Phenotypic Resistance to Amphotericin B in *Candida albicans*: Relationship to Glucan Metabolism

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The phenotypic resistance to amphotericin methyl ester (AME) of stationary phase cultures of *Candida albicans* was decreased by alkaline pH values and by treatment with 2-mercaptoethanol or glucanase preparations, and was increased by acid pH values, increased aeration, treatment with *N*-ethylmaleimide, or the presence of inhibitors of protein synthesis such as trichodermin. The effects of such treatments on endogenous glucanase activity and on the incorporation of glucose residues into the ‘glucan fraction’ of the organism were studied. The changes in the endogenous levels of lytic activities on laminarin [as a measure of the total (1→3)-β-δ-glucanase] and on *p*-nitrophenyl-β-δ-glucoside [reflecting the exo-(1→3)-β-δ-glucanase] were followed in *C. albicans* cells under a variety of conditions. Treatments which increased AME sensitivity stimulated both total and exo-(1→3)-β-δ-glucanase activities, while treatments which promoted resistance decreased the levels of both (1→3)-β-δ-glucanases. Changes in the ‘glucan fraction’ were followed by incubating suspensions of organisms in the presence of trace amounts of [U-14C]glucose. The rate of incorporation of radioactivity fell during the first 2–3 d of stationary phase culture and then rose to high values by 7–8 d; AME resistance increased throughout this period. The rate of incorporation was markedly stimulated by prior treatment of the organisms with 2-mercaptoethanol or glucanase and inhibited by trichodermin or treatment with *N*-ethylmaleimide.

The addition in the concentration range 0.3–3 mM of the glucose analogues β-δ-allose, 3-O-methyl-δ-glucose, 2-deoxy-δ-glucose or 5-thio-δ-glucose to cultures 24 h after inoculation prevented any further increase in AME resistance for the next 2–3 d and resulted in a decrease in the level of resistance established at the time of addition. Radioactivity from 14C- or 3H-labelled analogues added, 24 h after inoculation, to stationary phase cultures was incorporated into the ‘glucan fraction’ of the organisms.

The incorporation of glucose residues into the ‘glucan fraction’ is controlled by the activity of glucanases in producing glucose acceptor sites. The results reported confirm that there is a correlation between glucan metabolism, glucanase activity and resistance to AME, in that any factor leading to increased glucanase action also results in decreased resistance and vice versa, while incorporation of certain glucose analogues into the ‘glucan fraction’ delays the further increase in resistance.

**INTRODUCTION**

The sensitivity of *Candida albicans* towards amphotericin methyl ester (AME) can be measured in terms of the amount of antibiotic required to induce the release of K⁺ at a standard rate from suspensions of the organism (Gale, 1974). Organisms grown in batch

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culture are highly sensitive during the phase of exponential growth (0.1–0.3 µg AME ml⁻¹) but lose this sensitivity after the cessation of growth, so that organisms that have been in the stationary phase for several days may have a resistance two to three orders of magnitude greater than that of exponential phase organisms (Gale, 1974; Gale et al., 1975). Spheroplasts prepared from stationary phase organisms have the same sensitivity as those prepared from exponentially growing organisms, indicating that the difference in sensitivity shown by the intact organisms must result from changes in the cell wall (Gale et al., 1975). The development of such phenotypic resistance may be accelerated or prevented, and its extent modified, by controlling the culture conditions (pH, aeration, composition of the culture medium) or by treating the organisms with SH-reactive agents, inhibitors of protein synthesis, or cell-wall lytic enzymes (Gale et al., 1975, 1977, 1978, 1980). Analyses have shown that stationary phase organisms contain more lipid than those from exponential phase, but the differences in the antagonism to amphotericin B of lipid extracts from both kinds of organisms are not sufficient to account for the changes in sensitivity (Gale et al., 1975). Ultrastructural and analytical studies have shown that exponential and stationary phase organisms differ in wall thickness and layering, and that glucan is the cell-wall polysaccharide undergoing detectable changes after cessation of growth (Cassone et al., 1979). The involvement of the cell-wall glucan in the onset of polyene resistance in C. albicans is also suggested by the fact that treatment with (1→3)-β-D-glucanases of diverse origins is an effective method of reducing the resistance to AME of stationary phase and starved organisms (Gale et al., 1980).

(1→3)-β-D-Glucanases are believed to play a morphogenetic role in yeasts, especially in those where (1→3)-β-D-glucan is the component responsible for the rigidity and strength of the cell wall. These hydrolytic enzymes would create regions within pre-existing polysaccharide structures where glucose or newly synthesized oligosaccharides could be inserted by the biosynthetic enzymes (Johnson, 1968). Villa et al. (1980) have presented evidence for a reciprocal interdependence between endogenous (1→3)-β-D-glucanases and turnover of several cell wall components at the end of active growth in the yeast Pichia polymorpha. Preliminary results have shown that the production of endogenous cell-wall lytic enzymes in C. albicans, when growing in batch cultures at 37 °C, reaches a maximum during the early exponential phase of growth and then decreases continuously to almost undetectable levels in stationary phase organisms (Notario, 1982).

The possible correlation between decrease in endogenous cell wall lytic enzymes and decrease in antibiotic sensitivity, the changes in the glucan fractions of the cell walls in relation to AME resistance, and the efficacy of exogenous (1→3)-β-D-glucanases in decreasing the resistance to AME, suggest a possible role for the endogenous glucanases in the onset of the phenotypic resistance to amphotericin B. The present study describes the alterations in the endogenous levels of (1→3)-β-glucanases, and the changes that occur in the insertion of glucose residues into the 'glucan fraction', when the organism is submitted to treatments which modify its response to AME.

**METHODS**

**Organism.** *Candida albicans* strain 6406 was obtained from the Mycology Reference Laboratory of the London School of Hygiene and Tropical Medicine. Stock cultures were maintained on agar (2%, w/v) slopes of yeast nitrogen base (Difco; 6.7 g l⁻¹) containing 1% (w/v) glucose (YNBG medium).

**Culture conditions.** The organisms were subcultured from slopes into tubes containing 5 ml YNBG medium and incubated at 37 °C for 12 h. Each 5 ml subculture was used to inoculate a 2 litre flask containing 250 ml YNBG medium. The flasks were then incubated aerobically at 37 °C in an orbital shaker (Gallenkamp) at a setting of 200 rev. min⁻¹. Alternatively, organisms were grown in 1 litre of YNBG medium in a continuous-flow fermenter (FL101, AB Biotec, Stockholm, Sweden) under aerobic conditions (air flow 2.7 l min⁻¹) at 37 °C with a rate of stirring of 1000 rev. min⁻¹. These conditions, as well as the medium supply and pH, were monitored and controlled with an LP100 Process Instrumentation Panel (AB Biotec). After inoculation, the organisms were grown for 16 h;
continuous flow of medium \((D = 0.15 \text{ h}^{-1})\) was then started and continued for 36 h. The supply of medium was then stopped but aeration, stirring, temperature and pH control were continued for up to 8 d (Gale et al., 1977).

Estimation of K\(^+\) release and AME sensitivity. Washed suspensions of organisms (1 mg dry wt \(\text{ml}^{-1}\)) were suspended in 0-03 M Tris/HCl buffer pH 7.5 and the release of K\(^+\) was monitored by use of a K\(^+\)-sensitive electrode (Gale, 1974). Sensitivity is expressed in terms of standard release concentration (s.r.c.), i.e. the concentration, in \(\mu\text{g AME ml}^{-1}\), inducing the release of 1 nmol K\(^+\) min\(^{-1}\) (mg dry wt organisms\(^{-1}\)) above that in the control without AME. In experiments involving highly resistant organisms and the effect of enzymes thereon, estimations of the s.r.c. equivalent were made as described by Gale et al. (1980).

