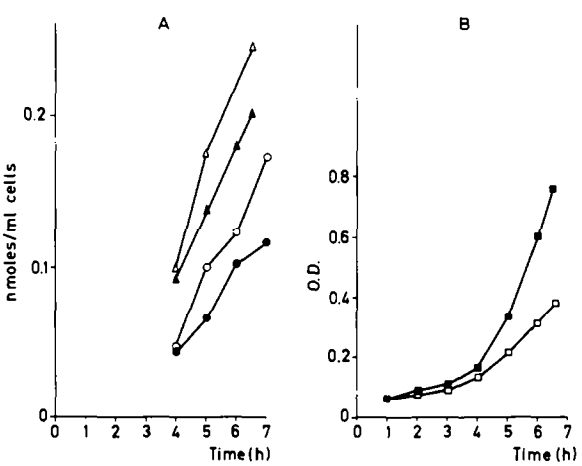

FIG. 5. Time course of effect of miconazole on 2'-deoxyadenosine incorporation into acid-soluble and -insoluble material (A) and on growth of *Candida* (B). Further details are presented in the legends to Figs. 2 and 4.

TABLE 6. EFFECT OF MICONAZOLE ON THE UPTAKE OF OROTIC ACID AND ON INCORPORATION OF RADIOACTIVITY FROM [5^3H]OROTIC ACID INTO DNA AND RNA OF *C. albicans**

Fraction†	Orotic acid (pmoles/mg dry wt per 4 hr) Control	Miconazole	Percentage of control
H	9·22	7·34	79·61
P	1·64	1·19	72·56
RNA	9·35	7·16	76·57
DNA	0·25	0·17	68·00

* Miconazole ($1{\cdot}04 \times 10^{-8}$ M) and orotic acid ($5{\cdot}7 \times 10^{-6}$ M) were added to a 3 hr-old culture (CYG-medium). Four hr later the radioactivity in the different fractions was measured.

† H = homogenate: P = Pool.

observed in controls. This indicates that the inhibitory effect of miconazole on the orotic acid uptake was much less pronounced than that on the adenine uptake.

Effects on the uptake and utilization of glucose and amino acids. The results shown in Table 7 indicate that the effect of miconazole on the accumulation of radioactivity derived from glucose, glycine and leucine in the Pool- and acid-insoluble fractions was not greater than that on growth. The incorporation of radioactivity derived from [^{14}C]L-glutamine into the acid insoluble fraction was somewhat more affected than growth when *Candida* was grown in CYG-medium and Neopeptone–glucose medium. In the latter medium the accumulation of ^{14}C in the Pool seemed to be

TABLE 7. EFFECT OF MICONAZOLE ON GROWTH AND ON THE ACCUMULATION OF SUBSTRATES AND DERIVATIVES IN THE POOL-AND ACID-INSOLUBLE FRACTIONS OF *C. albicans**

Substrate	Media†	Percentage of Control† Growth	Accumulation Pool	Acid-insoluble fraction
D-Glucose	CYG	63·2	64·9	62·7
Glycine	CYG	61·8	87·0	80·6
	NG	62·1	71·2	70·4
	YNBG	74·0	98·0	81·0
L-Glutamine	CYG	50·5	51·9	44·2
	NG	55·3	44·1	45·3
	YNBG	87·5	96·2	94·3
L-Leucine	CYG	63·7	74·3	86·7
	NG	55·6	49·9	52·7
	YNBG	72·0	82·0	73·0

* Miconazole ($1{\cdot}04 \times 10^{-8}$ M) and the substrates were added to the media immediately after inoculation with *Candida*; 6 hr later the radioactivity in the acid-soluble- (Pool) and acid-insoluble fractions was measured.

† CYG = casein hydrolysate + yeast extract + glucose; NG = neopeptone + glucose; YNBG = yeast nitrogen base + glucose.

