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The Lipid Composition of Azole-sensitive and Azole-resistant Strains of *Candida albicans*

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The lipid compositions of two azole-sensitive (A and B2630) and two azole-resistant (AD and KB) strains of the opportunistic fungal pathogen *Candida albicans* were studied by using several lipid extraction procedures: no differences were observed between the lipid content or total phospholipid/neutral lipid ratios of the four strains. All contained phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylserine as major phospholipids, with smaller amounts of phosphatidylglycerol and diphosphatidylglycerol; the relative proportions of these lipids differed between all four strains. The fatty acid composition of each major phospholipid within each strain differed, and there were also interstrain differences. A marked effect of culture growth phase in batch culture on lipid composition was observed. The major neutral lipids in each strain were triacylglycerol, non-esterified sterol and non-esterified fatty acid. The fatty acid compositions of the three fatty-acid-containing neutral lipids were distinct from each other and the phospholipids, and there were also interstrain differences. All strains possessed (lyso)phospholipase activity, which was non-specific. The proportions of triacylglycerol and non-esterified fatty acid did not vary between strains, but the azole-resistant strains AD and KB contained more non-esterified sterol, giving them a phospholipid/sterol ratio approximately half that of azole-sensitive strains. There appeared to be a relationship between the phospholipid/sterol ratio of exponentially growing sensitive strains and their ability to take up azole; this did not extend to the resistant strains, which either did not take up azole (AD and KB) or took it up at a faster rate (Darlington) than sensitive strains.

INTRODUCTION

Candida albicans is a widespread and troublesome opportunistic pathogen that causes a variety of superficial and deep-seated mycoses (Odds, 1979). Of the relatively few antifungal antibiotics available, those most commonly used to treat candidosis are the polyenes and the more recently introduced imidazoles (Speller, 1980). Polyenes work by complexing with membrane sterols (Hamilton-Miller, 1973). The primary action of imidazoles is probably inhibition of ergosterol synthesis that leads to an accumulation of 14α-methyl sterols which disrupt membrane structure and function (Van den Bossche *et al.*, 1982, 1983). However, imidazole actions are complex: they also inhibit a number of other yeast (and mammalian) membrane-bound enzymes (Uno *et al.*, 1982; Mason *et al.*, 1985), and at higher concentrations some of them affect yeast membranes by direct interaction with lipids (Cope, 1980; Brasseur *et al.*, 1983).

Clinical isolates of *C. albicans* vary considerably in their sensitivities to imidazoles (e.g. see Ryley *et al.*, 1984), and azole-resistant strains have been isolated (Holt & Azmi, 1978; Horsburgh & Kirkpatrick, 1983; Warnock *et al.*, 1983). The failure of two of these resistant strains (AD and

Abbreviations: YMA, yeast morphology agar; YNB, yeast nitrogen base.

KB) to accumulate the triazole antifungal ICI 153066 was suggested as a possible basis of their resistance (Ryley *et al.*, 1984). This might be due to an altered membrane lipid composition, which is known to affect membrane permeability (De Kruyff *et al.*, 1973). However, the lipid composition of these strains is unknown, as indeed is that of most *C. albicans* strains, and there is a dearth of information on lipid composition in this organism. This is particularly relevant in view of the mode of action of the two most important classes of antifungal compounds used to treat candidosis.

We report now a detailed comparison of the lipid composition of two azole-sensitive (A and B2630) and two azole-resistant (AD and KB) strains of *C. albicans*. The relationship between phospholipid/sterol ratios in these and two other strains, and the uptake of a triazole antifungal, is considered.

METHODS

Organisms and culture conditions. The following strains of *Candida albicans* were used: A (NCPF 3153), B (NCPF 3156), B2630, AD (NCPF 3302), KB (NCPF 3303) and Darlington (supplied by Dr C. F. H. Vickers). *C. albicans* A, B and B2630 are ketoconazole-sensitive, whereas AD, KB and Darlington are ketoconazole-resistant; details of the origins of these strains are given in Ryley *et al.* (1984). Stock cultures were maintained on slopes of yeast morphology agar (YMA; Difco Manual) and sub-cultured at monthly intervals. A loopful of cells from YMA slopes was used to inoculate 1 l of yeast nitrogen base (YNB; Difco Manual) containing 2% (w/v) glucose in a 2 l Erlenmeyer flask, which was shaken at 120 r.p.m. in an orbital incubator (Gallenkamp) for 16 h at 37 °C. The cell count of this culture was determined from its OD_{650} using a calibration curve (see below). This culture was used to inoculate portions of fresh YNB–glucose medium (1 l) in 2 l Erlenmeyer flasks to give 3×10^4 cells ml^{-1}; these were incubated for 16 h, when the cultures were in late exponential phase and contained approximately 10^8 cells ml^{-1}. The mean generation time for each strain was 70–75 min.

