Reduction of Amphotericin Resistance in Stationary Phase Cultures of *Candida albicans* by Treatment with Enzymes

By E. F. GALE,* JANE INGRAM, D. KERRIDGE, V. NOTARIO
AND FRANCES WAYMAN

Sub-Department of Chemical Microbiology, Department of Biochemistry,
University of Cambridge, Cambridge CB2 1QW

(Received 2 August 1979)

The resistance of *Candida albicans* to amphotericin B methyl ester increases rapidly as cultures enter the stationary phase of growth; organisms harvested after several days in the stationary phase may have a resistance two or three orders of magnitude greater than that of exponentially growing organisms. This resistance is decreased by incubation of the organisms with enzymes which attack components of the cell wall. Of the enzymes tested, (1→3)- β -D-glucanases are the most effective; incubation of 7 d batch cultures with exo-(1→3)- β -D-glucanase at a concentration of 10 μ g enzyme protein (mg dry wt organisms)⁻¹ for 24 h at 37 °C and pH 6.5 reduces the resistance of the organisms to a value approximating to that of exponentially growing organisms. Resistance is also decreased by treatment with chitinase, lipase, trypsin, α -mannosidase and (1→6)- β -D-glucanases but, on a specific activity basis, none of these enzymes is as effective as (1→3)- β -D-glucanase. The action of (1→3)- β -D-glucanase is markedly enhanced by the addition during incubation of chitinase, trypsin or lipase.

INTRODUCTION

The sensitivity to amphotericin B of *Candida albicans* grown in batch culture at 37 °C varies markedly with the growth phase (Gale, 1974; Hammond *et al.*, 1974). Judged by the concentration of amphotericin B methyl ester (AME) required to induce a standard rate of leakage of K⁺ under otherwise standard conditions, the sensitivity of *C. albicans* decreases from 0.1 to 0.2 μ g AME ml⁻¹ for organisms in the exponential phase to greater than 30 μ g AME ml⁻¹ for organisms in the late-stationary phase of growth. Protoplasts of stationary phase organisms have the same sensitivity to AME as those from exponentially growing organisms; the difference in sensitivity of the cells must therefore lie in alterations in the cell wall (Gale *et al.*, 1975). Ultrastructure studies (Cassone *et al.*, 1979) show that the walls of stationary phase organisms are thicker and do not show the pronounced electron-dense layering that can be seen in electron micrographs of walls of exponential phase organisms.

The resistance of stationary phase organisms can be decreased by a reduction of oxygen tension or by treatment with SH-reducing agents, or irreversibly increased by treatment with SH-binding agents such as iodoacetamide or *N*-ethylmaleimide (Gale *et al.*, 1975). Stationary phase cultures do not undergo a marked increase in resistance until endogenous H-donors, especially glutamate in the metabolic pool, approach exhaustion (Gale *et al.*, 1978). Stationary phase organisms contain more lipid than exponential phase organisms but the antagonism to AME displayed by lipid extracts from the two types of cell is not sufficiently different to explain the difference in their sensitivity (Gale *et al.*, 1975).

If phenotypic resistance of this nature is determined by the presence of specific compon-

Fig. 1. Diagrammatic representation of AME sensitivity profiles: A, sensitive organisms; B, sensitive organisms undergoing lysis; C, mixture of sensitive and resistant organisms; D, resistant organisms. *Candida albicans* was suspended at 1 mg dry wt organisms ml^{-1} in 0.03 M-Tris/HCl buffer at pH 7.5 and 20 °C and K^+ leakage was determined, by a K^+ -sensitive electrode, after adding AME at a final concentration of 1 $\mu\text{g ml}^{-1}$; if no leakage had occurred after 6 min, the AME concentration was raised to 2.5 $\mu\text{g ml}^{-1}$ and the process was repeated as described in Methods.

ents, or specific bonds between those components, in the wall, then an indication of their nature might be obtained by observing the effect on resistance of treating organisms with enzymes known to attack wall components. The present paper describes the reduction of AME resistance that can be obtained by treating stationary phase organisms with purified enzyme preparations and mixtures thereof.

