The Release of Potassium Ions from *Candida albicans* in the Presence of Polyene Antibiotics

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SUMMARY

The addition of amphotericin methyl ester (AME) to suspensions of *Candida albicans* is followed by a progressively increasing rate of release of K^+ from within the cell. The time taken for a given rate of release to be reached depends upon the AME concentration (the product of AME concentration and time being approximately constant), the pH and concentration of buffer, the temperature, the suspension density, and the phase of growth of the organisms. The sensitivity of *C. albicans* to AME decreases during the growth of a batch culture, rapid decrease occurring after the organisms have reached the stationary phase of growth. The induction of K^+ release does not occur at 0 °C and shows a high temperature coefficient over the range 15 to 30 °C.

Suspensions of mouse LS cells are 10 to 30 times less sensitive than *Candida albicans* towards AME, amphotericin B and nystatin; the two types of cell display little difference in sensitivity towards perimycin and candicidin, while mouse LS cells are more sensitive than *C. albicans* towards filipin.

Addition of sterols at the same time as AME increases the time taken for the release of K^+ to reach a given rate; of the sterols tested, zymosterol was the most effective in antagonizing AME, while ergosterol was approximately twice as effective as cholesterol on a molar basis. When organisms were exposed to AME for a short time, removed and resuspended in the absence of drug, the rate of K^+ release continued to increase; this increase was prevented by ergosterol: 2×10^{-5} M-ergosterol prevented further damage after organisms had been exposed to 10^{-6} M AME.

INTRODUCTION

The polyene antibiotics are effective against eukaryotic cells in general but are without action on bacteria. Their action can be antagonized by sterols (Gottlieb, Carter, Wu & Sloneker, 1960), of which the most effective is ergosterol (Zygmunt & Tavormina, 1966). The polyenes appear to disorganize the structure of natural membranes containing sterols (Lampen, Arnow, Borowska & Laskin, 1962), as shown particularly by the insensitivity of certain mycoplasmas (*Acholeplasma*) grown in the absence of sterol and rendered sensitive by growth in the presence of sterol (Lampen, Gill, Arnow & Magana-Plaza, 1963; Weber & Kinsky, 1965). Polyenes differ in the size of the lactone ring, the number of double bonded C atoms and the degree of substitution. All the polyenes tested produce a change in the permeability of sensitive organisms, such as *Candida* spp., but the smaller molecules produce greater damage than large ones; thus filipin with 35 C in the molecule induces leakage of K^+ , inorganic phosphate, amino acids and sorbose; nystatin with 46 C releases K^+ and phosphate but not sorbose, while succinylperimycin with 63 C releases K^+ only (Lampen, 1966; Borowski & Cybulska, 1967). Physical methods comparing the binding of polyenes to cholesterol have shown the binding affinity of polyenes to fall in the order:

filipin > amphotericin B > nystatin > lagosin (Bittman & Fischkoff, 1972; Norman, Demel, Kruffy, Guerts Van Kessel & Van Deenen, 1972; Schaffner, 1973). The reaction between polyenes and sterols depends upon the latter having (i) 3 β -hydroxy group on ring A, and (ii) 8 to 10 carbon side chain on C-17, and is affected by the degree of planarity of the sterol nucleus (Zygmunt & Tavormina, 1966; Bittman & Fischkoff, 1972). The permeability changes produced in natural membranes as a result of the interaction are attributed to micelle formation in the membrane followed by the formation of aqueous pores through the membrane (Finkelstein & Cass, 1968; Andreoli & Monahan, 1968; Cass, Finkelstein & Krespi, 1970; Holts & Finkelstein, 1970). Verkleij *et al.* (1973) have shown the appearance of aggregates 15 to 25 nm in diameter in acholeplasma membranes containing cholesterol and treated with filipin, and suggest that the mobilization of sterol and the formation of such aggregates would cause major disorganization of membranes whether pores were formed or not.

For the purposes of an investigation of the biochemistry of 'opportunistic' fungal infections and the use therein of polyene antibiotics, it was necessary to develop a rapid, quantitative method sufficiently accurate to assess the effects of differing growth conditions, alteration in sterol structure and composition, and modifications in polyene structure on the sensitivity of cells to polyenes. The instability of polyenes makes growth tests difficult to interpret but, since all polyenes tested induce loss of K^+ from sensitive organisms (Lampen, 1966; Zygmunt, 1966), it seemed that estimation of this loss with a sensitive K^+ -responsive electrode would provide a suitable method. A description is given in this paper of the use of such a method to study quantitatively the release of K^+ from *Candida albicans* and the conditions affecting that release, to assess the relative action of certain polyenes on *Candida* and mouse cells, and to study the time course of polyene action on the permeability of *Candida* membranes together with the effects of sterols on that action. Most of this work has been carried out with the water-soluble amphotericin methyl ester hydrochloride (Mechlinski & Schaffner, 1972); the structure of amphotericin B has been established by Mechlinski, Schaffner, Ganis & Avitabile (1970).

