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MODIFICATION OF K^+ TRANSPORT IN YEAST BY THE POLYENE ANTIFUNGAL ANTIBIOTIC N-ACETYLCANDIDIN

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Abstract—The action of the polyene antifungal, N-acetylcandidin (NAC), on K^+ retention and transport by *Saccharomyces cerevisiae* was examined, since K^+ loss is an early result of polyene binding by sensitive cells. Log-phase yeast in a K^+-free medium at 30° retained its K^+ relatively effectively in the presence of glucose, but slow losses occurred in the absence of an energy source. This loss was sharply increased by 20 μg NAC/mg cells; the effect was temporarily prevented by glucose. NAC-treated yeast could still accumulate added K^+ (30°; 0·1 M glucose) but to a lesser extent than did the untreated cells. This accumulation is energy dependent, since at 0° or when glucose was omitted, both cell types took up K^+ only with the concentration gradient. Adding 100 μg NAC/mg cells further increased the rate of K^+ loss and prevented the accumulation of K^+. The antibiotic does not appear to produce true holes in the cell membrane, since K^+ was retained at 0°, even by NAC-treated cells which had already lost a part of their K^+ at 30°.

Sodium enhanced both K^+ retention and accumulation in normal yeast cells. With NAC-treated cells, Na^+ increased K^+ loss and reduced K^+ accumulation when added with the K^+.

NAC did not seem to increase the permeability of the yeast cell membrane, nor did it completely prevent the active uptake of K^+ except at high antibiotic levels which eliminated energy-yielding reactions. The binding of NAC apparently alters the membrane, thus reducing indirectly the efficiency of ion transport, probably both into and out of the cell, and changing the usual Na^+–K^+ transport balance. This reduces the ratio of intracellular to extracellular K^+ which the treated cell can maintain.

It is proposed that this alteration of the transport system is an early, general effect of polyenes and that polyenes other than NAC produce additional changes which are important to, but not essential for, antifungal action.

THE polyene antifungal antibiotics are bound to the fungal cell membrane[1–3] and cause the loss of a variety of cellular constituents concurrent with their fungicidal action. The effects of nystatin,[1–7] filipin,[7, 8] and amphotericin B[1, 7] on *Saccharomyces* and *Candida* strains have been studied most intensively. Losses of K^+, NH_4^+, inorganic phosphate, carboxylic acids, sugar phosphates, nucleotides, and protein occur. The apparent importance of permeability alterations for the fungicidal action has been repeatedly emphasized.

At low levels of nystatin, the major alteration detected was a rapid fall in the K^+ content of the yeast cell.[4, 5] It thus appeared possible that the action of the polyenes on K^+ transport was relatively direct, particularly since the protection of glycolysis by NH_4^+ does not prevent the K^+ loss.[4] In the present communication this effect has been examined in more detail.

A series of N-acylated polyenes was recently described[9, 10] which, although less active than the unsubstituted polyenes, forms sodium salts that are readily soluble in water at neutral pH. One of these, the heptaene N-acetylcandidin (NAC), was selected for the present studies. Its inhibition of glycolysis was readily reversed by the addition of K^+ or NH_4^+ salts,[10] so that its action on K^+ transport might be relatively specific. Also, the solubility of NAC avoided the use of colloidal suspensions or the addition of a solvent—e.g. dimethylsulfoxide[4, 5]—which would have complicated interpretation of the observed effects on cell permeability.

METHODS

Saccharomyces cerevisiae

Strain LK2G12, grown on a slant of Penassay agar (Difco Laboratories, Inc.) containing 1% (w/v) glucose was transferred into 100-ml Wickerham's medium[11] (1% glucose) and incubated for 16 to 18 hr at 28° on a rotary shaker. Thirty ml of the stationary-phase culture was then introduced into 100 ml Wickerham's medium and incubated as before for 3 hr to obtain log-phase cells.

The log cells were washed three times in demineralized water. An estimate of cell concentration was obtained by cell count with a hemacytometer or optical density measurements with a Lumetron photometer at 420 mμ. Preliminary experiments on log cells provided data which permitted a correlation of optical density, dry weight, and cell number.

Potassium loss-uptake experiments

Washed log cell suspensions (ca. 2 mg/ml) were incubated at 30° or 0°. Since NAC is not bound at 0°,[2] it was necessary in these experiments to expose cells to the polyene at 30° for 10 to 20 min to permit binding and then drop the temperature to 0°. Agitation was provided by a shaking water bath or a Burrell shaker.

For aerobic experiments, compressed air was bubbled through the fluid in Erlenmeyer flasks open to the atmosphere. For anaerobic experiments, flasks with two side arms, similar to Warburg flasks, were used which permitted gassing with nitrogen and the anaerobic addition of fluids during experimentation. Polyethylene tubing penetrated a serum stopper sealing the flask and extended to the bottom of the flask. This served both as a means of nitrogen influx and for syringe removal of samples.

At various time intervals samples were obtained, centrifuged in chilled tubes, and the supernatant fluid removed. When KCl was present, the packed cells were resuspended in demineralized water at 0°, and the above procedure was repeated to remove K^+-containing medium trapped extracellularly.