‡ Mean values of at least duplicate determinations.

also more affected. No effect on glutamine uptake could be observed when the cells were grown in yeast nitrogen base glucose (YNBG) medium. It should be noted that in this medium miconazole affected growth much less than in the other media. This medium contains much more ammonia than the Neopeptone medium and is supplemented with histidine and tryptophan. The latter amino acid is also present in the Neopeptone medium. Since glutamine is a donor of nitrogen atoms for the synthesis of histidine it was thought that the effect of miconazole on the glutamine uptake was counteracted by histidine. However, the addition of histidine (1 mg/ml of incubation mixture) to the neopeptone medium did not affect the miconazole-induced inhibition of the glutamine uptake.

Distribution of miconazole and/or metabolites, in different fractions. The distribution of the radioactivity, derived from [^{3}H]miconazole, in different fractions of *Candida* cells 7 and 24 hr after incubation of the cells in CYG-medium is shown in Table 8. The results obtained indicate that in the log-phase cells (7-hr-old cultures) most of the radioactivity was found in the 16,000 *g* pellet (P1), whereas during the stationary phase (24 hr-old culture) the highest radioactivity was encountered in the 100,000 *g* pellet (P2). Electron microscopy revealed that the first pellet (P1) was composed almost exclusively of cell walls contaminated with fragments of plasmalemma, whereas the second pellet seemed to contain the microsomes. Preliminary results indicate that the latter fraction contained the cytochrome b-5 reductase (EC 1.6.2.2) an enzyme that in mammalian tissues participates in microsomal electron transport.[13] This finding supports the microsomal nature of this pellet.

DISCUSSION

The utilization of purines is usually a two-step process: the active uptake of the base into a pool of purine bases, and the conversion into the ribonucleotide.[14] Purine phosphoribosyltransferases have been found in yeast by Kornberg *et al.*,[15] i.e. an adenine (a)—(EC 2.4.2.7) and a hypoxanthine (b)—phosphoribosyltransferase (EC 2.4.2.8) which catalyse the following reactions:

$$\text{adenine} + \text{PRPP} \xrightarrow{(a)} 5'\text{-AMP} + \text{PPi}$$

$$\text{hypoxanthine} + \text{PRPP} \xrightarrow{(b)} 5'\text{-IMP} + \text{PPi}$$

$$(\text{guanine}) \qquad\qquad (5'\text{-GMP}).$$

Reactions in which purine bases react with ribose-1-phosphate to form nucleosides, seem to be absent in yeast.[16]

Adenosine can be converted into its 5'-nucleotide by an adenosine kinase (EC 2.7.1.20) following the reaction:

$$\text{adenosine} + 5'\text{-ATP} \rightarrow 5'\text{-AMP} + 5'\text{ADP}.$$

The above mentioned reactions indicate that free bases and nucleoside utilization might not involve the same mechanisms. Since miconazole irreversibly decreased the uptake of purines whereas it enhanced the uptake of nucleosides, it is possible that the drug competes with purines for phosphoribosyl pyrophosphate (PRPP) or for purine phosphoribosyltransferases. The increased utilization of nucleosides may present the cell a possibility to "by-pass" the inhibitory effect on purine phosphorylation. This is of course true when purines are present in the medium. The uptake of

TABLE 8. DISTRIBUTION OF MICONAZOLE IN THE *Candida* CELL

Incubation time (hr)	Percentage of Total*		
	P1	P2	S
7	75·4 ± 14·1	10·5 ± 6·7	14·2 ± 7·4
24	18·9 ± 2·1	57·1 ± 7·3	21·9 ± 8·3

* Results are expressed as a percentage of the total amount of miconazole taken up by the cells. *Candida* were grown in CYG-medium in the presence of $2{\cdot}08 \times 10^{-7}$M[^{3}H] miconazole. The figures given are mean values of three determinations ± S.D. P1 and P2 are the pellets obtained after centrifugation of the homogenates for 7 min at 16,000 *g* and 60 min at 100,000 *g* respectively. S is the supernatant of the 100,000 *g* centrifugation.

deoxyadenosine was also increased when *Candida* was grown in media from which purines were omitted (Neopeptone medium). Therefore the increased uptake of nucleosides may also have been due to the inhibition of nucleotide synthesis from endogenous purines.