METHODS

Organisms and methods of growth. Three strains of *Candida albicans*, 6406, 6431 and 18, were clinical isolates obtained from the Mycology Reference Laboratory of the London School of Hygiene and Tropical Medicine. Unless otherwise stated, experiments were carried out with strain 6406. The organisms were cultured on Bacto yeast nitrogen base medium (YNB), 6.7 g/l containing 0.5 % glucose; cultures were maintained on agar slopes of the same medium. For routine culture, the organisms were sub-cultured into tubes containing 5 ml YNB-glucose and incubated for 24 h at 30 °C; these were then used to provide inocula for 2 l conical flasks each containing 200 to 250 ml YNB-glucose. Flasks were incubated for 18 h in an orbital incubator at 37 °C, the inoculum size adjusted (usually 1 to 2 ml per flask) so that organisms were harvested at a suspension density of 0.2 to 0.6 mg dry wt/ml; exponential growth ceased around a suspension density of 1.2 to 1.4 mg/ml (see Fig. 4, below). Organisms were centrifuged down from the growth medium, washed twice at a density of approx. 5 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5) and re-suspended in that buffer at a density of 10 mg dry wt/ml. This suspension was then diluted as described below. Densities were determined on a Hilger Absorptiometer (Rank Precision Industries Ltd, Margate, Kent) calibrated against the organisms used.

Mouse fibroblasts, strain LS, were provided by Dr A. A. Newton and were grown in sus-

pension culture in Eagle's (1959) minimal essential medium (Biocult, Biocult Laboratories Ltd, Paisley, Scotland) supplemented with 5 % tryptose phosphate broth, 100 units penicillin/ml, 100 μ g streptomycin/ml, and 5 % ox serum. Cells were harvested from 200 ml while in the exponential phase of growth by centrifuging for 5 min at 1850 g. They were washed once by resuspension in 10 % sucrose solution, resedimented, and resuspended in 10 % sucrose to give a density of about 5×10^7 cells/ml; 1 ml of this suspension was added to 19 ml 0.1 M-tris-HCl buffer (pH 7.5) with 4 % sucrose as stabilizer, in the electrode vessel.

Materials. Amphotericin B, amphotericin methyl ester hydrochloride, candidin and perimycin were gifts from Dr C. P. Schaffner, filipin was a gift from Dr G. Whitfield, Upjohn Company, Kalamazoo, Michigan, U.S.A. Nystatin (4800 i.u./mg, 80 % pure), cholesterol and ergosterol were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A. Certain commercial preparations of amphotericin B, kept dry at -15°C , were found to lose all activity over a period of three to four months and have been discarded. Zymosterol was obtained from the Medical Research Council Steroid Reference Collection. Amphotericin methyl ester hydrochloride, nystatin and perimycin were soluble in water at the concentrations required; the other polyenes were dissolved in aqueous alcohol containing the minimum amount of ethanol necessary to effect solution, and controls containing equivalent amounts of ethanol were used with the *Candida* suspensions. Mouse LS fibroblasts were adversely affected by ethanol and aqueous suspensions of polyenes other than filipin were used in these experiments. Filipin was made up at 10 mg/ml in ethanol and the small amounts of ethanol then dispensed were tolerated by the cells. Antibiotic solutions were freshly prepared on the day of use.

Inhibition of growth. *Candida albicans* were inoculated (10^7 to 10^8 organisms) into 5 ml quantities of YNB-glucose medium in test-tubes and serial dilutions of drugs added. Growth was inspected after 24 h at 37°C and the minimum inhibitory concentration read by eye.

Estimation of K^+ release. Unless stated otherwise, organisms were suspended at a final density of 1.0 mg dry wt/ml in 20 ml of 0.03 M-tris-HCl buffer (pH 7.5) at room temperature (20°C) and stirred by a magnetic flea driven by an air-turbine. Potassium was estimated by the use of a K^+ -sensitive electrode (type BH115, Electronic Instruments Ltd, Richmond, Surrey) connected to measuring unit C33B-2 and Vibron electrometer 33B-2 (Electronic Instruments Ltd) recording on a potentiometric recorder (Servoscribe RE511-20, Smith Industries Ltd, Wembley, Middlesex). When not in use, the electrode was kept in 0.1 M-KCl and the 'bridge' filled afresh each day with 0.1 M-tris-HCl buffer (pH 7.5). The instrument was calibrated against standard solutions of KCl and could be used effectively to measure K^+ over the range 3×10^{-7} to 10^{-4} M. Using the instrument at highest sensitivity, for concentrations of the order of 10^{-6} M- K^+ , a change of 1 nmol K^+ /ml corresponded to approx. 4 cm displacement of the recorder pen. Suspensions of cells prepared as described above gave initial K^+ concentration readings of 10^{-6} M or less; such cells usually showed a small release of K^+ (although in some cases, especially with organisms freshly harvested early in the exponential phase of growth, uptake of the K^+ contaminating the suspension took place) and the suspension was left until the recorder showed a constant rate of release. Drug solutions, etc., were then added to the suspension and the record followed as described below.