Preparation of cell-free extracts. Samples of the cultures (approx. 30 g dry wt of organisms) were withdrawn at timed intervals, and the cells were collected by centrifugation at 2000 \(g\) for 10 min, washed twice with 100 ml 50 mm-acetic acid/sodium acetate buffer pH 5.5, and resuspended in the same buffer at a density of 200 mg dry wt \(\text{ml}^{-1}\). Portions (20 ml) were disrupted with 50 g of glass beads (0-45-0.50 mm diameter) in a Braun MSK homogenizer, with refrigeration, at 5000 oscillations min\(^{-1}\), for 90-120 s. The extent of cell breakage was greater than 98% in every case. After breakage, the remaining whole cells and cell walls were sedimented by centrifugation at 2000 \(g\) for 10 min; the supernatant fraction was centrifuged at 40000 \(g\) for 40 min. This final supernatant was dialysed overnight against approximately 81 5 mm-acetic acid/sodium acetate buffer pH 5.5 and then concentrated 10-fold by dialysis against polyethylene glycol flakes. The resulting preparation was used as the cell-free extract.

Cell wall purification. The first pellet obtained from the cell breakage was resuspended in 100 ml 0.5 m-sucrose, centrifuged 5 min at 800 \(g\), and washed twice with 100 ml of the same solution. The supernatants were pooled and centrifuged at 1500 \(g\) for 15 min, and the pellet was washed five times with 100 ml each of the following solutions: 0.5 m-sucrose, 2 m-NaCl and 0-1 m-NaCl, in that order, and 10 times with water. Cell wall purity was checked throughout the process by determining the nucleic acid content (as \(A_{260}\) of the supernatant after each washing, and examination under the phase-contrast microscope for absence of unbroken cells, decrease in the proportion of spherical bodies, and homogeneity.

Treatment with SH-reactive agents. Treatments were based on the procedure described by Gale et al. (1975). Either N-ethylmaleimide (NEM) or 2-mercaptopropanol (2-ME), at final concentrations of 1 mm and 0.2 m, respectively, were used to treat: (a) organisms under culture conditions in YNBG medium at different stages of growth; (b) organisms centrifuged out of the growth medium and resuspended at a density of 1 mg ml\(^{-1}\) in a medium containing 2 g KH\(_2\)PO\(_4\), 1 g (NH\(_4\))\(_2\)HPO\(_4\) and 10 g (NH\(_4\))\(_2\)HPO\(_4\), adjusted to a final pH of 6.5 (SY buffer); (c) cell walls purified from organisms harvested at different stages of growth, resuspended in SY buffer as above; and (d) crude cell-free extracts adjusted to pH 6.5. In all cases, incubations were carried out after addition of SH-reagents for up to 2 h at 37\(^\circ\)C. Samples were withdrawn at intervals and either washed twice with 50 mm-acetate buffer pH 5.5 \((a, b \text{ and } c)\) or adjusted to pH 5.5 with the same buffer \((d)\), before determination of \(\beta\)-glucanase activities. Treatment of enzymes with NEM was carried out under the same conditions, excess NEM being titrated back with 1-6 m-glutathione before use of the enzymes for incubation with \(C.\) albicans.

Enzyme treatments. Cultures grown in batches of 250 ml of YNBG medium under otherwise standard conditions for 7-8 d were adjusted to the optimum pH value for each enzyme. Culture samples containing 50 mg dry wt organisms were transferred to 250 ml flasks containing each of the enzyme preparations, to give a final concentration of 100 \(\mu\text{g enzyme protein (mg dry wt organisms)}^{-1}\), and then returned to the incubator. Samples were taken at timed intervals up to 24 h. The organisms were collected by centrifugation, and either \((a)\) washed twice with 50 mm-acetate buffer pH 5.5 and used to determine \(\beta\)-glucanase activities in situ, or \((b)\) washed twice in water and resuspended in 0-03 M-Tris/HCl buffer pH 7.5 for \(K^+\) release determinations.

Other treatments, (i) The exhaustion of endogenous substrates was delayed by growing the organisms in YNBG medium supplemented with sodium glutamate at 1, 3 or 5 mg ml\(^{-1}\) or with vitamin-free Casamino acids (Difco) at 3 or 10 mg ml\(^{-1}\); (ii) protein synthesis was inhibited by adding trichodermin (up to 20 \(\mu\text{g ml}^{-1}\)) to the culture or suspension medium; (iii) the effect of pH changes was followed by growing the organisms in YNBG medium with the pH adjusted by the addition of 6 m-HCl, 3 m-NaOH or 0-1 m-KH\(_2\)PO\(_4\)/NaOH buffer as required. All treatments were applied to organisms from different phases of culture; the organisms were collected by centrifugation, washed twice with 50 mm-acetate buffer pH 5.5, and used to determine \(\beta\)-glucanase activities.

Assay of \(\beta\)-glucanase activity. Activities on laminarin, as a measure of the total glucanase, and on \(p\)-nitrophenyl-\(\beta\)-D-glucoside (pNPG), as a measure of the exo-glucanase, were assayed at 37\(^\circ\)C (Notario, 1982). One unit of \(\beta\)-glucanase activity was defined as the amount of enzyme which liberated 1 nmol product min\(^{-1}\) under the assay conditions. Activities quoted represent the mean of at least three separate experiments.

In situ assay of \(\beta\)-glucanases. Both total and exo-(1\(\rightarrow\)3)-\(\beta\)-D-glucanases were determined in situ by a modification of the procedure described by Serrano et al. (1973). Samples of culture (approx. 20 mg dry wt organisms) were washed with 50 mm-acetate buffer pH 5.5 and resuspended in the same buffer to a final volume of 2 ml, and 0-1 ml toluene/ethanol \((1:1, v/v)\) was then added. The mixture was cooled in an ice bath, mixed for 5 min, diluted with two volumes of ice-cold buffer, kept in ice for 10 min and then centrifuged for 5 min at 2000 \(g\). Cells rendered permeable in this way were washed twice with 2-5 ml 50 mm-acetate buffer pH 5.5 and
resuspended in 1 ml of this buffer. These suspensions were used as enzyme source for assaying \( \beta \)-glucanase activities as described above. Reactions were stopped by centrifuging the cells down for 10 min at 2000 \( \times \) g and then the products were determined in the supernatants.

**Incorporation of radioactive substrates.** Samples of cultures (2-0 ml) of known density were adjusted to pH 4-5 or as stated elsewhere, and incubated in 10 ml centrifuge tubes at 37 \( ^{\circ} \)C. An amount of radioactive substrate was added and the contents of the tube were aerated on a Whirlimixer (Scientific Industries, U.K.) for 3-4 s. The incorporation of \([U-\text{\textsuperscript{14}}\text{C}]\text{glucose} \) (specific activity 280-325 mCi mmol\(^{-1} \); 10-4-12.0 GBq mmol\(^{-1} \)) was directly related to glucose concentration over the range 0-1-1.0 \( \mu \)Ci ml\(^{-1} \) unless otherwise stated, experiments were carried out at a final \([14\text{C}]\text{glucose} \) concentration of 0-2 \( \mu \)Ci ml\(^{-1} \) (about 0.6 \( \mu \)m). Specific activities, amounts and concentrations of labelled analogues are given in Tables 3 and 4. Incubation was stopped at required intervals by addition of 2-0 ml 10% (w/v) trichloroacetic acid (TCA) and the tubes plunged into ice. When the incubation continued for longer than 10 min, the tubes were re-aerated at 10 min intervals.