METHODS

Organism. *Candida albicans* strain 6406, obtained from the Mycological Reference Laboratory of the London School of Hygiene and Tropical Medicine, was maintained, cultured and prepared in washed suspension as previously described (Gale, 1974). For the experiments described below, stationary phase cultures with a high resistance to AME were required; in most cases batch cultures which had been incubated at 37 °C for 7 or 8 d were used.

Viable count. This was determined by diluting 1 ml of each sample in sterile saline and plating suitable dilutions on to Difco yeast morphology agar. The plates were incubated at 37 °C for 24 h and then the numbers of colonies per plate were counted.

Estimation of AME sensitivity by the K^+ release method. The terms 'AME sensitivity' and 'AME resistance' refer to the action of amphotericin B methyl ester in inducing the release of K^+ from suspensions of organisms, estimated by use of a K^+ -sensitive electrode (Gale, 1974). In previous papers, the sensitivities of exponential and stationary phase organisms have been measured by determining the concentration of AME (s.r.c. = standard release concentration) required to induce a release of K^+ (above that in the control without AME) equal to 1 nmol min^{-1} (mg dry wt organisms) $^{-1}$ in 8 min at 20 °C. The release is time- and AME concentration-dependent and, within certain limits, the product (time $\times$ concentration) is constant (Gale, 1974) so that the s.r.c. is readily determined by two or three estimations per sample of organisms, especially when previous work enables a reasonable guess to be made of the probable value. However, for the present work where highly resistant organisms are used initially, followed by treated samples of unknown resistance, the method is time-consuming as several tests may be needed before the s.r.c. is found. The procedure that was adopted was to set up the electrode in the buffered suspension of organisms as usual and, when the control rate of leakage had been established, add a small amount of AME (usually a final concentration of 1 $\mu\text{g ml}^{-1}$) and follow the leakage for 6 min. If, at the end of that time, there had been little or no increase in the rate of leakage, the concentration of AME was increased to 2.5 $\mu\text{g ml}^{-1}$ and the leakage was followed for another 6 min. This process was repeated, doubling the AME concentration every 6 min until a measurable increase in leakage was obtained, and continued until the induced leakage rose to 3 $\text{nmol K}^+ \text{min}^{-1}$ or higher. A typical experiment is shown in Fig. 3: in this experiment, the untreated organisms did not show an increased leakage until AME had been added to a final concentration of 20 $\mu\text{g ml}^{-1}$.

Table 1. *Effect of Cytophaga L1 preparation on the AME resistance of a stationary phase culture of Candida albicans*

A 7 d culture was adjusted to pH 6.5 and incubated for 24 h at 37 °C with Cytophaga L1 preparation at the concentration shown. The standard release concentration equivalent (s.r.c.e.) was determined as described in Methods.

Cytophaga L1 [μg (mg dry wt organisms) $^{-1}$]	S.r.c.e. (μg AME ml $^{-1}$)	S.r.c.e. $^{-1}$	S.r.c.e. $^{-1}$ minus control
0	ca 30	0.033	—
50	3.2	0.313	0.28
100	1.74	0.573	0.54
200	0.81	1.233	1.20

Four main types of 'sensitivity profile' are obtained, as shown in Fig. 1: profiles A and D are those obtained with sensitive and resistant organisms, respectively; profile C indicates a heterogeneous suspension with a proportion of sensitive organisms and is the type of response obtained when enzyme action is decreasing resistance but has not been continued for long enough; profile B is that obtained when the cells have been damaged by treatment sufficiently for much of the internal K^+ to be lost. Responses C and B could be distinguished by determining the K^+ content of a sample of the suspension of organisms after heating to 100 °C for 10 min. The organisms used, prepared as previously described (Gale, 1974), had an initial K^+ content of 200 to 350 nmol K^+ (mg dry wt organisms) $^{-1}$.