Cells were harvested by centrifugation for 5 min at 3024 $g_{av.}$ ($r_{av.}$ = 30·0 cm), resuspended in distilled water, recentrifuged and the cell pellet resuspended in a known volume of distilled water by vigorous vortex mixing. Portions were removed for determination of cell number, dry weight, and protein and DNA content, and the remainder was used for lipid extraction as described below.

Cell number. Diluted portions of yeast suspensions in distilled water were counted in a Neubauer haemocytometer (Deverall, 1981). A calibration curve of cell number versus OD_{650} (Cecil CE272 spectrophotometer) was constructed for more rapid, routine estimations of cell number.

Dry weight. Portions of yeast suspensions in distilled water in pre-weighed Pyrex glass vials were dried to constant weight at 100 °C.

Protein and DNA. The protein and DNA contents were measured in homogenates of yeast cells broken by three passages through a French pressure cell (American Instrument Co. Inc., Silver Springs, MD 20910, USA) at $1·3 \times 10^5$ kPa. Cell breakage was >96%, determined by cell counts. Protein was measured by the Lowry method and by the Coomassie blue method of Bradford (1976), using a bovine serum albumin standard; the methods agreed to within 2%. DNA was measured by the method of Leyva & Kelley (1974).

Lipid extraction. Yeast cell suspensions in water were centrifuged for 5 min at 707 $g_{av.}$ ($r_{av.}$ = 15·8 cm). The cell pellets were resuspended in propan-2-ol and incubated at 70 °C for 45 min; this step was included in order to inactivate degradative enzymes such as phospholipases. The suspension was cooled, recentrifuged and the supernatant retained. The pellets were extracted routinely using the method of Bligh & Dyer (1959). In some experiments the method of Folch *et al.* (1957) was used (see text). The propan-2-ol wash and chloroform extracts were combined to give a crude total lipid extract; the solvent was removed by rotary evaporation under reduced pressure, and the lipid redissolved in water-saturated-chloroform/methanol (19 :1, v/v). Non-lipid contaminants were removed using Sephadex G-25 (Wells & Dittmer, 1963). The amount of purified total lipid was established gravimetrically and stored under N_2 as a concentrated solution in chloroform/methanol (2 :1, v/v) at −40 °C.

Isolation of polar and neutral lipids. Total lipid was fractionated into various components by TLC using precoated 20 × 20 cm plates of silica gel H (Merck); the thickness of the silica gel was 0·25 mm for analytical work and 2 mm for preparative work.

Total phospholipid was separated from neutral lipid by one-dimensional TLC using light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (85 :15 :1, by vol.) as developing solvent; this system leaves total phospholipid at the origin, and separates the individual neutral lipids. Individual phospholipids were fractionated by two-dimensional TLC developed in the first dimension with chloroform/methanol/28% (w/v) aqueous ammonia (13 :5 :1, by vol.); the thin-layer plate was dried in a fume cupboard until no smell of ammonia could be detected, and was then developed in the second dimension with chloroform/acetone/methanol/acetic acid/water (6 :8 :2 :2 :1, by vol.). It is difficult to achieve complete separation of phosphatidylserine and phosphatidylinositol in two-dimensional TLC systems which resolve other phospholipids. Thus separate additional one-dimensional

thin-layer chromatograms were developed using chloroform/methanol/40% (v/v) aqueous methylamine (13 : 7 : 2, by vol.) to resolve phosphatidylserine and phosphatidylinositol for accurate quantitation (Harrington *et al.*, 1980). Lipids on thin-layer chromatograms were visualized by spraying with 0·2% (w/v) 8-anilino-1-naphthalene sulphonic acid in ethanol, and viewing under a UV lamp at 350 nm.