Harris and Thompson[17] found a link between the uptake of adenine and glucose metabolism in yeast, which may have been due to a combination of the purine with a dissimilation product of glucose, possibly PRPP, to form nucleotides. The fact that miconazole did not affect glucose uptake makes it doubtful that the decreased purine uptake should have been the result of a decreased PRPP synthesis.

The studies of Sarachek[18] on *C. albicans* clearly indicate that adenine must be freed from adenosine as well as from deoxyadenosine before utilization. The entry of the adenine moiety of adenosine and of deoxyadenosine into cellular purine metabolism will be achieved by a reaction catalysed by a nucleosidase (EC 3.2.2.1):

$$\text{adenosine} + H_2O \rightarrow \text{adenine} + \text{D-ribose}$$

Thus the different effects of miconazole on the adenine and adenosine uptake can not be due to the utilization of different mechanisms for the formation of 5′-AMP.

Based on the above mentioned studies it is believed that the decreased incorporation of purines and of orotate did not result from an interference of miconazole with PRPP or with the phosphoribosyltransferases. Since miconazole failed to exhibit an effect on glycine uptake and only slightly and under certain conditions affected the glutamine uptake, it may be suggested that miconazole does not interfere with the synthesis of nucleotides.

The results of chromatographic analyses of pool- and RNA-fraction described in this study indicate that the drug-induced decrease in incorporation of purines into nucleic acids may be a consequence of an inhibition of the rate of absorption of the purines by *C. albicans*. The fact that our results indicate that miconazole is bound to the cell membranes further points at a possible interference with transport systems.

The effects of miconazole on purine uptake were observed in log-phase cells (up to 7 hr of cultivation) at a concentration between 10^{-9} and 10^{-8} M. Preliminary results indicate that increasing the drug concentration up to 4×10^{-7} M and prolonging the cultivation time up to 24 hr resulted in an almost complete inhibition of the cytochrome b-5 reductase. Since miconazole was found in the 100,000 *g* pellet and inhibited the cytochrome b-5 reductase it may be assumed that miconazole also

affects microsomal membranes. Although there is no reason to doubt that the endoplasmic reticulum plays an important role in bud formation and also seems to be involved in cell wall synthesis[19] we need more precise information in order to correlate the observed inhibitory effect and localization of miconazole with the fungicidal action of this compound.

The effect of miconazole on transport systems must be selective. This can be concluded from the different effects on purine and nucleoside transport systems and also from the fact that it did not affect the uptake of glucose, glycine and leucine. The inability to affect the uptake of the latter amino acids may indicate that miconazole does not interfere with protein synthesis. One would expect that the combination of purines with a pentose to form nucleosides would markedly change their permeability. These differences may well explain the different effects of miconazole on purine and nucleoside uptake.

An effect on adenine uptake is of course disastrous for adenine requiring mutants. However, in wild strains of yeast, the purine biosynthetic pathway appears to be identical with that described for other microorganisms and animal cells.[16] The presence of moderate amounts of exogenous purines in the culture medium represses purine synthesis *de novo* in *Escherichia coli*[20,21] ascites tumor cells,[22] *Schizosaccharomyces pombe*[23] and also in soluble supernatant solutions of pigeon liver homogenates.[24] Since a feedback control of purine formation *de novo* operates in a great number of organisms it seems reasonable to assume that it also operates in *C. albicans*. Therefore, in media where purines are present, a decreased uptake of purines can affect growth.