Protoplasts of *Candida albicans* 6406 were prepared by Mr M. Marriott by the method of Garcia-Mendoza & Villanueva (1962) using streptomyces enzyme kindly given by Professor Villanueva. Organisms were harvested in mid-exponential phase and stabilized during enzyme treatment with 0.6 M-KCl. KCl could not be used for stabilization during

Fig. 1. Time course of release of K^+ from *Candida albicans* in the presence of amphotericin methyl ester (AME). *C. albicans* 6406 was harvested in mid-exponential growth, washed twice and suspended at a density of 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer at pH 7.5 (20 ml) in the electrode vessel as described in Methods. Release of K^+ followed until a steady rate obtained and AME at the final concentrations indicated on the curves was added. The diagram represents the series of traces collected and normalized for the time of AME addition (at arrow). The K^+ scale is approximate only, as calibration varies to some extent with each curve. The trace was stopped when standard rate of release was obtained in each case.

release tests and it was found satisfactory to wash the protoplasts three times and finally suspend them in 0.1 M-tris-HCl buffer (pH 7.5) containing 0.8 M-sorbitol as stabilizing agent; the protoplast suspension was made at a density corresponding to 1.0 mg dry wt of initial cells/ml.

RESULTS

Release of potassium in the presence of amphotericin methyl ester (AME)

Time course and AME concentration. Fig. 1 reproduces a collection of recorder tracings obtained when AME at varying concentrations was added to a suspension of *Candida albicans* 6406. After addition of antibiotic, there was a period before the rate of K^+ release increased and this lag period depended upon the AME concentration. The rate then increased progressively to values of 20 to 30 nmol K^+ /min/mg cells until (although this is not shown in Fig. 1) the K^+ content of the cells had equilibrated with that in the medium. In order to use the rate of release as a means of studying the action of the drug quantitatively, the following terms were defined. Standard rate of release (SR): release of 1 nmol K^+ /min/mg dry wt of cells above the release in control cells without drug. Standard release time (SRT): the time taken to attain the standard rate of release. Standard release concentration (SRC): the concentration of drug (μ g/ml) which gives rise to SR in a given time; times chosen were either 6 or 8 min and indicated by SRC (6) and SRC (8) respectively.

Table 1 shows the relation between the time and drug concentration for organisms harvested at different growth densities; the product time $\times$ drug concentration is approximately constant, especially for longer times, for any given culture and can be used as a guide in the determination of SRC. SRT can be used conveniently to study changes in

Table 1. Relationship between AME concentration and SRT for leakage of K⁺ from *Candida albicans*

Candida albicans 6406 was suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer at pH 7.5.

Culture density at harvesting (mg/ml)	AME concn (μg/ml)	SRT (min)	AME concn × SRT
0.46	2.0	5.2	10.4
	1.0	8	8
	0.5	14.6	7.3
	0.3	19	5.8
	0.2	24	4.8
	0.15	31.5	4.7
	0.1	46	4.6
0.98	4.5	14	62
	2.0	27	54
	1.5	38	58
	Nystatin concn (μg/ml)		Nystatin concn × SRT
0.98	5.0	22	110

SRT, time required for rate of K⁺ release to attain 1 nmol/min/mg over that in control without drug; AME, amphotericin methyl ester.

Table 2. Effect of suspension density and amphotericin methyl ester (AME) concentration on rate of K⁺ release from *Candida albicans*

C. albicans was suspended at the densities shown below in 0.03 M-tris-HCl buffer pH 7.5; AME at the concentrations shown below was added and release followed until the rate was greater than 3.0 nmol K⁺/min. Times taken to reach rates of release of 0.3, 1.0 and 3.0 nmol K⁺/min were determined.