Identification of lipids. Phospholipids on analytical thin-layer plates were identified using the phosphorus reagent spray of Dittmer & Lester (1964). Specific phospholipids were identified by their content of free amino groups with ninhydrin, or choline with the Dragendorf reagent, or vicinal hydroxyl groups with Schiff's reagent (Kates, 1972), and by comparison of their R_F values with those of authentic standards. To confirm their identity each phospholipid was isolated by preparative two-dimensional TLC as described above, deacylated by mild alkaline hydrolysis and the head-groups subjected to paper chromatography; their R_F values were compared with authentic standards and published values (Kates, 1972).

The identity of neutral lipids was established by comparison of R_F values with those of authentic standards, and by GLC analysis which was also used to quantify them (see below). Sterol identification was made by GC–MS: trimethylsilyl derivatives were fractionated using a Dani gas chromatograph equipped with a 25 m fused silica column (int. diam. 0·22 mm) of CP Sil (Chrompak) the outlet of which was connected directly to a Varian 7070 mass spectrometer. Fatty acids were separated as described previously (Russell & Volkman, 1980) and identified by their retention times compared with authentic standards and by GLC analysis after catalytic hydrogenation (Kates, 1972).

Quantitation of lipids. Phospholipids were quantified by estimation of their phosphorus content using the method of Marinetti (1962) or fatty acid content using GLC analysis with an internal standard (Lloyd & Russell, 1984). The two methods gave excellent agreement to within <2%.

Triacylglycerols and sterol esters were quantified by GLC analysis of their constituent fatty acids after conversion to fatty acid methyl esters by transmethylation directly on silica gel using 2·5% (v/v) H_2SO_4 in dry, redistilled methanol (Christie, 1982). The same method was used to methylate non-esterified fatty acids. All fatty acids were quantitated using 15 : 0 as the internal standard and electronic integration (Russell & Harwood, 1979).

Non-esterified sterols were routinely quantified by GLC analysis as follows. Non-esterified sterols were eluted from silica gel with three washings of chloroform/methanol (2 : 1, v/v), dried over anhydrous Na_2SO_4, and the solvent evaporated *in vacuo*. Sterols were converted to trimethylsilyl derivatives (Christie, 1982) and analysed by GLC on 1·5 m × 5 mm (int. diam.) columns packed with 3% (w/w) SE-30 or OV-17 (Phase Separations, Queensferry, Clwyd, UK) supported on 80/100 mesh Gas Chrom Q operated isothermally at 275 °C in a Perkin Elmer F33 gas chromatograph with injector and detector temperatures of 300 °C. The carrier gas was N_2 with a flow rate of 30 ml min^{-1}. The sterols (largely ergosterol) ran as a single peak which coincided with an ergosterol standard, and could be quantified using an internal standard of cholesterol. Sterol compositions were determined by GC–MS as described above.

In some experiments sterol esters were also quantified by GLC analysis of trimethylsilyl derivatives of their constituent sterols obtained by saponification of sterol esters with 15% (w/v) KOH in 90% (v/v) ethanol at 80 °C for 1 h. There was excellent agreement (<2% variation) between the GLC measurements of sterol esters by sterol and fatty acid contents.

Measurement of azole uptake. Uptake of the ^{14}C-labelled triazole ICI 153066 was measured at 37 °C. Washed cell suspensions (10^9 cells ml^{-1}) were shaken in 0·1 M-potassium phosphate buffer, pH 5·5, containing 5·66 × 10^{-7} M-[U-^{14}C]ICI 153066 [specific radioactivity 19·4 Ci mg^{-1} (718 GBq mg^{-1})]. Samples (1 ml) were removed and added to 10 ml ice-cold deionized, glass-distilled water. Cells were sedimented by centrifugation and washed in 10 ml of water as above. The cells were transferred to a scintillation vial in 10 ml of scintillation fluid (Luma Gel, May and Baker) and radioactivity was measured by scintillation counting using the external standards ratio method for determination of the counting efficiency. Control experiments using autoclave-killed cells or incubation at 4 °C, and blank assays lacking cells, were done regularly in order to establish the amount of drug binding to cells. These amounts were reproducible for each strain, and did not exceed 25% of the radioactivity incorporated.

Materials. Phospholipid and neutral lipid standards were purchased from Sigma. Solvents and other chemicals were of analytical grade.