Miconazole in some way also affected glutamine uptake. This amide plays an important role in cell metabolism as a donor of nitrogen atoms for the synthesis of amino acids, nucleotides and glucosamine-6-phosphate. The latter hexosamine is a precursor of mucopolysaccharide, glycoproteins and bacterial cell-wall polysaccharides. Although more experiments are needed to establish the effects of miconazole on glutamine uptake and/or utilization it is clear that an interference with the uptake of this amide can be involved in the mode of action of miconazole.

In conclusion it can be said that at low concentrations miconazole appears to act primarily on the yeast cell membranes inducing selective permeability changes. Higher doses and longer incubation periods also seem to alter the activities of the endoplasmic reticulum.

Biochemical and electron microscopic studies to test the hypothesis that the membranes are the sites of the critical action of miconazole are in progress.

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REFERENCES

1. E. F. GODEFROI, J. HEERES, J. VAN CUTSEM and P. A. J. JANSSEN, *J. med. Chem.* **12,** 784 (1969).
2. J. M. VAN CUTSEM and D. THIENPONT, *Chemotherapy* **17,** 392 (1972).
3. J. P. BRUGMANS, J. M. VAN CUTSEM and D. THIENPONT, *Arch. Derm.* **102,** 423 (1970).
4. A. A. BOTTER, *Mykosen* **14,** 187 (1971).
5. P. GODTS, P. VERMYLEN and J. VAN CUTSEM, *Arzneimittelförsch.* **21,** 256 (1971).

6. G. SCHMIDT and S. J. THANNHAUSER, *J. biol. Chem.* **161,** 83 (1945).
7. N. DABROWA, D. H. HOWARD, J. W. LANDAU and Y. SCHECHTER, *Sabouraudia* **8,** 163 (1970).
8. K. RANDERATH and E. RANDERATH, in *Methods in Enzymology* (Eds. L. GROSSMAN and K. MOLDAVE), Vol. 12A, p. 339. Academic Press, New York (1967).
9. D. GILBERT, *Biochem. Pharmac.* **21,** 2933 (1972).
10. K. W. GILES and A. MYERS, *Nature, Lond.* **206,** 93 (1965).
11. D. W. HATCHER and G. GOLDSTEIN, *Anal. Biochem.* **31,** 42 (1969).
12. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
13. P. STRITTMATTER, in *Biological Oxidations* (Ed. T. P. SINGER), p. 171. Interscience Publishers, New York (1968).
14. W. R. PICKERING and R. A. WOODS, *Biochim. biophys. Acta* **264,** 45 (1972).
15. A. KORNBERG, I. LIEBERMAN and E. S. SIMMS, *J. biol. Chem.* **215,** 417 (1955).
16. H. DE ROBICHON-SZULMAJSTER and Y. SURDIN-KERJAN, in *The Yeasts* (Eds. A. H. ROSE and J. S. HARRISON), Vol. 2, p. 338, Academic Press, London (1971).
17. G. HARRIS and C. C. THOMPSON, *Biochim. biophys. Acta* **56,** 293 (1962).
18. A. SARACHEK, *Antonie van Leeuwenhoek* **30,** 289 (1964).
19. PH. MATILE, H. MOOR and C. W. ROBINOW, in *The Yeasts* (Eds. A. H. ROSE and J. S. HARRISON), Vol. 1, p. 242. Academic Press, London (1969).
20. M. L. MAY and A. L. KOCH, *Archs Biochem. Biophys.* **109,** 330 (1965).
21. J. S. GOTS and J. GOLDSTEIN, *Science N.Y.* **130,** 622 (1959).
22. J. F. HENDERSON, *J. biol. Chem.* **237,** 2631 (1962).
23. J. E. CUMMINS and J. M. MITCHISON, *Biochim. biophys. Acta* **136,** 108 (1967).
24. J. B. WIJNGAARDEN, H. R. SILBERMAN and J. H. SADLER, *Ann. N.Y. Acad. Sci.* **75,** 45 (1958).