Dry weight cells (mg/ml)	...	...	0.3	0.3	1.0	3.0	3.0
AME (μg/ml)	...	...	0.3	1.0	1.0	1.0	3.0
K ⁺ release rate (nmol/min/ml)	Time (min)						
0.3			11.7	5.8	9.1	10.6	5.1
1.0			16.1	8.1	11.0	13.5	7.3
3.0			23.5	11.5	14.8	17.8	9.1
K ⁺ release rate (nmol/min/mg organisms)							
0.3			9.5	3.5	9.1	13.2	6.9
1.0			11.6	5.9	11.0	17.9	9.2
3.0			15.7	7.9	14.8	—	—

response of the order of 2 to 3 times but larger variations are studied by estimation of SRC.

Effect of suspension density. Table 2 shows that the rate of K⁺ release depends upon both drug concentration and suspension density. Decreasing or increasing the suspension density threefold decreases or raises respectively the time taken to reach a given rate of release (1.0 or 3.0 nmol/min/ml) by 20 to 25 %. If the release is calculated on the basis of nmol K⁺/min/mg dry wt of organisms, it can be seen that the same rate of release is obtained if the ratio of suspension density to drug concentration remains the same. In a similar experiment in which the drug concentration required to produce a release of 1 nmol K⁺/min/ml was determined in the presence of suspension densities of 0.3, 1.0 and 3.0 mg dry wt/ml,

Fig. 2

Fig. 3

Fig. 2. Effect of concentration of tris-HCl buffer on the rate of K^+ release. Conditions were as for Fig. 1 except for the concentration of the buffer; $0.25 \mu\text{g}$ AME was added/ml and SRT determined (i.e. time taken to give a release of $1 \text{ nmol } K^+/\text{min}/\text{mg}$ organisms in excess of that in the control without drug).

Fig. 3. Effect of pH on the rate of release of K^+ from *Candida albicans* in the presence of AME (in the presence and absence of 1.6 nmol ergosterol) and nystatin. Organisms were suspended in 0.03 M -tris buffer at the pH values indicated. (●), $0.4 \mu\text{g}$ AME/ml added at time 0; (○), $0.4 \mu\text{g}$ AME + 1.6 nmol ergosterol/ml added at time 0; (▲), $1.0 \mu\text{g}$ nystatin/ml added at time 0.

the values obtained were 0.65 , 1.0 and $1.45 \mu\text{g}$ AME/ml respectively. In all other experiments, the suspension density has been standardized at 1.0 mg dry wt/ml .

Effect of pH and strength of buffer. Fig. 2 shows that the time taken to induce the SR increases with the strength of tris-HCl buffer at pH 7.5, while Fig. 3 shows that, both for AME and nystatin, the SRT increases slowly over the pH range to 8.0 but more rapidly at more alkaline values; the increase at values greater than pH 8.0 is greater for nystatin than for AME. In other experiments quoted, release was studied in 0.03 M -tris-HCl buffer at pH 7.5.

Effect of 'age of culture'. Fig. 4 shows the variation of SRC for AME with the time of incubation of *Candida albicans* 18 culture. The sensitivity decreases progressively throughout the growth, the rate of decrease being much greater once the culture has entered the stationary phase. Similar variations are obtained with *C. albicans* 6406, where the SRC is around 0.2 to $0.5 \mu\text{g}$ AME/ml for organisms harvested in the early exponential phase but may rise to $10 \mu\text{g/ml}$ for organisms harvested in the stationary phase of growth. For routine work, organisms were harvested at a culture density of 0.2 to 0.6 mg dry wt/ml .

Effect of temperature. Lampen *et al.* (1962) found that the uptake of nystatin by *Saccharomyces cerevisiae* was temperature dependent. Table 3 shows that the rate of release of K^+ from *Candida albicans* in the presence of AME is also markedly affected by temperature, a change of 10°C over the range 10 to 30°C altering the value of SRT by six to seven times. No release, over that without drug, could be observed at 0°C up to 60 min.

Fig. 4. Effect of 'age of culture' on sensitivity of *Candida albicans* 18 suspensions to AME. *C. albicans* was grown on a rotary shaker at 37 °C as described in Methods except that the glucose concentration in the medium was reduced to 0.1 % to limit growth within the experimental period. Organisms were harvested at intervals as shown, washed twice and suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5). The standard release concentration [SRC(8)] was determined for each culture (i.e. that concentration of AME, in µg/ml, inducing a release of 1 nmol K^+ /min/mg organisms, over that in the control without drug, in 8 min at 20 °C). ●—●, SRC(8); ○---○, culture density (mg dry wt/ml) at time of harvest.

Table 3. Effect of temperature on release of K^+ from *Candida albicans* in the presence of AME

C. albicans was suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5) in electrode vessels maintained at temperatures as below. AME was added and SRT determined.

AME (µg/ml)	Temperature (°C)	SRT (min)
1.0	20	8
	15	30
	10	65
	0	No release at 60
0.3	30	20
	20	3.5

For abbreviations, see Table 1.