**Estimation of radioactivity.** The incorporation of \( ^{14} \text{C} \)-labelled substrates was estimated in preliminary experiments by plating samples containing not more than 2 mg dry wt of a given fraction on metal discs, 2 cm diameter, and assaying the radioactivity using an end-window Geiger-Müller tube and associated scaler. \( ^{14} \text{C} \)-labelled and \( ^{3} \text{H} \)-labelled materials were otherwise estimated by mixing samples with 5-0 ml scintillant, consisting of 0-4%, (w/v) 2,5-bis\( \beta \)-tert-butylbenzoxazolyl-(2')-thiophen, 750 ml toluene and 250 ml Triton X-100, and assaying the radioactivity in a Packard Liquid Scintillation Spectrometer model 3385.

**Preparation of the ‘glucan fraction’.** Following the termination of incubations in which isotope incorporation was measured, pellet A, obtained by centrifugation after standing the reaction tubes containing 5% (w/v) TCA for 1 h at 0 \( ^{\circ} \)C, was resuspended in 3-0 ml 0.5 m-perchloric acid. After 90-120 min at 38 \( ^{\circ} \)C the insoluble residue was centrifuged down and washed twice by resuspension in SY buffer followed by centrifugation. This material (pellet B) was then suspended in 2-0 ml SY buffer containing 500 \( \mu \)g Cytophaga L1 preparation (mg original dry wt organisms\(^{-1} \)) (Gale et al., 1980) and incubated at 37 \( ^{\circ} \)C overnight with shaking. The mixture was then separated by centrifugation and the radioactivity of the supernatant (‘glucan fraction’) and the pellet (pellet C) were separately determined. In key experiments, the ‘glucan fraction’ was released by incubation with a purified preparation of exo-(1-\( \alpha \)-3)-\( \beta \)-D-glucanase obtained from Basidiomycete QM806 and provided by Dr T. G. Villa of Salamanca University, Spain (see Gale et al., 1980). In no case was there a significant difference in the radioactivity released from pellet B by the two preparations. Extraction of pellet B with Cytophaga L1 or (1-\( \alpha \)-3)-\( \beta \)-D-glucanase, as above, released between 60 and 82% of the radioactivity according to the conditions during the incorporation. In a series of six experiments in which the ‘glucan fraction’ contained 62% (s.d. \( \pm \)4%) of the radioactivity present in the pellet, a second overnight extraction of the pellet with pronase, lipase, chitinase or (1-\( \alpha \)-3)-\( \beta \)-D-glucanase, each at 200 \( \mu \)g protein (mg dry wt initial organisms\(^{-1} \)) released a further 20\( \pm \)4\%, 8\( \pm \)3\%, 7\( \pm \)3\% and 4\( \pm \)1%, respectively. For the purposes of the present study, the radioactivity released by (1-\( \alpha \)-3)-\( \beta \)-D-glucanase or, less precisely, Cytophaga L1 under the conditions described is referred to as the ‘glucan fraction’ and expressed in terms of the dry weight of the original sample of organisms.

**Chemical fractionation.** The polysaccharides of pellet B (see above) were fractionated according to the method of Herbert et al. (1971). In a series of seven experiments with cultures grown for 24 h and then incubated for 30 min with \([14\text{C}]\text{glucose} \) under standard conditions, the distribution of radioactivity between the polysaccharide fractions was: alkali-soluble glucan 31\( \pm \)5\%, acid-soluble glucan 31\( \pm \)6\%, insoluble residue 8\( \pm \)2% and mannan 30\( \pm \)7%. The corresponding values for 6-7 d cultures were 15\( \pm \)6\%, 26\( \pm \)12\%, 38\( \pm \)7\% and 20\( \pm \)5\%, respectively.

**Hydrolysis of polysaccharide fractions.** Samples were evaporated to dryness in a vacuum evaporator and 1 m-H\(_2\)SO\(_4\) (1 ml (mg preparation\(^{-1} \)) was added. The sample was hydrolysed by autoclaving at 120 \( ^{\circ} \)C for 1 h and then neutralized to pH 4-5 by gradual addition of BaCO\(_3\). The precipitate was removed by centrifuging and the supernatant passed through a 5 cm column of Amberlite LR-120H to remove residual barium.

**Chromatography of sugars.** Sugars were chromatographed on Whatman no. 1 paper developed in ethyl acetate/pyridine/water (8:2:1, by vol.) as described by Notario et al. (1976). Radioactive sugars were located by autoradiography.

**Analytical measurements.** Reducing sugars were determined by the method of Nelson (1944), using glucose as standard. Free glucose was determined by using glucose oxidase coupled with peroxidase as described by Bruss & Black (1978). Protein was determined either by the Lowry method, using bovine serum albumin as standard, or by measuring \( A_{280} \).

**Chemicals.** \(|-\text{\textsuperscript{14}}\text{C}]\text{Glucose}, 3-O-\text{\textsuperscript{14}}\text{C}[\text{methyl-}\text{D-}\text{glucose}, 3-O-\text{methyl-}\text{D-}\text{[\text{\textsuperscript{14}}\text{C}]glucose}, 2-\text{deoxy-}\text{D-}\text{[\text{\textsuperscript{14}}\text{H}]glucose} and 2-\text{deoxy-}\text{D-}\text{[\text{\textsuperscript{14}}\text{C}]glucose} were purchased from Amersham. \(|-\text{\textsuperscript{14}}\text{H}]\text{Allose} was prepared at Amersham and purified by crystallization as the tetra-acetate. Laminarin, \( \text{n-p-nitrophenyl-}\text{\beta-D-glucose, } \beta-D\text{-allolose, } \text{d-} \text{Gallose, } \text{d-} \text{D-talose, } 3-O-\text{methyl-}\text{D-glucopyranose and 2-} \text{deoxy-}\text{D-glucose} were purchased from Sigma, and 5-thio-}\text{D-glucose from Aldrich Chemical Co., Milwaukee, U.S.A. Amphotericin B methyl ester was kindly donated by Dr W. E. Brown of E. R. Squibb & Sons, Princeton, N.J., U.S.A. and trichodermin was a gift from Leo Pharmaceutical Products, Denmark. Enzymes were purchased from sources described by Gale et al. (1980).
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RESULTS

Studies on β-glucanase activity in Candida albicans

Production of β-glucanases. When organisms were incubated in batch cultures on limiting glucose, the cell-associated amounts of both laminarinase and pNPG-ase first showed a small decrease followed by an increase in specific activities. For both activities maximum levels were reached during the exponential phase of growth, when 75–85% of the organisms were budding – after about 5–6 h for pNPG-ase and 7–8 h for laminarinase. At the maximum, the laminarinase activity was 5–6 times higher than that of pNPG-ase (Fig. 1). When cultures passed into the late-exponential/early-stationary phase of growth there was a clear decline in the levels of both activities, which became almost undetectable after the complete cessation of active growth.

The activities in starved cultures, aerated in a fermenter, were about 10 times lower than those in exponential cultures. The maximum laminarinase was attained on the first day of starvation and then decayed slowly but continuously. The pNPG-ase activity increased rapidly during starvation, reached a maximum after 2 d and then declined to undetectable levels after 6 d of starvation. In contrast to actively growing cultures, the levels of activity on pNPG in starved organisms were greater than those on laminarin, being about two-fold greater at the maximum.