RESULTS

Efficacy of lipid extraction and lipid content

The amount of total lipid extracted from *Candida albicans* A using the method of Bligh & Dyer (1959) was 2·72 ± 0·20 mg (g dry wt)$^{-1}$, or 6·70 ± 1·47 mg per 10^{10} cells, or 39·25 ± 0·95 μg (mg total cellular protein)$^{-1}$, or 2·26 ± 0·2 mg (mg total cellular DNA)$^{-1}$ (n = 3). Corresponding values for another azole-sensitive strain (B2630), and two azole-resistant strains (AD and KB) were not significantly different (P > 0·05), irrespective of the method of expressing lipid

Table 1. *Phospholipid composition of four strains of C. albicans*

Values are means ± SD of at least two phosphorus and two fatty acid analyses of phospholipids of three separate cultures, except for strain A/A* for which the values are the means of two fatty acid analyses of two separate cultures (values varied by <2%).

	Composition (percentage by wt ± SD) of *C. albicans* strain:				
Phospholipid	A	A*	B2630	AD	KB
Phosphatidylcholine	25·4 ± 1·3	32·1	21·5 ± 1·4	24·5 ± 2·0	27·4 ± 1·9
Phosphatidylethanolamine	26·6 ± 1·8	22·9	16·9 ± 1·9	20·0 ± 1·7	20·7 ± 1·9
Phosphatidylinositol	19·9 ± 1·7	21·6	27·7 ± 1·8	22·8 ± 0·9	21·1 ± 0·8
Phosphatidylserine	17·6 ± 0·8		10·6 ± 0·7	11·4 ± 1·1	11·4 ± 1·2
Phosphatidylglycerol	3·2 ± 1·3	2·6	3·9 ± 0·2	5·6 ± 0·3	3·8 ± 1·0
Diphosphatidylglycerol	7·3 ± 1·8	20·8	19·8 ± 2·3	14·0 ± 1·8	15·7 ± 0·9
Sphingomyelin	<1·0	<1·0	<1·0	<1·0	<1·0

* Stationary phase culture of strain A.

content. These values of total lipid contents determined gravimetrically were essentially the same as the sum of the separated components measured individually by the various procedures reported here; thus, for *C. albicans* A the sum of the weights of the individual lipids was 96·3 ± 2·2% (n = 3) of the total lipid gravimetric weight. Although the total lipid content of *C. albicans* spp. is generally low compared with other yeasts and fungi (Weete, 1980), a second extraction procedure was used to check the above results. Using the extraction procedure of Folch *et al.* (1957), the total lipid content of *C. albicans* A was 2·45 mg (g dry wt)$^{-1}$, which is in good agreement with the value obtained using the Bligh & Dyer (1959) method. Furthermore, when the solvent-extracted residues were subjected to additional vigorous extractions using various boiling solvent mixtures no further lipid was recovered; nor did acidification of the solvents affect lipid yields. In addition, using the methods of Gonzales & Parks (1977) no further sterol (or sterol ester) could be extracted. It was concluded, therefore, that the procedures used were adequate to extract the total lipid.

Polar lipid composition

Polar lipids comprised 78·6 ± 0·3% of the total lipid in *C. albicans* A, and consisted entirely of phospholipids in all four strains. The phospholipid compositions (percentage by wt) are summarized in Table 1; they did not vary with the type of lipid extraction used. The major phospholipids in all strains were phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, with smaller amounts of phosphatidylglycerol and diphosphatidylglycerol and a trace (<1%) of sphingomyelin (Table 1).

The main fatty acids in each phospholipid of *C. albicans* A were 16:0, 16:1, 18:1 and 18:2, although there were differences as reflected by the different values of percentage unsaturated fatty acid, double bond index and C18/C16 ratio (Table 2). Phosphatidylcholine was the most unsaturated phospholipid, due mainly to its quite different relative proportions of 16:0 and 16:1 giving a bigger difference in percentage unsaturated fatty acid than double bond index compared with other phospholipids, whose proportions of polyunsaturated fatty acids were very similar (Table 2). In this respect percentage unsaturated fatty acid is probably a better guide to lipid unsaturation than is double bond index, especially since introduction of the first double bond has most effect on lipid fluidity (Coolbear *et al.*, 1983).

The fatty acid compositions of phospholipids in the other three strains also differed, whether comparisons were made of phospholipids within a strain or between strains. These differences are summarized in Table 3. Although the total phospholipid fatty acid compositions for each strain differed (data not shown) and there were differences in their percentage unsaturated fatty acid and C18/C16 ratios, their double bond indices were very similar (Table 3).