Effect of metabolic inhibitors. The marked effect of temperature on the rate of release of K^+ might be explained by a requirement for metabolic energy for either the uptake of the antibiotic or the subsequent membrane disorganization process. Table 4 shows that inhibitors such as iodoacetamide and dinitrophenol increase the time required for standard release in the presence of a given concentration of drug. It was not possible to extend the studies to sodium azide, arsenate or cyanide as the electrode was not efficient in the presence of these inhibitors at the concentrations required to affect metabolism. Lampen *et al.*

Table 4. *Action of metabolic inhibitors*

Candida albicans was suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5); standard release time after addition of 1.0 µg AME/ml was determined in the absence and presence of inhibitors as below.

Inhibitor	Concentration (mM)	Standard release time (min)
None	—	6.5
Iodoacetamide	3	8
	10	15
	30	27.5
Na azide	1	7
2,4-Dinitrophenol	1	9
	3	12

Table 5. *Sensitivity of Candida albicans cells and protoplasts to amphotericin methyl ester (AME)*

C. albicans 6406 was harvested in early exponential growth (0.05 to 0.1 mg/ml culture medium) and suspended at a density of 1.0 mg dry wt/ml in 0.1 M-tris-HCl buffer (pH 7.5) with additions as below; protoplasts were produced by strepzyme treatment and suspended at a density corresponding to 1.0 mg initial cells/ml, with 0.8 M-sorbitol as stabilizer. AME was added and standard release concn (µg/ml) determined for 8 min release [SRC (8)].

Treatment of cells	SRC (8)	
	Expt 1	Expt 2
(1) Suspended in 0.1 M-tris-HCl	0.10	0.15
(2) As (1) + 0.8 M-sorbitol		0.45
(3) Protoplasts formed and suspended in 0.1 M-tris-HCl + 0.8 M sorbitol	0.85	0.6
(4) Cells treated as (3) less protoplasting enzyme; suspended in 0.1 M-tris-HCl + 0.8 M-sorbitol		0.8

(1962) noted that digitonin, which reacts with sterols, extracted nystatin from yeast membranes; 10 µM-digitonin doubled the SRT when added directly to the organisms at the same time as AME; when the organisms were treated with 10 µM-digitonin for 4 h at 20 °C before the addition of AME they were then resistant to AME (SRC > 20 µg/ml).

Sensitivity of protoplasts and resistant strain of Candida albicans. The minimum growth inhibitory concentrations of AME for the three strains of *Candida albicans*, 6406, 18 and 6431, were determined by the conventional growth method using serial dilutions of the drug and inocula of approx. 10⁷ to 10⁸ organisms/ml; values of 0.4 µg/ml were obtained for strains 6406 and 18, and 1.2 µg/ml for 6431. The SRC was then determined for K⁺ release from suspensions of early-exponential phase of the three cultures; values obtained were 0.3, 0.7 and 9.5 µg AME/ml for strains 6406, 18 and 6431, respectively.

Table 5 shows the sensitivity of protoplasts of strain 6406. Protoplasts were first made by the strepzyme method (Garcia-Mendoza & Villanueva, 1962) and showed a marked increase in resistance compared with the parent cell suspension (Table 5, Expt 1); however, when organisms were taken through the treatment involved in the protoplast formation, omitting the enzyme, and suspended in tris-HCl buffer containing 1.2 M-sorbitol as stabilizer, it was found that there were no significant differences in the sensitivities of protoplasts and the organisms from which they were formed (Table 5, Expt 2).

Sensitivity of Candida albicans and mouse LS cells. Table 6 shows the relative sensitivity

Table 6. *Sensitivity of Candida albicans and mouse LS cells to polyene antibiotics*

Cells suspended at 1.0 mg dry wt/ml in 0.1 M-tris-HCl buffer (pH 7.5) with, for mouse LS cell suspensions, 4% (w/v) sucrose added. Standard release concentration [SRC (8)] determined for antibiotics as below.