Inspection of such sensitivity profiles readily shows changes in AME sensitivity that have taken place as a result of enzyme treatment but it is desirable to have a simpler method of recording changes to compare treatments. As described above, the product ($C \times T$) of the time T required to induce a standard rate of K^+ release (1 nmol K^+ min $^{-1}$) and the concentration C of AME inducing that release is approximately constant; the s.r.c. of previous papers in this series is defined as that value of C inducing the standard release of K^+ in 8 min. An estimate of the response in a profile such as Fig. 1(D) or those in Figs 3 and 4 is given by the sum of the CT values from $T = 0$ until K^+ is released at 1 nmol min $^{-1}$. This value can be related to the s.r.c. by dividing by 8 and $\Sigma CT/8$ defines the s.r.c. equivalent; in the example shown by Fig. 3 (24 h) the s.r.c.e. is equal to the s.r.c. High values of s.r.c.e. indicate resistance and increasing sensitivity is shown by decreasing values of s.r.c.e.; the range falls between 0.1 and $> 100 \mu\text{g}$ ml $^{-1}$. The s.r.c.e., obtained after a series of additions of AME, was compared with the s.r.c. value obtained (in the light of the s.r.c.e.) by a single addition and agreement within 15 to 20% was obtained for experiments involving three or four serial additions of AME. However, within any one series of estimations, the same dose-time sequence was followed for all samples. Since we are concerned with increases in sensitivity rather than resistance as such, this is conveniently expressed by the reciprocal of s.r.c.e. for the treated organisms less that for the untreated control. Table 1 illustrates that increases in sensitivity, expressed in this way, for resistant organisms treated with Cytophaga L1 preparation are directly correlated with the concentration of that preparation used in the incubation medium.

Enzyme treatment. Preliminary experiments showed that reproducible results were obtained only if the cultures were manipulated as little as possible. Cultures were grown in batches of 250 ml medium dispensed in 2 l Erlenmeyer flasks rotated at 200 rev. min $^{-1}$ on a rotary incubator (Gallenkamp) at 37 °C for 7 to 8 d. Such a culture was removed from the incubator, its pH was adjusted if necessary by addition of sterile NaOH, and it was then divided into samples, each equivalent to 50 mg dry wt organisms; these were added to 250 ml Erlenmeyer flasks containing the enzyme preparation, and returned to the rotary incubator. In the first instance, enzymes were tested at a final concentration of 100 μg protein (mg dry wt organisms) $^{-1}$. After the required time, the suspensions were prepared for determination of the s.r.c.e. as previously described (Gale, 1974).

Carbohydrate analysis. Quantitative determination of the major carbohydrate constituents of *C. albicans* was carried out as described by Herbert *et al.* (1971). Cassone *et al.* (1979) found significant differences in the structure and thickness of walls as seen in electron micrographs of whole cells or wall preparations. Consequently the present analyses were carried out on whole cells.

Enzyme preparations. Cytophaga L1, obtained from B.D.H., was used as a reference preparation. According to Marshall (1972, 1973*a, b*) and the Glaxo patent (1966) the preparation contains a mixture of (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)- β -D-glucanases as major components together with other glucanases, isoamylase and traces of laminarinase, keratinase, elastase, protease, lipase and chitinase.

Specific glucanases. Non-lytic (1 $\rightarrow$ 6)- β -D-glucanase (EC 3.2.1.75) from *Bacillus circulans* with an activity

of 5.45 pustulan units (mg protein)⁻¹ was a gift from Dr T. G. Villa of Salamanca University. Lytic (1→6)- β -D-glucanase from *Bacillus circulans* with an activity of 117 pustulan units (mg protein)⁻¹ was also a gift from Dr T. G. Villa. Exo-(1→3)- β -D-glucanase (EC 3.2.1.39) was prepared from an unidentified basidiomycete culture by Dr T. G. Villa. A series of endo- and exo-(1→3)- β -D-glucanases was extracted from *Candida albicans* strain 6406 and purified (V. Notario, unpublished).

Zymolase-60000, obtained from Kirin Brewery Co., Miyahara, Takasaki, 370-12 Japan, is a (1→3)- β -D-glucanase obtained from *Arthrobacter luteus*; the preparation 'may contain traces of protease and mannanase'. The activity – 60000 units g⁻¹ – relates to a unit defined in terms of decrease in A_{800} of a suspension of Brewer's yeast; 1 unit lyses 3 mg dry wt yeast.