Table 2. *Fatty acid composition of the total phospholipid and the major individual phospholipids in C. albicans A*

Values are means ± SD of at least two GLC analyses of fatty acids in phospholipids isolated from three separate cultures. Abbreviations: PL, phospholipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; ND, not detected; UFA, unsaturated fatty acid; DBI, double bond index.

	Composition (percentage by wt ± SD)				
Fatty acid	Total PL	PE	PI	PS	PC
14:0	0·3 ± 0·0	ND	0·2 ± 0·1	0·5 ± 0·0	ND
16:0	26·8 ± 0·1	24·7 ± 0·6	39·3 ± 0·3	26·8 ± 0·1	12·7 ± 1·2
16:1	17·3 ± 0·2	22·0 ± 2·6	8·5 ± 0·5	12·7 ± 0·3	25·3 ± 0·6
16:2	2·7 ± 0·1	3·4 ± 1·2	2·2 ± 0·0	2·9 ± 0·0	4·0 ± 1·0
18:0	2·0 ± 0·3	3·0 ± 0·0	3·8 ± 0·2	6·1 ± 0·1	3·0 ± 0·0
18:1	25·6 ± 0·4	25·0 ± 1·0	22·9 ± 0·4	29·7 ± 0·3	21·0 ± 1·0
18:2	22·4 ± 0·1	18·7 ± 0·6	20·8 ± 0·3	18·0 ± 0·5	20·7 ± 0·6
18:3	2·7 ± 0·3	2·7 ± 0·6	0·8 ± 0·2	1·4 ± 0·3	3·0 ± 1·0
UFA (%)	70·7	72·0	55·2	64·7	74·0
C18/C16 ratio	1·12	0·98	0·96	1·32	1·13
DBI	1·01	0·99	0·80	0·89	1·00

Table 3. *Summary of total phospholipid fatty acid unsaturation and chain length in four strains of C. albicans*

Data for *C. albicans* A is taken from Table 2. See Table 2 for abbreviations.

	Strain			
Fatty acid parameter	A	B2630	AD	KB
UFA (%)	70·7	68·8	73·7	72·8
C18/C16 ratio	1·12	1·03	1·21	1·04
DBI	1·01	1·00	1·02	1·02

The interstrain differences in phospholipid and fatty acid compositions of exponentially growing cultures were not due to different growth rates of the strains, which were very similar (generation time 70–75 min). However, when the lipid compositions of stationary phase cultures were examined, there were marked differences in polar and neutral lipid compositions compared with those of corresponding exponentially growing cultures. There was a decrease in polar lipid and an increase in neutral lipid; for example, in strain A the polar lipid content decreased from 78·6% to 44·4% of the total lipid. There were also differences in phospholipid composition, and data for strain A are included in Table 1. The major change is the large increase in diphosphatidylglycerol in stationary phase cultures; there is no corresponding decrease in phosphatidylglycerol. There is a small rise in phosphatidylcholine and a fall in phosphatidylethanolamine, indicating a conversion of phosphatidylethanolamine to phosphatidylcholine as cultures enter stationary phase. There are also differences in the fatty acid compositions of all phospholipids from stationary phase cultures, the major change being an increase in 18:1 and a decrease in 18:2, reflected in the lower double bond index of 0·78 (cf. Table 3 for exponential phase cultures) for total phospholipid.

In view of these differences, great care was taken to standardize the growth and harvesting conditions of exponentially growing cultures (see Methods). The same was true of neutral lipid compositions. We did not find any differences in lipid compositions when Sabouraud's Dextrose Broth was used as the growth medium instead of YNB plus glucose, as in all the experiments reported in this paper.

Table 4. *Neutral lipid composition of four strains of C. albicans*

Values are means ± SD of at least two GLC analyses of neutral lipids extracted from three separate cultures, except for A* for which the values are the means of two GLC analyses of two separate cultures (values varied by <2 %).

Neutral lipid	Composition (percentage by wt ± SD) of *C. albicans* strain: A	A*	B2630	AD	KB
Non-esterified sterol	28·3 ± 0·6	8·3	30·6 ± 1·5	35·3 ± 1·5	35·4 ± 1·5
Non-esterified fatty acid	19·7 ± 2·9	6·8	19·7 ± 1·5	19·3 ± 1·5	19·8 ± 0·6
Triacylglycerol	39·9 ± 5·5	55·7	37·0 ± 2·0	35·4 ± 0·9	34·8 ± 0·9
Sterol ester	8·1 ± 0·9	28·9	7·7 ± 0·6	7·5 ± 1·3	7·5 ± 1·8
Squalene	6·1 ± 1·4	<1·0	4·7 ± 1·5	2·5 ± 1·8	3·5 ± 1·3

* Stationary phase culture of strain A.