Effect of pH of the culture medium on β-glucanase production. The pH value for optimum activity for both glucanases was 5.5, with 95 or 75% of the activity retained at pH 7.0 for laminarinase and pNPG-ase respectively (Notario, 1982). Four different conditions were chosen for the study of the effect of pH on the production of glucan-hydrolysing enzymes: (i) standard culture condition (control); (ii) YNBG medium initially adjusted to pH 7.0 with 3 M-NaOH; (iii) YNBG medium buffered at pH 7.0 with 0.1 M-KH2PO4/NaOH buffer; or (iv) YNBG medium adjusted to pH 3.0 with 6 M-HCl. The initial pH values of the media in (i) and (ii) were 5.5 ± 0.2 and 7.0 ± 0.1, respectively, but dropped to about 3.0 during growth in both cases. In media (iii) and (iv) the pH remained roughly constant at the desired values.

The change in both laminarinase and pNPG-ase activities with time followed the same pattern in all cases. The highest activity was displayed by organisms in the culture maintained at pH 7.0. In cultures initially adjusted to pH 7.0 but falling to pH 3.0 during growth, the activity was greater at all stages than that in the control under standard culture conditions. Conversely, the activity in cultures initially adjusted to pH 3.0 was less than that in the control.

Fig. 1. Production of β-glucanases by C. albicans in batch culture. Activities on laminarin (○) and p-nitrophenyl-β-D-glucoside (pNPG) (●) were determined in situ from samples (10 ml) of cultures taken at times shown; □, growth; ■, glucose concentration in the culture medium.
The effect of pH on cultures starved and aerated in the fermenter was similar. When the pH value was maintained at 3.0 both activities were smaller than at pH 7.0 (about 56% and 34% lower for laminarinase and pNPG-ase, respectively) for up to 6 d. After 7-8 d starvation the amounts of both activities were so low that the differences were not significant.

**Effect of changes in the glutamate content of the pool.** Gale et al. (1978) found that the resistance of stationary phase organisms was correlated with the glutamate content of the low molecular weight pool of the cells, and that the onset of resistance could be delayed by the addition of glutamate to the initial growth medium. Supplementation of YNBG medium with either sodium glutamate or vitamin-free Casamino acids affected the pattern of production of glucan-hydrolysing enzymes during growth of C. albicans. Compared with control cultures, the following differences were noted for the supplemented ones: (1) the initial increase in specific activities of both laminarinase and pNPG-ase was slower; (2) although maximal activity occurred at the same time in all cultures, that of the supplemented ones was less than that of the control; (3) the loss of activity after the peak was slower than in the control; (4) after the end of growth a second peak of both activities occurred, the height of this second peak being inversely related to the glutamate concentration; and (5) the levels of both activities remained higher than those of control cultures until exhaustion of glutamate from the extractable pool of the organisms.

**Effect of trichodermin.** Trichodermin at concentrations up to 20 µg ml⁻¹ was added at intervals to cultures growing in YNBG medium (which contains 1% glucose). In 4 or 7 h cultures, laminarinase and pNPG-ase activities were 50% inhibited by 60 min exposure to 2.5 and 20 µg trichodermin ml⁻¹, respectively; in 24 h cultures laminarinase activity was 50% inhibited by 120 min exposure to 10 µg ml⁻¹ while pNPG-ase activity was not significantly affected by 20 µg ml⁻¹.

**Effect of SH-reactive agents on endogenous β-glucanases in C. albicans.** Cells, cell wall preparations and cell-free extracts were tested for glucanase activity before and after treatment with 2-ME or NEM in 0-1 M-phosphate buffer pH 6-5 as described in Methods. With all three preparations, 2-ME promoted an almost instantaneous increase in laminarinase activity which reached a peak after about 10 min treatment, then decayed and increased again slowly (Fig. 2). In general, the older the cultures, the greater the 2-ME stimulation. Thus, after 10 min treatment with 2-ME the laminarinase present in cell-free extracts from cultures 6 d old was increased about 15-fold and therefore comparable to that exhibited by cultures 7-8 h old (Fig. 1). NEM invariably decreased the levels of laminarinase but its action was much slower than that of 2-ME, requiring 2-3 h to induce about 50% inhibition (Fig. 1). The effects of both 2-ME and NEM on pNPG-ase activity in whole cells, cell walls and cell-free extracts were qualitatively similar but quantitatively different. The extent of 2-ME stimulation or NEM inhibition was less than for laminarinase, and the rate of each process was slower.

**Treatment with exogenous enzymes.** Figure 3 depicts the effect on the cell-associated laminarinase activity of treatment of 7 d old organisms with exogenous lipase, trypsin or chitinase. Similar results were obtained for pNPG-ase. All the exogenous enzymes, separately or in mixtures, increased both endogenous activities over the first 4 h of treatment. After a quick decline (between 4 to 6 h) the activities increased progressively for at least 24 h. Chitinase alone, or in combination with lipase or trypsin, was the most effective enzyme after 24 h treatment.

Gale *et al.* (1980) described several β-glucanase preparations as the enzymes most effective in reducing the AME resistance of stationary phase organisms. Attempts were made to test their efficacy in increasing the levels of endogenous glucanases. Preliminary experiments showed that the exogenous β-glucanases bound to the cells, making it impossible to distinguish between exogenous and endogenous activities after treatment. However, assuming that the amount of exogenous enzyme bound to the cells remained constant throughout the 24 h incubation, and that the endogenous enzymes were not released into the medium, it was
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Fig. 2 Fig. 3

Fig. 2. Effect of β-mercaptoethanol (○) and N-ethylmaleimide (□) on laminarinase activity in cell-free extracts prepared from organisms grown for 72 h under standard conditions. Results are expressed as percentages of the initial (t = 0) activity of untreated extract (●), which was 1.35 units glucanase (mg dry wt organisms)^{-1}.

Fig. 3. Enzymic treatments of amphotericin-resistant organisms (7 d old cultures). The activity on laminarin was measured after treatment with chitinase (●), lipase (□) or trypsin (■). Untreated organisms (○) were used as control. Treatment conditions are given in the text.

estimated that Cytophaga L1 and Basidiomycete QM806 β-glucanase were, respectively, 2 and 6–7 times more effective than chitinase.

Incubation of resistant organisms with glucanases: effects of SH-reactive agents. Gale et al. (1980) have shown that incubation of resistant cultures of C. albicans in the stationary phase of growth with Cytophaga L1 or other (1→3)-β-D-glucanase preparations brings about a marked reduction in the resistance of the organisms to AME. ‘Sensitivity profiles’ (Gale et al., 1980) were determined on organisms from a 7 d old batch culture (initial s.r.c. value, 80) incubated with 50 µg Cytophaga L1 preparation (mg dry wt cells)^{-1} for 24 h at an initial pH of 6.5. As previously described, the resistance decreased to an s.r.c. value of 4.5. Treatment of the enzyme preparation with NEM prior to the experiment (see Methods), had no effect. Incubation of the organisms without added enzyme resulted in a decrease in resistance from the initial s.r.c. value of 80 to a value of 24. If the cells were treated with NEM prior to enzyme treatment (see Methods), then this decrease in resistance of the control cells was abolished but the Cytophaga L1 preparation still brought about a marked decrease. Prior treatment of the organisms with 2-ME markedly increased the subsequent reduction of resistance by the Cytophaga L1 preparation. The experiment was repeated with purified (1→3)-β-D-glucanase with essentially the same results.