'Laminaranase' was obtained from Calbiochem; it is described as a glucanase obtained 'ex mollusca, with activity releasing 30 mg glucose from laminarin g⁻¹ min⁻¹'.

Chitinase (EC 3.2.1.14) was obtained from Calbiochem; its activity is stated as 1 mg yields 0.48 mg glucose from chitin.

Trypsin (EC 3.4.21.4), crystalline preparation, was obtained from Boehringer.

Lipase (EC 3.1.1.3), from wheat germ, was obtained from B.D.H.; its activity is 'at least 1 EU mg⁻¹ (Singer & Hofstee, 1948)'.

α -Mannosidase (EC 3.2.1.24) was prepared from *Arthrobacter* sp. according to the method of Jones & Ballou (1969); the final preparation had an activity of 130 mannosidase units mg⁻¹. No mannosidase activity was detected in *C. albicans*.

Amphotericin B methyl ester was used as the water-soluble aspartate preparation kindly given to us by Dr W. E. Brown of E. R. Squibb & Sons, Princeton, N.J., U.S.A.

RESULTS

Preliminary work

Gale *et al.* (1977) found that lowering the oxygen tension in stationary phase cultures resulted in a rapid decrease in resistance; cultures with resistance greater than s.r.c. values of 15 to 20 μ g ml⁻¹ were less affected by reducing conditions. Consequently, experiments on the effects of enzyme treatment were carried out with cultures grown for 7 d or longer (s.r.c. > 30 μ g ml⁻¹) and the cultures were subjected to as little disturbance of aeration conditions as possible. The organisms were not removed from the culture, but the culture, after pH adjustment if necessary, was divided into smaller samples, enzymes were added and the samples were returned to the rotary incubator as soon as possible (see Methods).

pH during digestion

Figure 2 shows the effect of pH on the sensitivity of organisms incubated for 24 h in the presence or absence of various enzymes. The Cytophaga L1 preparation was most active at pH 7.0, but as the resistance of the control culture (without added enzyme) also decreased at pH 7 more than at pH 6.5 or 7.5, the net effect was greatest at pH 6.5. Apart from lipase, which was most effective at pH 5, the other enzymes were most active at pH 6.5 which was adopted as the standard pH for further experiments. All the activities decreased abruptly at pH values above 7 and results became variable.

Digestion with the Cytophaga L1 lytic enzyme preparation

Figure 3 shows the effect of the time of incubation of 7 d cultures of *C. albicans* with the Cytophaga L1 preparation [final concentration 200 μ g (mg dry wt organisms)⁻¹] at pH 6.5. The s.r.c.e. values after 1, 6 and 24 h were 11, 1.4 and 0.55 μ g ml⁻¹, respectively, while the control without enzyme had an s.r.c.e. value of > 20 μ g ml⁻¹ after 24 h. There was no loss of viability after 24 h treatment under the conditions shown. The activity of the Cytophaga L1 preparation varied from batch to batch and the susceptibility of the 7 d culture also varied; in a series of 14 experiments carried out over a period of 4 months the value of the increase in s.r.c.e.⁻¹ (see Methods) was 0.467 ± 0.106 for 24 h digestion at pH 6.5 with the

Fig. 2. Effect of pH on the reduction of AME resistance in stationary phase organisms by incubation with enzymes. Samples of a 7 d batch culture of *C. albicans*, pH adjusted as shown, were incubated for 24 h at 37 °C with enzyme preparations at a final concentration of 100 μg protein (mg dry wt organisms)⁻¹. Organisms were harvested, washed twice and resuspended in 0.03 M-Tris/HCl buffer pH 7.5 for estimation of the standard release concentration (s.r.c.). Sensitivity is expressed as s.r.c.⁻¹. Enzyme preparations added: Cytophaga L1 (○); lipase (△); chitinase (□); trypsin (×); none (●).