Neutral lipid composition

The neutral lipid of exponentially growing cultures of *C. albicans* A comprised 21·5 ± 0·3% of the total lipid, and consisted mainly of triacylglycerol, non-esterified sterol and non-esterified fatty acid with smaller amounts of sterol ester and squalene in all four strains (Table 4). As noted above for polar lipids, there were also changes in neutral lipid composition of cells from stationary phase cultures; data for strain A are included in Table 4. The major differences are an increase in the relative amounts of triacylglycerol and esterified sterol, and a decrease in the relative amounts of non-esterified fatty acids and sterol; this probably reflects increasing amounts of stored lipids in stationary phase cells. There were also changes in the fatty acid compositions of non-esterified fatty acids, triacylglycerol and esterified sterol, which were all more saturated in stationary phase cultures. In contrast with the phospholipids, for exponentially growing cultures there were no significant differences ($P > 0·1$) in the relative proportions of different neutral lipids between strains, except for non-esterified sterol; *C. albicans* AD and KB, the two azole-resistant strains, contained 25% more non-esterified sterol than did *C. albicans* A and B2630 (Table 4). Similar differences in non-esterified sterol have been observed in polyene-resistant strains of *C. albicans* (Hammond, 1977; Pierce *et al.*, 1978) but no comparative data exist for other azole-resistant strains. The non-esterified sterol in all strains was comprised of ergosterol, with only traces of other components. In contrast, there were marked differences in the sterol ester compositions of the four strains; ergosterol was the major component in strain AD with only traces of other sterols, whereas in the other strains there were approximately equal amounts of ergosterol and zymosterol together with other components, some of which were not identified.

The fatty acid compositions of the three fatty-acid-containing neutral lipids in *C. albicans* A were distinct (Table 5), and also differed from that of the phospholipids (cf. Table 2). There were also differences in the neutral lipid fatty acid compositions between the four strains (see below).

The triacylglycerols were characterized by a relatively large proportion of 18 : 1 and the greatest degree of unsaturation amongst the neutral lipids (Table 5). The double bond indices of triacylglycerols in *C. albicans* B2630, AD and KB were 0·82, 0·99 and 0·79 respectively.

The sterol esters contained a large proportion of 18 : 0, but smaller amounts of 18 : 2, and 18 : 3 were absent (Table 5), thus giving them a double bond index and percentage unsaturated fatty acid that was lower than other neutral lipids or phospholipids (cf. Tables 2 and 5). The sterol esters in *C. albicans* B2630, AD and KB also lacked 18 : 3 and contained 14·3%, 13·0% and 11·6% 18 : 2 respectively, and had double bond index values of 0·84, 0·64 and 0·82 respectively.

All four strains contained significant amounts of non-esterified fatty acids (Table 4). The non-esterified fatty acid composition was similar to the total phospholipid fatty acid composition, except that it contained significantly ($P < 0·001$) greater proportions of 16 : 2 in *C. albicans* A (cf. Tables 2 and 5) and the other three strains.

Table 5. *Fatty acid composition of non-esterified fatty acids and acyl neutral lipids in C. albicans A*

Values are means ± SD of at least two GLC analyses of neutral lipids isolated from three separate cultures. Abbreviations: NEFA, non-esterified fatty acid; TAG, triacylglycerol; SE, sterol ester; ND, not detected; UFA, unsaturated fatty acid; DBI, double bond index.

	Composition (percentage by wt ± SD)		
Fatty acid	NEFA	TAG	SE
16:0	30·0 ± 1·0	11·7 ± 1·1	23·4 ± 0·5
16:1	10·0 ± 1·0	18·4 ± 1·1	6·6 ± 0·9
16:2	7·0 ± 0·9	3·0 ± 0·0	0·6 ± 0·0
18:0	6·3 ± 0·6	3·7 ± 1·5	29·0 ± 0·6
18:1	24·0 ± 1·0	35·0 ± 1·0	35·5 ± 1·0
18:2	15·0 ± 0·5	20·7 ± 0·6	4·5 ± 0·5
18:3	3·7 ± 0·5	3·3 ± 0·6	ND
UFA (%)	59·7	80·7	47·2
C18/C16 ratio	1·04	2·48	2·25
DBI	0·90	1·10	0·52