The decrease in resistance during incubation involved two effects: an endogenous system, and an action of added glucanase. Treatment of the cells with NEM abolished the former, but had little effect on the action of either Cytophaga L1 or the (1→3)-β-glucanase preparation used. The question remained whether the reduction of resistance in the absence of the added enzyme preparations was due to the action of endogenous glucanases which are inhibited by NEM. Table 1 shows the effect on the sensitivity to AME of incubating the organisms in SY buffer adjusted to various pH values. Organisms were harvested from the growth medium at pH 3; incubation at pH 6 or 7 resulted in decreased resistance while subsequent incubation at pH 3 raised resistance to the original value or higher. Incubation at pH 8 resulted in highly resistant organisms which did not respond to subsequent incubation at
Table 1. Effect of pH on AME resistance of C. albicans in the stationary phase of growth

Cultures were grown for 4 d in batches on a rotary incubator and harvested, and the standard release concentration (s.r.c.) was determined. Samples of cultures were taken, the pH was adjusted as below, and incubation was continued for 24 h at 37 °C with shaking; the organisms were then harvested and the s.r.c. was determined. In the table below, each arrow indicates an incubation of 24 h at the pH value indicated at the head of the arrow; s.r.c. values were determined after the incubation period in each case. The pH of the initial medium (4 day culture) was 3.

<table>
<thead>
<tr>
<th>Incubation at pH:</th>
<th>s.r.c. (µg AME ml⁻¹)</th>
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<tbody>
<tr>
<td>3</td>
<td>27→4.7</td>
</tr>
<tr>
<td>6</td>
<td>27→16→3.5</td>
</tr>
<tr>
<td>7</td>
<td>16→(3.5)*</td>
</tr>
<tr>
<td>8</td>
<td>28→(3.5)*</td>
</tr>
<tr>
<td>25</td>
<td>25→4.2</td>
</tr>
<tr>
<td>32</td>
<td>32→(4.2)*</td>
</tr>
<tr>
<td>&gt;50</td>
<td>&gt;50→50</td>
</tr>
</tbody>
</table>

* Samples incubated for 24 h at pH 7, pH then readjusted to 6 or 3 and incubation continued for a second 24 h.

low pH values. Notario (1982) has shown that C. albicans glucanase activity is increased by 2-ME, is inhibited by NEM, has a broad pH optimum at pH 5.5–6.0, retaining 75–95% of the activity at pH 7.0, and is inactivated at pH 8 or higher.

Incorporation of radioactivity from [¹⁴C]glucose into the ‘glucan fraction’

In these experiments C. albicans was grown under aerobic conditions in YNB medium initially containing 1% glucose as the main source of carbon and energy. The glucose of the medium was exhausted after about 12 h incubation and the organisms then went into stationary phase. The phenotypic increase in AME resistance occurs during this phase. Previous work, described above, suggested that changes in cell wall glucan play a key role in producing AME resistance and that attention should be concentrated on the metabolism of glucan after the cessation of active growth. Glucan synthesis could be monitored by following incorporation of labelled glucose, but the addition of more than trace amounts of glucose would result in increased reducing conditions and cell growth. Johnson (1968) and Villanueva et al. (1976) have proposed that glucose residues can be inserted into glucan polymers at the position of breaks produced by glucanase action. The method adopted in these experiments was therefore to incubate organisms with trace amounts of glucose of high specific activity and follow the incorporation of radioactivity into the ‘glucan fraction’ as defined in Methods. This is the fraction released by treatment either with the Cytophaga L1 preparation or exo-(1→3)-β-D-glucanase under the conditions used by Gale et al. (1980) for decreasing the AME resistance of stationary phase C. albicans. Radiochromatography of the ‘glucan fraction’ obtained from organisms labelled as described below and the pellet B (see Methods) then extracted with exo-(1→3)-β-D-glucanase showed that the radioactivity ran as a single spot corresponding to a glucose marker with a small amount of activity remaining at the origin. After hydrolysis, all the radioactivity ran as a single spot corresponding to glucose with a small spot corresponding in position to mannose.

Conditions for glucose incorporation. Since the AME resistance of stationary phase cultures can be markedly affected by environmental changes, the cultures were manipulated as little as possible. Culture samples were taken and the pH was adjusted directly by addition of 1 M-NaOH. The optimum pH for incorporation was about 4.5, at which value 0.2–35 pmol glucose (mg dry wt organisms)⁻¹ (assuming no dilution with endogenous sugar) was incorporated into the ‘glucan fraction’ in 10 min at 37 °C. Since radiochromatography
AME resistance and glucan metabolism in C. albicans

Fig. 4. Variation with incubation time of the sensitivity to AME and the rate of incorporation of \[14C\]glucose into the glucan fraction of C. albicans. The organisms were grown in YNBG medium in flasks in a rotary incubator (A, B) or under starved and aerated conditions in a fermenter (C, D) as described in the text. AME sensitivity (B, D) was estimated as the standard release concentration (s.r.c.) in \(\mu g\) AME ml\(^{-1}\). For estimations of glucose incorporation (A, C), samples of culture were taken, the pH was adjusted to 4.5, 0.2 \(\mu\)Ci \([U-14C]\)glucose ml\(^{-1}\) was added (sp. act. 325 mCi mmol\(^{-1}\); final concentration 0.6 \(\mu\)M), and samples were taken at intervals over 20 min at 37 °C. The ‘glucan fraction’ was extracted as described in Methods. Results were calculated from 12 separate flask and three fermenter experiments; the bars represent standard deviations.

showed that the radioactivity of the extracts was located solely in glucose, results were expressed in terms of pmol glucose incorporated, except in cases where some variation in metabolism might have affected the nature of the incorporation.

Variation with phase of growth. AME resistance and the rate of glucose incorporation into the ‘glucan fraction’ varied with the time for which the culture had previously been incubated (Fig. 4). Curves A and B in Fig. 4 relate to batch cultures grown in Erlenmeyer flasks on a rotary incubator, curves C and D to cultures grown, starved and aerated in a fermenter as described by Gale et al. (1977). The first estimations were made 24 h after inoculation of the batch culture or 24 h after the fermenter culture had been switched to conditions of starvation and high aeration; the main difference between the two systems was that the rate of aeration in the fermenter was greater than that in the orbital system. In both cases, the 24 h culture had a relatively high rate of incorporation and low resistance to AME. On continued incubation the AME resistance increased as previously described while the rate of incorporation at first fell to a low value and then rose rapidly. After 5 d in the fermenter culture or 7 d in the batch culture, the resistance had risen to values too great to be determined and the rate of incorporation began to fall. The relative progress of resistance and
incorporation was the same in both cultures but the changes occurred more rapidly in the highly aerated fermenter culture than in the batch culture.

It was shown in the first section that glucanase activities in C. albicans are high during exponential growth and decrease as the culture passes into the stationary phase; in the later stages of stationary phase, glucanase activity can be evoked by reduction of the organisms through metabolic activity (see Gale et al., 1978) or treatment with 2-ME. Glucanase activities are lower in the fermenter than in the batch cultures.