Polyene	Standard release concentration (8 min) (μ g polyene/ml)						Ratio <i>b/a</i>
	<i>Candida albicans</i> 6406			Mouse LS cells			
	Range	No.*	Mean (<i>a</i>)	Range	No.*	Mean (<i>b</i>)	
Amphotericin methyl ester (AME)	0.45-1.0	8	0.7	12.5-18	5	15.6	22
Nystatin	1.5-5.0	5	3.3	28-35	3	30	9
Candicidin	0.55-1.1	3	0.9	1.2-2-3	3	1.7	2
Perimycin	1.5	1		2.0	1		1.3
Filipin	10-12	5	11	2-3	3	2.5	0.23
Amphotericin B	1.8-5.0	5	3.0	>45	1		>13

* Number of different suspensions used for determination of mean value.

to a range of polyene antibiotics of *Candida albicans* suspension harvested in mid-exponential growth and mouse LS fibroblast cells. In both cases the sensitivity is affected by the stage of growth of the cells and attempts have been made to standardize the conditions as far as possible. Detailed investigations have not been carried out with the fibroblast suspensions and batches have varied in stability; in two instances the suspensions were stable in 0.1 M-tris-HCl buffer containing 0.5% sucrose, but other batches required 4% sucrose to prevent rapid K^+ release in the absence of drug. AME was included as one of the antibiotics in all experiments. Results with amphotericin B have proved variable, probably due to the insolubility of the preparation. The ratio of SRC for yeast and fibroblast cells gives a guide to the selectivity of the drug for the systems used; clear-cut differences in sensitivity have been consistently found for amphotericin methyl ester and nystatin, whereas the two cell types have similar sensitivities for perimycin and candicidin while the mouse LS cells were markedly more sensitive to filipin than *C. albicans*.

Effect of sterols on K^+ release from Candida albicans in the presence of AME or nystatin

Effect of sterols added at the same time as the polyene. Preliminary work showed that the addition of cholesterol or ergosterol at the same time as AME reduced the rate of the induced release of K^+ but the degree of reduction varied widely from experiment to experiment. To carry out these tests, AME was made up in mM solution in water and then diluted to 10^{-4} M in the presence of sterol and samples of the AME/sterol mixture used for the determination of SRT. The sterol solutions contained ethanol and the alcohol content was adjusted to the same value in all mixtures including the AME control. Fig. 3 shows that the pH of the sterol/AME mixture affects the degree of antagonism subsequently obtained; the results quoted in Table 7 were obtained with sterol/AME mixtures and organisms at pH 8.0 in 0.03 M-tris-HCl buffer. Table 7 shows that the SRT for a given AME concentration is increased by a variety of sterols; the most effective polyene antagonist of the sterols tested was zymosterol (5 experiments). Ergosterol was approximately twice as effective as cholesterol at the same sterol/AME ratio. It can be seen from Table 7 that ergosterol had some antagonism towards nystatin whereas cholesterol had no significant action under the conditions used.

Table 7. *Effect of sterols on the release of K⁺ from Candida albicans in the presence of polyenes*

C. albicans at a density of 1.0 mg dry wt/ml was suspended in 0.03 M-tris-HCl buffer (pH 8.0). Drug solutions were made up in 0.03 M-tris-HCl buffer (pH 8.0) containing 10⁻⁴ M-AME (mol. wt. 972) or 2 × 10⁻⁴ M-nystatin (mol. wt. 929), sterol as below, and 10% (v/v) ethanol; samples were taken and added to the electrode vessel (20 ml mixture) to give final drug concentrations as below.

AME (μM)	...	...	...	0.8	1.4	1.6	0.5	—
Nystatin (μM)	...	...	...	—	—	—	—	3
	Molar ratio:		Standard release time (min)					
	Sterol							
Sterol	Polyene		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	
None	0		10.3	7.5	6.5	12.0	16.5	
Cholestane	3		—	—	10.1	—	—	
1,4-Pregnadiene- 11β,17,21-triol- 3,20-dione	3		—	—	10.8	—	—	
Cholesterol	2		13.2	10.0	—	—	—	
	3		—	—	13.0	—	—	
	4		15.6	15.5	—	16	—	
	6		18.5	—	—	—	17.0	
Stigmasterol	3		—	—	14.0	—	—	
Ergosterol	2		14.3	13.0	—	—	—	
	3		—	—	21.0	—	—	
	4		20.2	20.3	—	20	21.0	
	6		26.4	—	—	—	23.0	
Zymosterol	4		—	—	—	31	—	

Effect of ergosterol added after removal of AME. Fig. 1 indicates that damage to the membrane of *Candida albicans* by AME is progressive and raises two questions: will the rate of K⁺ release continue to increase if the organisms are removed from the drug after a short period of exposure, and can the damage be stopped or reversed by the presence of sterol after removal of the drug? Organisms have been suspended under the usual conditions and the SRC determined for AME; further samples have then been suspended in the presence of the SRC and higher concentrations of AME, shaken for 1 to 4 min at room temperature, the organisms centrifuged, the supernatant medium removed as completely as possible and the organisms resuspended at a density of 1.0 mg/ml in 0.03 M-tris-HCl buffer (pH 7.5) in the electrode vessel (dilution of drug left in the pellet > 100 times); readings were taken as soon as the assembly had equilibrated. If organisms were resuspended after initial treatment with drug for 2 min plus the time required for centrifuging, draining and resuspension – a total of 8 min – readings could be taken from 10 min (2 min after resuspension) and experiments showed that linear increase in the rate of K⁺ release then occurred. Decreasing the initial period of treatment gave inconsistent results. Fig. 5 shows the rates of release obtained after resuspension of organisms (SRC = 0.7 μg AME/ml) after initial exposure to different concentrations of the drug: values for the rates of release in organisms resuspended after the same treatment in the absence of drug have been subtracted in all cases (see Fig. 6a). Organisms resuspended after initial treatment with SRC showed a small release over that of control after some 20 min. Higher initial concentrations of drug gave rise to faster increases in release rate on resuspension and an initial concentration 1.5 to 2 times SRC was adopted for further experiments. It is clear that