Fig. 4. AME sensitivity profiles obtained after incubating 7 d batch cultures of *C. albicans*, pH adjusted to 6.5, for 24 h at 37 °C with enzymes at 100 µg protein (mg dry wt organisms)⁻¹ unless otherwise shown: 1, *Cytophaga* L1 (200 µg mg⁻¹); 2, *Cytophaga* L1; 3, α -mannosidase; 4, chitinase; 5, lipase; 6, trypsin; 7, 'Laminaranase'; 8, none.

Figure 4 shows the increase in sensitivity towards AME induced in 7 d cultures of *C. albicans* incubated with various enzymes each at a concentration of 100 µg protein (mg dry wt organisms)⁻¹ for 24 h at pH 6.5 and 37 °C. 'Laminaranase' was inactive, lipase and trypsin had little activity, while chitinase and α -mannosidase had activities between 40 and 75% of the *Cytophaga* L1 preparation. A variety of glucanases was also tested (Fig. 5) and all had activity which in many cases was greater than that of the *Cytophaga* L1 preparation. Exo-(1 $\rightarrow$ 3)- β -D-glucanase from an unidentified basidiomycete was highly active, 10 µg protein (mg dry wt organisms)⁻¹ being as active as 100 µg *Cytophaga* L1 (mg dry wt organisms)⁻¹. Zymolase, containing (1 $\rightarrow$ 3)- β -D-glucanase, was about three times as active as the *Cytophaga* L1 preparation while at least three (1 $\rightarrow$ 3)- β -D-glucanases extracted from the *Candida albicans* strain 6406 had activities of the same order as *Cytophaga* L1.

Higher concentrations of the (1 $\rightarrow$ 3)- β -D-glucanase preparations were more effective in reducing resistance but had a lytic action on the organisms (see Fig. 5). Two preparations of (1 $\rightarrow$ 6)- β -D-glucanase had little effect on AME resistance although both preparations were highly active against pustulan.

Effects of mixtures of enzymes

Table 2 shows that the addition of trypsin, lipase or chitinase markedly enhanced the action of either *Cytophaga* L1 or exo-(1 $\rightarrow$ 3)- β -D-glucanase (basidiomycete) in reducing the resistance of *C. albicans* towards AME.

Recovery from treatment with Cytophaga L1 preparation

There was no loss of viability in 7 d cultures treated for 24 h at pH 6.5 and 37 °C with *Cytophaga* L1 preparation at concentrations of 1 to 200 µg (mg dry wt organisms)⁻¹. If the organisms were then removed from the incubation mixture by centrifuging and re-suspended in either enzyme-free medium obtained from a control suspension or SY medium (Gale, 1974) adjusted to pH 3, then their resistance to AME increased. Table 3 shows that when a culture whose s.r.c.e. value had been reduced to 0.41 µg AME ml⁻¹ by 24 h treatment with *Cytophaga* L1 was transferred to enzyme-free medium, its resistance increased to a

Fig. 5. AME sensitivity profiles obtained after incubating 7 d batch cultures of *C. albicans*, pH adjusted to 6.5, for 24 h at 37 °C with enzymes at the concentrations shown: 1, Cytophaga L1 [200 μg (mg dry wt organisms) $^{-1}$]; 2, 3 and 4, exo-(1 $\rightarrow$ 3)- β -D-glucanase, from an unidentified basidiomycete [50, 30 and 10 μg (mg dry wt organisms) $^{-1}$, respectively]; 5, Cytophaga L1 [100 μg (mg dry wt organisms) $^{-1}$]; 6, (1 $\rightarrow$ 6)- β -D-glucanase, non-lytic (4.7 pustulan units); 7, (1 $\rightarrow$ 6)- β -D-glucanase, lytic (9.4 pustulan units); 8, none.

Table 2. *Effect of treatment with enzyme mixtures on the AME resistance of stationary phase cultures of Candida albicans*

A 7 d culture was adjusted to pH 6.5 and dispensed in samples equivalent to 50 mg dry wt organisms; enzyme preparation was added at the concentrations indicated in parentheses [μg protein (mg dry wt organisms) $^{-1}$] and the samples were returned to the incubator at 37 °C for 24 h. Sensitivity profiles (standard release concentration equivalent, s.r.c.e.) and increase in sensitivity (s.r.c.e. $^{-1}$ minus value for control without enzyme) were determined as described in Methods. (A) and (B) represent two separate experiments.