Table 6. *Ratio of total phospholipid to non-esterified sterol and triazole ICI 153 066 uptake in strains of C. albicans*

All lipid analyses and uptake experiments were done with exponentially growing cultures, except for A*. Values are means ± SD of at least two analyses by each method of lipids from three separate cultures, except for strain A/A* for which the values are means of two analyses of two cultures (values varied by <2%). Phospholipid contents have been corrected for loss by degradation during isolation (see text). Values of uptake of ^{14}C-labelled ICI 153 066 are the means of two independent experiments; values varied by <10%. The resistant strains AD and KB do not take up the triazole. Abbreviations: PL, total phospholipid; S, non-esterified sterol.

Strain	PL/S ratio (by wt)	Triazole uptake (pmol min^{-1} per 10^9 cells)
Sensitive		
A	15·4 ± 0·6	2·52
A*	8·6	7·62
B	23·0 ± 3·0	3·55
B2630	11·6 ± 1·3	2·14
Resistant		
AD	7·8 ± 0·7	–
KB	7·5 ± 0·4	–
Darlington	16·3 ± 1·5	6·20

* Stationary phase culture of strain A.

Polar lipid/neutral lipid ratio

The weight ratios of total phospholipid to neutral lipid in *C. albicans* A, B2630, AD and KB were 3·61 ± 0·15, 3·97 ± 0·25, 3·37 ± 0·17 and 3·31 ± 0·11 ($n = 3$ for each strain) respectively. The values for the sensitive strains (A and B2630) were not significantly different ($P > 0·1$); the same was true when the resistant strains AD and KB were compared ($P > 0·1$). The ratios for the sensitive strains were significantly greater than those of the resistant strains, but were only just outside the 95% confidence limit ($0·05 < P < 0·1$). The ratio for stationary phase cultures of strain A was 0·77, reflecting the large increase in stored neutral lipid after the end of exponential growth.

The weight ratios of total phospholipid to non-esterified sterol of exponentially growing cultures of the azole-sensitive strains were significantly greater ($P < 0·02$) than those of the azole-resistant strains (Table 6). This Table includes data for a third sensitive strain, *C. albicans*

B, which had a ratio that was higher than that of the other two sensitive strains, and the only other resistant strain available, *C. albicans* Darlington, which had a ratio similar to that of the sensitive strain A. The ratio of this latter strain decreased twofold when cultures were in the stationary phase.

Triazole uptake

The rates of uptake of triazole ICI 153066 by the three sensitive strains and the resistant strain *C. albicans* Darlington are shown in Table 6. The three sensitive strains took up triazole at similar rates. In strain A this rate increased in stationary phase cultures. The two resistant strains AD and KB do not take up the triazole (Ryley *et al.*, 1984).

DISCUSSION

The lipid contents of all strains, both sensitive and resistant, used in the present study were very similar, and are similar to the value of 3·2 mg (g dry wt)$^{-1}$ obtained by Combs *et al.* (1968) for *C. albicans* ATCC 10231 grown in similar media. These data are for exponentially growing cultures. We found that the lipid content of cells increased when cultures went into stationary phase. For example, in strain A the total lipid content was 8·5 mg (g dry wt)$^{-1}$ compared with 2·7 mg (g dry wt)$^{-1}$ in exponentially growing cultures, due largely to a rise in the amount of stored lipid. Comparison with data from other studies is made difficult not only by there being differences in culture age and phase, but also by the fact that different media are used which can affect lipid content (and composition) markedly (e.g. see Getz *et al.*, 1970). Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were the most abundant phospholipids in our strains, as observed previously in other *C. albicans* strains (Nishi *et al.*, 1973; Ballman & Chaffin, 1979) and other *Candida* spp. (Kates & Baxter, 1962). However, as noted above, detailed comparison of phospholipid compositions with other studies is difficult and not very meaningful because of the different growth conditions used by other workers.