**Effect of trichodermin and treatment with SH-reactive agents.** Treatment of the organisms with NEM prior to incubation with [14C]glucose almost abolished incorporation into the 'glucan fraction' (Fig. 5). Prior treatment with 2-ME markedly stimulated the incorporation. In three separate experiments, the standard treatment of the cell pellet, after extraction with cold TCA and perchloric acid, with (1→3)-β-D-glucanase released 65% (s.d. ±5%) of the radioactivity incorporated by the control organisms and 82±3% of that incorporated by 2-ME-treated organisms. It was shown above that NEM inhibits C. albicans glucanases while 2-ME stimulates both their activities *per se* and their release from latent forms. In the course of this work it was observed that whereas treatment of organisms with 2-ME for 1 h produced the usual decrease in AME resistance and increase in glucose incorporation, prolonged
AME resistance and glucan metabolism in C. albicans

Table 2. Effect of trichodermin on the incorporation of [14C]glucose into the 'glucan fraction' of C. albicans in the stationary phase of growth before and after treatment with Cytophaga L1

C. albicans was grown (a) in batch culture on a rotary incubator or (b) in a fermenter at high aeration rate. In (a) trichodermin at a final concentration of 20 μg ml⁻¹ was added after 24 h growth; in (b) trichodermin at the same final concentration was added at the time medium flow was stopped and the culture entered a condition of starvation (see Methods). Samples were taken at 24 h intervals and the pH was adjusted to 4.5; [14C]glucose (sp. act. 296 mCi mmol⁻¹, final concentration 2 μCi ml⁻¹ – 0.6 μM) was added and the incorporation into the 'glucan fraction' estimated after 5 and 10 min at 37 °C. Parallel samples were taken and pretreated with 300 μg Cytophaga L1 mg⁻¹ for 3 h at 37 °C and pH 6.5, washed and resuspended at pH 4.5 for estimation of glucose incorporation.

Glucose incorporation (pmol (5 min)⁻¹ (mg dry wt organisms)⁻¹)

<table>
<thead>
<tr>
<th>Time after</th>
<th>Before treatment</th>
<th>After Cytophaga L1 treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>trichodermin addition (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Batch culture</td>
<td>0</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>(b) Fermenter culture</td>
<td>0</td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Treatment (24 h or longer) with 2-ME gave organisms with high resistance and decreased incorporation.

The presence of trichodermin during incubation with glucose inhibited incorporation into the 'glucan fraction' (Fig. 5). At a final concentration of 5 μg ml⁻¹, trichodermin inhibited glucose incorporation by about 30%; at 20 μg ml⁻¹ the inhibition rose to 50% but remained at this value for concentrations up to 40 μg ml⁻¹. The inhibition produced by trichodermin was pH-dependent; the antibiotic had no inhibitory action at pH 7.5. Trichodermin has no effect on the activity of cell-free glucanases from C. albicans, but cells grown in the presence of trichodermin lose glucanase activity within 24 h.

Reversal of glucose incorporation. Attempts were made to demonstrate a reversal or chase of radioactivity incorporated into the 'glucan fraction'. Organisms were pre-labelled either by short-term incubation with [14C]glucose, as above, or by overnight growth in YNBG medium containing [14C]glucose. The organisms were then washed and resuspended in SY buffer pH 4.5, either alone or in the presence of excess unlabelled glucose. In no case was it possible to show a significant loss of radioactivity from the 'glucan fraction'.

Pretreatment of the organisms with exogenous glucanase. The schemes proposed by Johnson (1968) and Villanueva et al. (1976) for the insertion of glucose residues into glucan imply that such insertion would be limited by the availability of breaks in the glucan chain. One limiting factor in glucose incorporation into the glucan of stationary phase organisms could therefore be the endogenous glucanase activity. Figure 6 shows the effect on glucose incorporation of pretreating the organisms with the Cytophaga L1 preparation. Such treatment can result in 10- to 20-fold increase in the rate and amount of glucose incorporation into the 'glucan fraction'. For the culture tested, pretreatment with 250 μg Cytophaga L1 (mg dry wt organisms)⁻¹ was about twice as effective as that with 100 μg mg⁻¹, whereas increasing the Cytophaga L1 preparation to 500 μg mg⁻¹ was less effective (Fig. 6). Further experiments were carried out with pretreatment of organisms with 300 μg mg⁻¹. The pH optimum for glucose incorporation into 'glucan' under these conditions remained at about 4.5. The pretreatment also greatly increased the rates of incorporation for organisms harvested at various times throughout the growth period in batch fermenter cultures. It would thus appear that glucose incorporation into 'glucan' is limited by the glucanase activity of the organisms under all the experimental conditions so far studied.
Fig. 7. Effect of D-allose on the development of AME resistance in stationary phase cultures of C. albicans. Organisms were grown in YNBG medium (initial glucose concn 1%) at 37 °C; samples were taken daily and AME resistance was estimated as the standard release concentration (s.r.c.) (see Fig. 4). D-Allose was added 24 h after inoculation (arrow) to final concentrations of 0 (●), 0.3 (○), 1 (□), or 3 mM (■).

**Effect of trichodermin on incorporation of [14C]glucose into stationary phase organisms before and after treatment with Cytophaga L1.** Table 2 shows the effect of adding trichodermin to the incubation medium on the subsequent ability of the organisms, before and after treatment with Cytophaga L1, to incorporate [14C]glucose into the ‘glucan fraction’. Experiments were carried out both on batch cultures in which the trichodermin was added 24 h after inoculation, and on cultures starved and aerated in the fermenter, the trichodermin being added at the beginning of starvation. In both experiments, the rate of glucose incorporation decreased rapidly, so that organisms examined 24 h after the addition of the antibiotic had a rate of incorporation 10% or less of that of the initial culture. Incorporation fell to a negligible value after a further 24 h. In organisms tested after treatment with Cytophaga L1 (releasing the uptake from limitation by the endogenous glucanase), the incorporation also fell with time but remained significantly active after 48 h exposure of the culture to antibiotic.

**Effects of glucose analogues**

The major glucan polymer in the wall of C. albicans is (1→3)-β-linked, with occasional (1→6)-β-D linked side chains (Chattaway et al., 1968). The evidence presented so far suggests that breaking the (1→3)-β linkages results in a decrease in AME resistance and an increase in the rate of glucose incorporation into the ‘glucan fraction’. If AME resistance is associated with the formation of long-chain glucan polymers, then an increase in resistance should be prevented by incorporation of hexose residues with the 3 position either blocked or altered, or, alternatively, by the incorporation of residues which would alter the properties of the glucan, including its susceptibility to breakdown by glucanase. Studies were therefore made of the effect on the AME resistance of C. albicans of 3-O-methyl-α-D-glucopyranose, β-D-allose (the 3-enantiomorph of glucose), 2-deoxy-D-glucose, and 5-thio-D-glucose. None of these sugars supported the growth of C. albicans.

**Addition of glucose analogues to growth medium.** To avoid interference with essential glucose metabolism during active growth, the analogues were added at the end of 24 h incubation, when the glucose in the medium was exhausted and growth had ceased. Figure 7 shows the effect on AME resistance of adding allose in the range 0.3–3 mM to the medium at
AME resistance and glucan metabolism in C. albicans

Table 3. Incorporation of radioactivity from labelled glucose analogues into the 'glucan fraction' of C. albicans in the stationary phase of growth

C. albicans was grown for 24 h in medium YNBG (initial glucose concentration 1%) at 37 °C on a rotary incubator. Analogue was then added to the final concentration shown and incubation continued; at 24 h intervals samples were taken and radioactivity was determined in the 'glucan fraction'. Incorporation was calculated on the assumption that no degradation of analogue occurred; means ± S.D. are shown.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Label</th>
<th>Concentration (mM)</th>
<th>Specific activity (mCi mmol⁻¹)</th>
<th>Incubation period:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-O-Methyl-d-glucose</td>
<td>[¹⁴CH₃]</td>
<td>0.3</td>
<td>1.0</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td>[U-¹⁴C]</td>
<td>0.1</td>
<td>27.6</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>9.8</td>
<td>48 h</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>[¹³H]</td>
<td>0.1</td>
<td>247</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>83</td>
<td>48 h</td>
</tr>
<tr>
<td>D-Allose</td>
<td>[U-³H]</td>
<td>0.1</td>
<td>800</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>800</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>800</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>800</td>
<td>72 h</td>
</tr>
</tbody>
</table>

Analogue incorporated into 'glucan fraction' [pmol (mg dry wt organisms)]⁻¹

this time: the resistance which had developed by the time of addition decreased during the next 24 h and no significant increase occurred until day 4 of the incubation. There was little difference in the effects of allose within the concentration range tested but, by day 4, when resistance began to rise, the higher concentrations of allose were more effective in delaying this rise. The effect broke down at this stage and all cultures had become highly resistant by day 5. The effect of adding allose (3 mM) to the culture at time 0, 24 or 48 h was studied. Allose was very effective in preventing the onset of resistance when added at time 0 and organisms harvested after 24 h were found to have a high rate of leakage of K⁺ in the absence of added AME. Again the resistance rose rapidly by day 4. Addition of allose 24 or 48 h after inoculation confirmed that resistance established by the time of the addition subsequently decreased. On two occasions allose (3 mM) was added to cultures on day 5, when the s.r.c. value was >100: in one culture the s.r.c. value dropped to 60 after 24 h but no effect on resistance was observed in the other.