Fig. 5. Rate of K^+ release from *Candida albicans* 6406 exposed to AME and resuspended in the absence of drug. *C. albicans* was suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5) containing AME as below; after 2 min at 20 °C, organisms were centrifuged, the supernatant was removed quantitatively and the pellet resuspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5); K^+ release was studied over the next 30 min. The concentrations of AME present during the initial period of 8 min were as follows ($\mu\text{g/ml}$): ($\square$), 0.7; ($\blacksquare$), 0.8; ($\bullet$), 1.0; ($\times$), 1.25; ($\circ$), 1.35; ($\triangle$), 1.50. ($\blacktriangle$), Initial AME concentration 0.7 $\mu\text{g/ml}$; cells resuspended in buffer containing 0.7 μg AME/ml.

damage initiated during the 8 min exposure to AME progresses after removal of the drug from the medium.

The effect of the presence of ergosterol in the medium after resuspension was then tested. Fig. 6(a) shows the release obtained after resuspension of organisms, pretreated in the presence or absence of AME, in buffer alone or with the addition of 2×10^{-5} M-ergosterol. The addition of ergosterol to resuspended control organisms results in a transient increase in the rate of K^+ release and Fig. 6(b) shows that, when the appropriate controls are taken into account, 2×10^{-5} M-ergosterol largely prevents the increase in release that takes place in drug-treated organisms. Table 8 shows the effect of ergosterol concentration; under the conditions used in these experiments 2×10^{-5} M-ergosterol gave the greatest protection against further damage initiated by approx. 10^{-6} M-AME. Smaller or higher concentrations of ergosterol were less effective and it has not been possible to demonstrate any reversal of AME damage in the sense of a decreased rate of release after resuspension in the presence of ergosterol. An interesting finding is that at high concentrations (10^{-4} M) ergosterol increased the rate of release in AME-treated organisms but had no progressive effect on control organisms.

Fig. 6. Effect of ergosterol on the release of K⁺ from *Candida albicans* 6406 exposed to AME and then resuspended in buffer without drug. (a) Conditions were as for Fig. 5. Organisms were initially suspended in 0.03 M-tris-HCl buffer (pH 7.5) with or without 1.35 μg AME/ml (1.39 μM); they were centrifuged and resuspended as follows: (●), Initial treatment, no drug; resuspended in buffer. (▲), Initial treatment, no drug; resuspended in buffer containing 2 × 10⁻⁵ M-ergosterol. (△), Initial treatment, 1.35 μg AME/ml; resuspended in buffer containing 2 × 10⁻⁵ M-ergosterol. (○), Initial treatment, 1.35 μg AME/ml; resuspended in buffer. (b) (○), Release of K⁺ from organisms pretreated with AME and resuspended in the presence of 2 × 10⁻⁵ M-ergosterol (curve △, Fig. 6a) corrected for control (curve ▲, Fig. 6a). (●), Release of K⁺ from organisms pretreated with AME (curve ○, Fig. 6a) corrected for control (curve ●, Fig. 6a).

Table 8. Effect of ergosterol on the rate of release of K⁺ from *Candida albicans* after treatment with AME

Organisms were suspended at 1.0 mg dry wt/ml in 0.03 M-tris-HCl buffer (pH 7.5) with or without 1.25 μg AME/ml, and shaken at 20 °C for 2 min; the organisms were centrifuged, the supernatant removed and the organisms resuspended at 1.0 mg/ml in 0.03 M-tris-HCl buffer, pH 7.5 (8 min from first suspension); ergosterol was added to give a final concentration as below and readings were started after a further 2 min (see Fig. 6a for nature of time course). Results for AME-treated organisms are corrected for controls without drug treatment.