Enzyme preparation	S.r.c.e. (μg AME ml^{-1})	Sensitivity (s.r.c.e. $^{-1}$)	Increase in sensitivity	
			Single enzyme	Mixed enzymes
(A) Cytophaga L1 (100)	2.41	0.415	0.365	
Trypsin (100)	9.0	0.111	0.061	
Lipase (100)	5.5	0.182	0.132	
Chitinase (100)	3.5	0.285	0.235	
Cytophaga L1 (100)+ Trypsin (100)	1.0	1.000		0.950
Cytophaga L1 (100)+ Lipase (100)	0.5	2.000		1.950
Cytophaga L1 (100)+ Chitinase (100)	0.35	2.85		2.80
Trypsin (100)+ Lipase (100)	5.9	0.170		0.12
Control	20	0.05		
(B) Exo-(1 $\rightarrow$ 3)- β -D-glucanase (10)	2.7	0.370	0.327	
Trypsin (100)	10.8	0.092	0.049	
Lipase (100)	6.1	0.164	0.121	
Chitinase (100)	4.5	0.222	0.179	
Glucanase* (10)+Trypsin (100)	0.75	1.333		1.290
Glucanase* (10)+Lipase (100)	0.91	1.100		1.057
Glucanase* (10)+Chitinase (100)	1.07	0.935		0.892
Control	23	0.043		

* Exo-(1 $\rightarrow$ 3)- β -D-glucanase.

Table 3. *Changes in the AME resistance and carbohydrate content of Candida albicans during digestion with, and after removal of, Cytophaga L1 preparation*

A 7 d batch culture was adjusted to pH 6.5 and incubated for 24 h at 37 °C with *Cytophaga* L1 preparation at 200 µg (mg dry wt organisms)⁻¹; at the end of that time, the organisms were separated by centrifuging, resuspended in SY medium at pH 3, and returned to the incubator at 37 °C. Samples were taken at 24 h intervals for estimation of AME resistance (s.r.c.e.) and carbohydrate content (values quoted as % of total carbohydrate content of the organisms)

Estimation	Initial culture	After 24 h digestion	Recovery in SY medium		
			24 h	48 h	72 h
S.r.c.e. (µg AME ml ⁻¹)	20	0.41	2.0	4.5	15
TCA*-soluble carbohydrate (%)	1.2	19.7	13.4	8.2	5.0
Mannan (%)	25	24	37.5	24.3	20.4
Glucan: acid-soluble (%)	36.3	25	18.7	27.5	28
Glucan: alkali-soluble (%)	11	5	—	10.8	16.2
Insoluble residue (%)	27	25	29.5	29	30

* TCA, Trichloroacetic acid.

value of 15 µg AME ml⁻¹ after 72 h at 37 °C. Estimation of the carbohydrate fractions of the cells (Herbert *et al.*, 1971) showed major decreases in the acid-soluble and alkali-soluble glucan fractions during enzyme treatment followed by a resynthesis of these fractions during the period of recovery of resistance. There was a major increase in the trichloroacetic acid-soluble carbohydrate fraction during enzyme treatment, followed by disappearance of much of the material in the fraction during the recovery period.

DISCUSSION

Removal of the wall from stationary phase organisms results in a decrease in the resistance of those organisms to a value approximating to that of exponentially growing cultures (Gale *et al.*, 1975). The results set out in this paper show that it is not necessary for the wall to be removed to reduce the resistance. Any attack on major components of the wall – e.g. by trypsin, lipase, α -mannosidase or chitinase – results in a decrease in resistance while the action of (1→3)- β -D-glucanase is markedly more effective on a specific activity basis than that of any other enzyme tested. This could be an indication that glucan plays a major role in phenotypic resistance towards AME. It is interesting that the action of (1→3)- β -D-glucanase is markedly potentiated by the presence of other enzymes, e.g. trypsin, chitinase or lipase. *Candida albicans* produces a range of (1→3)- β -D-glucanases some of which are highly active in reducing AME resistance and it is possible that the action of added enzymes could be due to activation of these endogenous glucanases. In this connection it is of interest that resistance of organisms grown at pH 3 to 4 for 7 d is markedly reduced by incubation in the culture medium after adjustment to pH 7.5 and that the presence of trypsin or chitinase apparently ‘inhibits’ this reduction (see Fig. 2).