5-Thioglucose, 2-deoxyglucose and 3-O-methylglucose, tested over the range 0.3–3 mM, had similar effects to allose and were some 2–3 times as effective on a molar basis. No increase in resistance occurred until day 4 when a rapid increase occurred. With 5-thioglucose or 2-deoxyglucose, the presence of sugar at 1–3 mM resulted in a rapid rise in resistance after day 3 so that the s.r.c. value was greater than that of the control culture on day 4 (not illustrated); viable counts showed a large decrease in viability. With 1 mM-thioglucose, viability on day 4 was 0.01%, and with 1 mM-2-deoxyglucose between 1 and 10%, of that in the control culture. 3-O-Methylglucose at 3 mM had no effect on viability up to day 5 while in three cultures grown in the presence of 3 mM-allose for 4 d the viable count was 25±15% that in the control culture without added sugar.

A few experiments were carried out with other analogues of D-glucose which do not support growth of C. albicans. These included D-altrose (enantiomorphic in positions 2 and 3), D-gulose (enantioimorphic in positions 3 and 4) and D-talose (enantioimorphic in positions 2 and 4). All three prevented an increase in AME resistance after addition to the growth medium. Altrose and gulose gave rise to a loss of viability similar to that obtained with 2-deoxyglucose.

Incorporation of radioactivity from labelled analogues into the 'glucan fraction'. Labelled preparations of 3-O-methyl-D-glucose, 2-deoxy-D-glucose and D-allose were added, at final
Table 4. Incorporation of radioactivity from glucose analogues into the polysaccharides of C. albicans in the stationary phase of growth

Conditions were as for Table 3. Labelled analogues were added to cultures 24 h after inoculation and organisms harvested after a further 24 h at 37 °C; polysaccharide fractions were separated according to Herbert et al. (1971), and the radioactivity of each fraction was determined. The extent of incorporation was calculated on the assumption that radioactivity lay in analogue residues. The values shown represent mean and standard deviation for three separate experiments in each case.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration (mM)</th>
<th>Specific activity (mCi mmol⁻¹)</th>
<th>Analogue incorporation into polysaccharide fraction (pmol (mg dry wt organisms)⁻¹)</th>
<th>Distribution of radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Alkali-insoluble glucan</strong></td>
<td><strong>Acid-sol.</strong></td>
</tr>
<tr>
<td>3-O-Methyl-D-[U-¹⁴C]-glucose</td>
<td>0.3</td>
<td>9.8</td>
<td>24.5 ± 4.1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>2-Deoxy-D-[U-¹⁴C]-glucose</td>
<td>0.3</td>
<td>8.55</td>
<td>168 ± 22</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>D-[U-³H]-Allose</td>
<td>0.3</td>
<td>800</td>
<td>9.6 ± 6.2</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

concentrations in the range 0.1–3 mM, to cultures 24 h after inoculation (i.e. in the stationary phase); samples were harvested at 24 h intervals for the next 3 d. Radioactivity was found in the 'glucan fraction' (Table 3). Assuming that this radioactivity was present in residues corresponding to those of the analogue added, then growth in the presence of 0.3 mM-3-O-methyl-D-glucose led to the incorporation of 21.5 ± 0.4 pmol analogue residues (mg dry wt organisms)⁻¹. The corresponding values for incorporation of 2-deoxy-D-glucose and D-allose were 151 ± 9 and 7.8 ± 0.2 pmol mg⁻¹, respectively. In the three series of experiments, increasing the concentration of analogue in the medium led to increased incorporation in the 'glucan fraction'; there was little increase in the incorporation after the first 24 h (Table 3). The action of analogues in delaying the onset of AME resistance was obtained in the concentration range 0.3–3 mM (higher concentrations were not tested) and, assuming that glucan represents about 20% of the cell dry weight (see Gale et al., 1980), the calculated incorporation of 2-deoxyglucose at 0.3 mM is about 1 residue per 8000 glucose residues in glucan. The incorporation of allose and 3-O-methylglucose is less, although it is improbable that the whole of the glucan was uniformly involved, nor can it be assumed that the whole of the radioactivity measured lies in analogue residues. When 2-deoxy-D-[U-¹⁴C]glucose (0.3 mM, sp. act. 8.55 mCi mmol⁻¹) was added to a culture grown for 24 h, the organisms harvested after a further 24 h, and the alkali-insoluble glucan fraction prepared, hydrolysed and chromatographed, a single radioactive peak was obtained, corresponding to the 2-deoxyglucose marker.

Table 4 shows the distribution of radioactivity, obtained after 24 h incubation with labelled analogues, between the polysaccharide fractions separated by the method of Herbert et al. (1971). The distribution differed in each case; radioactivity from 3-O-methylglucose was found mainly in the alkali-soluble glucan fraction while that from allose was distributed evenly between the glucan fractions with negligible amounts in mannan.

**Discussion**

We start from the assumption, based on experiments with intact cells and spheroplasts derived therefrom, that the resistance to AME displayed by stationary phase cultures of C. albicans is due to a component of the cell wall which impedes penetration of the antibiotic to its site of action in the cytoplasmic membrane. The barrier effect increases with oxidizing
conditions or treatment of the cells with NEM, and decreases with reducing conditions. A simple explanation would be that the barrier is an SH-compound held in open configuration by reducing conditions and closed by S-S bridges under oxidizing conditions or by acetylation by NEM treatment. Studies on the penetrability of polyethyleneglycols (D. Kerridge, unpublished) do not support this idea, nor is it readily compatible with the effects of prolonged treatment with 2-ME. An alternative hypothesis is that the barrier is a component which is constantly modified in situ, the effects of reduction, etc. lying in their actions on breakdown and synthesis of that component.

Studies on treatment of resistant cells with various enzymes (Gale et al., 1980) suggest that glucan, or a particular form of glucan, forms part, at least, of the barrier. If this were the case, then alterations in resistance would depend upon the balance between synthesis and breakdown of that glucan. Notario (1982) found that (1→3)-β-D-glucanases can be extracted from the cell walls of C. albicans, and Dickerson et al. (1979) have found that purified glucan preparations from yeast contain firmly bound proteins, including (1→3)-β-D-glucanases. The glucanases of C. albicans are inhibited by NEM and stimulated by 2-ME; in stationary phase organisms, 2-ME treatment also releases glucanase activity from inactive precursors. Further, treatment with enzymes such as trypsin and chitinase – which also decrease AME resistance in stationary phase cells – liberates glucanase activity. The rapid loss of activity in organisms grown in trichodermin suggests also that glucanase, once activated, loses activity, and overall activity will then depend on further synthesis and activation.