Pretreatment AME (μg/ml)	Ergosterol added after resuspension at 8 min (M)	K ⁺ release rate (nmol/min/mg organisms)		
		2 min after resuspension	22 min after resuspension	Increase (2 to 22 min)
0	0	0.15	0.13	—
	10 ⁻⁵	0.37	0.26	—
	2 × 10 ⁻⁵	0.45	0.30	—
	4 × 10 ⁻⁵	0.68	0.33	—
	10 ⁻⁴	0.49	0.42	—
1.25 (1.28 μM)	0	0.32	1.38	1.06
	10 ⁻⁵	0.32	0.88	0.56
	2 × 10 ⁻⁵	0.25	0.53	0.28
	4 × 10 ⁻⁵	0.24	1.03	0.79
	10 ⁻⁴	0.25	1.67	1.42

DISCUSSION

Exposure of *Candida albicans* to AME results in progressive damage to the cell membrane as judged by the rate of release of K^+ from inside the organism. The release takes place at an increasing rate even if the organisms are removed from AME-containing medium and it would appear that changes in membrane organization take place during and after the organisms have taken up the drug. Cass *et al.* (1970) suggested that micelle formation between polyene and sterol molecules takes place and that the joining of micelles on opposite sides of the membrane can give rise to aqueous pores through that membrane. The steady development of increasing permeability towards K^+ could be a consequence of the changes in formation and distribution of micelles in the membrane after uptake of the drug. An alternative explanation would be that AME initially binds to non-specific surface structures and then redistribution occurs; the action of ergosterol in preventing further changes of permeability in organisms resuspended in the absence of drug would then be due to removal of AME from non-specific sites before redistribution occurs.

The electrode method described in this paper enables an assessment to be made of the sensitivity of cells of different species and its variation under different conditions. Cells classified as resistant as a result of conventional growth tests require a higher concentration of AME to produce a given change in K^+ permeability than do cells classified as sensitive. Mouse LS cells were 10 to 30 times more resistant to amphotericin, AME and nystatin than *Candida albicans* suspensions; little difference in sensitivity was found for perimycin and candididin whereas mouse cells proved more sensitive to filipin than the yeast.

Lampen *et al.* (1962) found that labelled nystatin or *N*-acetylcandidin was rapidly taken up at 0 °C by isolated cell walls or protoplast membranes from *Saccharomyces cerevisiae*, whereas the binding sites were relatively inaccessible or unreactive in intact organisms so that binding was slow at 0 °C. Table 3 confirms the absence of response at 0 °C for *Candida albicans* and shows that the release of K^+ has a high temperature coefficient. The release of K^+ must be the result of a complex series of events following uptake of the drug, and immobility of the membrane lipid at low temperatures may be a further factor in the absence of response at 0 °C. Lampen *et al.* (1962) suggested that metabolic energy might be required to alter the accessibility of sites or to move antibiotics to them; Table 4 shows that inhibitors such as iodoacetamide and dinitrophenol markedly increase the time taken for AME to affect the permeability but no metabolic inhibitor has so far been found which completely prevents the effect of AME.

The inhibition of growth produced by polyene antibiotics can be antagonized by addition of sterols to the test medium (Gottlieb *et al.* 1960; Zygmunt & Tavormina, 1966; Lampen, 1966) while sterols reduce the rate of K^+ release in the presence of AME or nystatin as shown above. However, Table 7 shows that ergosterol added to the suspension at a concentration 4 to 6 times that of AME reduces the action of the latter by not more than 60 to 75 %. The complete antagonism reported in growth tests must rest on a number of factors, including delay in the onset of damage in the presence of sterol, rapid inactivation of some polyenes under the conditions of the growth tests, and the action of ergosterol in preventing further damage to the cells after the drug has been removed (Fig. 6). It has not been possible to reverse or repair damage in cells exposed to AME by subsequent addition of ergosterol, and Lampen *et al.* (1962) reported that labelled nystatin, once bound, could not be displaced by excess cold drug.

Results given here and by others indicate that polyene antibiotics react with sterols and their action can be antagonized by sterols. It is of interest that the immunosuppressive

drug 1,4-pregnadiene-11 β ,17,21-triol-3,20-dione reduces the action of AME (Table 7). Of the sterols tested, zymosterol was the most effective in reducing the action of AME, and ergosterol was about twice as active as cholesterol on a molar basis. The interpretation of 'antagonism' data must await further experiments on the absorption of the drug in the presence and absence of sterols but it would seem probable that ergosterol has a higher affinity for AME and nystatin than does cholesterol. Since ergosterol forms the major sterol in *Candida albicans* membranes (M. Marriott, unpublished) while cholesterol is the main sterol of mammalian cells, such a difference in affinity could provide the basis for the selective effect shown in Table 6 and the reason why AME, amphotericin B and nystatin can be used for the treatment of *Candida* infections in Man.

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