There was a significant difference ($P < 0·05$) in the phospholipid compositions of the two imidazole-sensitive strains, with *C. albicans* A containing larger proportions of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine compared with *C. albicans* B2630 (Table 1). In contrast, there was no significant difference ($P > 0·05$) between the phospholipid compositions of the two imidazole-resistant strains (AD and KB). The two resistant strains contained higher proportions of phosphatidylcholine and diphosphatidylglycerol compared with *C. albicans* A, but otherwise the differences in the relative amounts of each phospholipid between imidazole-sensitive and imidazole-resistant strains were complex and did not follow a pattern. The differences in fatty acid composition between phosphatidylcholine and its metabolic precursor phosphatidylethanolamine may indicate that phosphatidylcholine is a better substrate than phosphatidylethanolamine for 'palmitate desaturase', assuming that phospholipid is the substrate for desaturation in *C. albicans* as in other yeasts (Kates *et al.*, 1984).

The combined amount of triacylglycerol and sterol ester both in sensitive and resistant strains, as well as their mean unsaturation values, were very similar – e.g. double bond index values of *C. albicans* A, B2630, AD and KB were 0·81, 0·83, 0·81 and 0·82 respectively. If the sterol esters are used to provide energy (although it has been suggested that they are membrane components, Grover & Kushley, 1979) this would indicate that despite individual differences in triacylglycerol and sterol ester amounts and their acyl compositions, together they represent comparable potential energy stores in each strain grown under the same conditions.

The neutral lipid fraction of sensitive and resistant strains contained non-esterified fatty acids which are usually regarded as being the products of phospholipase digestion. *C. albicans* A and B2630 (Barrett-Bee *et al.*, 1985) and some other strains (Pugh & Cawson, 1975; Banno *et al.*, 1985) contain and secrete phospholipases and a lysophospholipase. The non-esterified fatty acid composition did not correspond to that of one type of phospholipid, thus ruling out a specific phospholipase, unless, for example, the *sn*-2 position is enriched in 16 : 2 and the enzyme is a phospholipase A2. In *C. albicans* A and B2630 the lysophospholipase is very much more active

than is the phospholipase (Barrett-Bee *et al.*, 1985), which explains why lysophospholipids were never detected on two-dimensional thin-layer chromatograms in the present study, despite a careful search using several solvent systems. That all four strains used in this study did contain (lyso)phospholipase activity was confirmed by control experiments in which the boiling propan-2-ol step (see Methods) was omitted; under these conditions 39% (by wt) of the phospholipid was lost compared with the usual extraction procedure. The degradative enzyme(s) is unlikely to be a lipase or an acyl hydrolase (Harwood & Russell, 1984) because there was no change in sterol ester and triacylglycerol content when the boiling propan-2-ol step was omitted. Assuming that during the routine extraction procedure all non-esterified fatty acid is derived from phospholipid breakdown gives a value of 14% (by wt) loss of phospholipid. Comparison with control experiments shows that approximately 35% of the degradative enzyme(s) was not inactivated by the boiling propan-2-ol step. Repeated attempts, using a variety of solvents and heating steps, to inhibit residual phospholipid degradative activity were unsuccessful. However, the phospholipid fatty acid compositions in the parallel experiments were the same, indicating that the degradative enzyme(s) lacked specificity for a particular phospholipid. It is assumed, therefore, that phospholipid and fatty acid compositions were not distorted by phospholipid degradation, and valid conclusions can be drawn from the data.

The marked difference in ratio of total phospholipid to non-esterified sterol is interesting because in other membranes (e.g. red blood cells, Owen *et al.*, 1982) this ratio has a profound effect on their physical and biochemical properties. Sterols reduce the permeability of natural (De Kruyff *et al.*, 1973) and synthetic (de Gier *et al.*, 1968; Connolly *et al.*, 1985) membranes. It could be particularly significant, therefore, that the azole resistance of *C. albicans* AD and KB was shown recently to be due to their impermeability to triazole antifungals rather than to the insensitivity of ergosterol biosynthesis (Ryley *et al.*, 1984).

All sensitive strains took up triazole at a rate which was proportional to their phospholipid/sterol ratio, as long as exponentially growing cultures were used as the source of washed cell suspensions (Table 6). Exponentially growing cultures of the resistant strain Darlington took up triazole at a rate higher than that of the sensitive strain A; the phospholipid/sterol ratios of these two strains are very similar – i.e. strain Darlington does not have a ratio like that of the other two resistant strains (Table 6), and clearly its azole-resistance cannot be due to a lack of permeability. Together, these data are consistent with there being a relationship between triazole permeability and membrane lipid composition in exponentially growing sensitive strains, but it does not explain the resistance of strain Darlington. However, it remains to be established which particular cellular membrane is involved, and we are exploring further the basis of resistance in strain Darlington.

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