Both endo- and exo-β-glucanases have been described as glycoproteins (Villa et al., 1978) possessing 10–15% glutamic acid in their protein moieties and SH groups presumably involved in maintaining their catalytic activity and/or structure (Notario et al., 1976), as exhibiting optimum pH values about 5.5 but retaining 70–85% of the activity at pH 7.0 while being almost completely inactivated at pH 3.0 or 8.0 (Notario, 1982), and as enzymes whose synthesis could be modified by inhibitors such as trichodermin (Santos et al., 1978). The action of most of the agents used in this study could therefore be explained as a direct effect on the enzymes themselves. However, the action of exogenous cell wall lytic enzymes, the effect of changes in the oxygen tension of the medium and the effect of other agents on starved cultures may be more easily understood within the context of the interrelationship between glucanases and cell-wall glucan described above.

Johnson (1968) demonstrated that the glucan of yeast cell walls is in a state of metabolic change and postulated that such metabolism is necessary for growth and expansion of the wall. Villanueva et al. (1976) formulated a cycle of glucan synthesis and breakdown in which insertion of glucose residues into a glucan polymer would be dependent on the provision by glucanase action of breaks or acceptor sites in that polymer. The present series of experiments has used the incorporation of radioactivity from [U-14C]glucose into the 'glucan fraction' as a measure of glucan metabolism as suggested by this scheme. Table 5 shows that the factors which activate cell-free glucanases also increase glucose incorporation and vice versa. Such incorporation is markedly increased by treatment with exogenous glucanase, presumably by increasing the number of glucose acceptor sites, indicating that the process in the untreated cell is limited by the endogenous glucanase activity. Further, those factors which increase glucanase activity and glucose incorporation also increase AME sensitivity, while inhibition of glucanase is associated with increased AME resistance.

Gale et al. (1977) noted that the addition of trichodermin, an inhibitor of protein synthesis, to the growth medium resulted in a rapid development of AME resistance, faster than in the control without antibiotic. The incorporation of glucose into the ‘glucan fraction’ decreased rapidly after addition of trichodermin to the cultures (Table 2); this agrees with the finding that glucanase activity is rapidly lost under these conditions, so that continued activity must be dependent on continued resynthesis and activation of glucanase. Although glucose incorporation also decreased with time in organisms pretreated with Cytophaga L1, it nevertheless continued after incorporation had ceased in untreated organisms (Table 2).
Table 5. Relationship between endogenous $\beta$-glucanase activity and resistance to AME in C. albicans

<table>
<thead>
<tr>
<th>Condition or treatment</th>
<th>$\beta$-Glucanase activity</th>
<th>Glucose incorporation into 'glucan fraction'</th>
<th>AME resistance</th>
<th>References*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth into stationary phase</td>
<td>Decrease</td>
<td>See Fig. 4</td>
<td>Increase</td>
<td>1, 2</td>
</tr>
<tr>
<td>pH increase 3–7</td>
<td>Increase</td>
<td>Increase</td>
<td>Decrease</td>
<td>1, 5, 7</td>
</tr>
<tr>
<td>pH maintained at 8.0</td>
<td>Inactivation</td>
<td>Inhibition</td>
<td>Irreversible increase Decrease</td>
<td>1, 7</td>
</tr>
<tr>
<td>Growth medium supplemented with glutamate (stationary phase cells)</td>
<td>Increase</td>
<td>—</td>
<td>Decrease</td>
<td>1, 5</td>
</tr>
<tr>
<td>Oxygen saturation of medium</td>
<td>decreased</td>
<td>Increase</td>
<td>Increase</td>
<td>1, 4</td>
</tr>
<tr>
<td></td>
<td>Increased</td>
<td>Increase</td>
<td>Decrease</td>
<td>1, 4</td>
</tr>
<tr>
<td>Trichodermin added to medium</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Increase</td>
<td>1, 4</td>
</tr>
<tr>
<td>Incubation with 2-ME</td>
<td>Increase</td>
<td>Increase</td>
<td>Decrease</td>
<td>1, 3, 5</td>
</tr>
<tr>
<td>Incubation with NEM</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td>Irreversible increase Decrease</td>
<td>1, 3, 5</td>
</tr>
<tr>
<td>Incubation with chitinase</td>
<td>Increase</td>
<td>Increase</td>
<td>Decrease</td>
<td>1, 6</td>
</tr>
<tr>
<td>Incubation with $\beta$-glucanase</td>
<td>(Increase)$^\dagger$</td>
<td>Increase</td>
<td>Decrease</td>
<td>1, 6</td>
</tr>
</tbody>
</table>

* References: 1, this paper; 2, Gale (1974); 3, Gale et al. (1975); 4, Gale et al. (1977); 5, Gale et al. (1978); 6, Gale et al. (1980); 7, Notario (1982).

$^\dagger$ See text.

Although the system is obviously complex, this behaviour is consistent with the possibility that, although glucanase activity is rapidly lost in the presence of an inhibitor of protein synthesis, the loss of synthetic activity occurs more slowly so that the balance between breakdown and synthesis is tipped in favour of synthesis. Under these conditions, the resistance to AME would be expected to increase more rapidly than in control cultures.

The inhibitory effect of trichodermin on glucose incorporation observed in cultures grown in the absence of antibiotics (Fig. 5) suggests that, even in short-term experiments under the conditions used, continued synthesis of glucanase is important. The effect is pH-dependent and trichodermin has little or no inhibitory action when the pH is in the region of high glucanase activity.

The evidence produced so far in this and previous papers provides a strong prima facie case for the hypothesis that a polyglucan constitutes the cell-wall component controlling AME resistance in C. albicans. It is shown above that allose, 3-O-methylglucose, 2-deoxyglucose, and 5-thioglucose added to cultures in stationary phase prevented any further increase in AME resistance for 2–3 d. In many experiments, the resistance, established by the time the analogue was added, decreased during the next 24–48 h. The analogues had diverse effects on the incorporation of glucose into the 'glucan fraction', possibly due to different effects on different glucan fractions. These will be dealt with in a later paper.

The incorporation studies described in this paper have been referred to as involving the 'glucan fraction'. In most experiments this has been the fraction released from cell pellets (after extraction with trichloroacetic and perchloric acids) by incubation with the Cytophaga L1 preparation. This is a commercial preparation containing $(1\rightarrow3)$-$\beta$-glucanases as the major enzymes but containing traces of other enzymes (see Gale et al., 1980) and, as such, is a readily available, reasonably consistent source of glucanase for experiments such as those described here. In key instances experiments have been repeated with estimations of the fractions released by exo-$(1\rightarrow3)$-$\beta$D-glucanase from Basidiomycete QM806 (see Gale et al., 1980), but there have been no significant differences in the results obtained by the two preparations. Nevertheless it is possible that the radioactive components were not released...
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entirely from (1→3)-β-glucan by extraction; hence the extracts have been referred to as ‘glucan fraction’. This ‘glucan fraction’ of C. albicans and the enzymes involved in its breakdown and synthesis in stationary phase cultures require further study. It is probably important that the effects of the analogues on AME resistance lasted for 2–3 d only, and that resistance then increased very rapidly. This period covers the time during which glucose incorporation in untreated batch cultures falls and then begins to rise again (Fig. 4). The highest rates of incorporation are obtained in cultures 6–7 d old, when the resistance has risen to unmeasurable values. It would appear that new metabolic processes begin around the 3rd to 4th day of batch cultures – after the free glutamate content of the cell pool has fallen to a negligible value (Gale et al., 1979). All these processes, and the substrates involved, must be studied in more biochemical detail before their effects on AME resistance can be properly understood.

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