Davies & Pope (1977, 1978) have found that mycolytic enzymes will act synergistically with amphotericin B against *Candida albicans* and *Aspergillus* spp. both *in vitro* and *in vivo*. They suggest that enzymic degradation of the wall probably allows the drug increased access to the cell membrane. This hypothesis is in accord with the results described in this paper and the potentiating effect of enzyme digestion on AME sensitivity of organisms in a state of phenotypic resistance may also play a part in the results obtained by Davies & Pope.

This work was supported by a grant from the Medical Research Council. One of us (V.N.) is indebted to the Spanish Ministry of Education for a personal grant.

REFERENCES

- CASSONE, A., KERRIDGE, D. & GALE, E. F. (1979). Ultrastructural changes in the cell wall of *Candida albicans* following cessation of growth and their possible relationship to the development of polyene resistance. *Journal of General Microbiology* **110**, 339–349.
- DAVIES, D. A. L. & POPE, A. M. S. (1977). Lytic enzymes as antifungal agents – a new approach to antimycotic therapy. *Bulletin of the British Mycological Society* **11**, 144.
- DAVIES, D. A. L. & POPE, A. M. S. (1978). Mycolase, a new kind of systemic antimycotic. *Nature, London* **273**, 235–236.
- GALE, E. F. (1974). The release of potassium ions from *Candida albicans* in the presence of polyene antibiotics. *Journal of General Microbiology* **80**, 451–465.
- GALE, E. F., JOHNSON, A. M., KERRIDGE, D. & KOH, T. Y. (1975). Factors affecting the changes in amphotericin sensitivity of *Candida albicans* during growth. *Journal of General Microbiology* **87**, 20–36.
- GALE, E. F., JOHNSON, A. M. & KERRIDGE, D. (1977). The effect of aeration and metabolic inhibitors on resistance to amphotericin in starved cultures of *Candida albicans*. *Journal of General Microbiology* **99**, 77–84.
- GALE, E. F., JOHNSON, A. M., KERRIDGE, D. & MILES, E. A. (1978). Phenotypic resistance to amphotericin B in *Candida albicans*: the role of reduction. *Journal of General Microbiology* **109**, 191–204.
- GLAXO GROUP LTD (1966). Preparation of an enzyme complex. *Netherlands Patent appl.* 6,506,419 Nov.29, 1965: *Chemical Abstracts* **65**, 2972a.
- HAMMOND, S. M., LAMBERT, P. A. & KLIGER, B. N. (1974). The mode of action of polyene antibiotics; induced potassium leakage. *Journal of General Microbiology* **81**, 325–330.
- HERBERT, D., PHIPPS, P. J. & STRANGE, R. E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology* **5B**, 209–344.
- JONES, G. H. & BALLOU, C. E. (1969). Studies on the structure of yeast mannan 1. Purification and some properties of an α -mannosidase from an *Arthro-bacter* species. *Journal of Biological Chemistry* **244**, 1043–1051.
- MARSHALL, J. J. (1972). Facile purification of β glucanhydrolase by chromatography on DEAE-cellulose. *Journal of Chromatography* **71**, 367–385.
- MARSHALL, J. J. (1973a). Nature of the binding of a β (1 $\rightarrow$ 4) glucanhydrolase to ion exchangers. *Journal of Chromatography* **76**, 257–260.
- MARSHALL, J. J. (1973b). Behaviour of β glucanhydrolase on ion exchangers. *Analytical Chemistry* **53**, 191–198.
- SINGER, T. P. & HOFSTEE, B. H. J. (1948). Studies on wheat germ lipase. 1. Methods of estimation, purification and general properties of the enzyme. *Archives of Biochemistry* **18**, 229–259.