Warburg experiments

Washed log cells, 1·19 and 1·36 mg (dry weight)/3 ml fluid volume, were used respectively for aerobic and anaerobic experiments. Each flask contained 0·06 M Tris or Na^+-Tris buffer, 0·125 M glucose, and, where indicated, 0·01 M KCl and 30 μg of N-acetylcandidin. Potassium chloride was added to the flask initially, or from the side arm at 70 and 90 min, respectively, in anaerobic and aerobic experiments. Glucose and NAC were tipped in at 0 min from the other side arm. As the direct method[12] was used for the aerobic experiments, 0·1 ml 40% KOH was added to the center wells

of duplicate flasks. Air and nitrogen were used for aerobic and anaerobic experiments. The temperature was 30°.

Potassium analyses

Samples of packed cells were resuspended in known volumes of demineralized water. The tubes were then placed in a boiling water bath for 5 to 10 min to extract the potassium. On cooling, the samples were centrifuged to remove particulate matter. The Beckman model B flame spectrophotometer with oxygen-acetylene burner was used to analyze the supernatants for potassium.

Buffers

(a) Tris buffer, 0·3 M, pH 7·5 and 8·8; 26 ml 1:1 (v/v) solution of 0·3 M tartaric acid and 0·3 M succinic acid were added to 60 ml 0·3 M Tris to obtain pH 7·5 buffer. Five ml of a 1:1 (v/v) solution of 0·3 M tartaric acid and 0·3 M succinic acid were added to 60 ml 0·3 M Tris to obtain pH 8·8 buffer.

(b) Na^+–Tris buffer, 0·3 M, pH 7·5; 45 ml of a 1:1 (v/v) solution of 0·3 M sodium tartrate and 0·3 M succinic acid were added to 50 ml 0·3 M Tris to obtain pH 7·5 buffer.

(c) Na^+ buffer, 0·26 M, pH 7·5; 15·8 ml of 1·0 M NaOH was added to 26 ml of a 1:1 (v/v) solution of 0·3 M tartaric acid and 0·3 M succinic acid and the final volume made up to 90 ml with demineralized water to give a buffer of pH 7·5 in which NaOH has replaced Tris.

N-Acetylcandidin

This substance was graciously supplied by E. Borowski and C. P. Schaffner of this Institute. Five mg NAC was dissolved in 1 ml 0·3 M Tris buffer, pH 8·8. The pH was immediately lowered by the addition of 1·5 ml 0·1 M HCl and 5·5 ml of 0·3 M Tris buffer, pH 7·5, and the final volume was adjusted to 10 ml with demineralized water. This compound is unstable at high pH; solubilization must therefore be done quickly and with care.

RESULTS

Preliminary experiments (not included) showed that 20 μg of NAC/mg dry weight of cells produced a moderate rate of K^+ loss. This was chosen as the lower level and 100 μg/mg as the higher level in subsequent investigations.

Sodium effect

Results from early experiments indicated that the K^+ changes were dependent both on NAC and on the presence of Na^+. Normal log-phase cells in a K^+-free medium retained cellular K^+ effectively, losing* it only slowly to the external medium (Table 1). In most instances this small loss was reduced in the presence of Na^+ (cf. Table 2), although with certain batches of cells Na^+ caused a substantial initial drop which was subsequently reversed (cf. Table 1). NAC-treated cells lost K^+ rapidly to the external medium. This loss was accelerated in the presence of Na^+.

* Changes in cellular ion concentrations are net flux changes, as individual influx and efflux were not measured. The terms sodium and potassium carriers as used herein refer to the two carriers thought respectively to extrude sodium and take up potassium against an ion-concentration gradient.

TABLE 1. EFFECT OF NAC AND SODIUM ON POTASSIUM RETENTION*

Buffer cation	NAC (μg/mg† cells)	K^+ content (mg/g† yeast cells) 0 min	15 min	30 min	60 min
Tris	0	20·6‡	18·6	19·9	18·6
	22	20·4‡	15·5	16·0	11·7
Na^+	0	15·8	11·1	10·8	14·0
	29		7·1	0·0	0·0

* Cells incubated aerobically at 30° in 0·06 M Tris or Na^+ buffer, pH 7·5, with 0·11 M glucose. Glucose and NAC were added before the 0-min sampling.
† Dry weight.
‡ Five min samples.

TABLE 2. POTASSIUM UPTAKE IN THE PRESENCE OF NAC AND SODIUM*

Na^+ present	K^+ added at 60 min	NAC (μg/mg† cells)	K^+ content (mg/g† yeast cells) 0 min	60 min	90 min	120 min	K^+ at equilibrium‡
–	–	0	12·6	6·1	5·6	5·6	
–	–	21		2·3	1·4	0·7	
–	–	106		0·9	0·5	0·5	
–	+	0		6·5	8·0	8·9	3·8
–	+	21		2·3	6·1	5·1	3·8
–	+	106		0·9	2·3	2·3	3·8
+	–	0	11·3	9·8	10·4	11·8	
+	–	21		0·9	0·7	0·5	
+	–	104		0·7	0·5	0·7	
+	+	0		9·3	19·7	26·8	3·8
+	+	21		0·9	9·1	10·0	3·8
+	+	104		0·7	2·3	2·1	3·8

* Cells incubated aerobically at 30° in 0·06 M Tris or Na^+–Tris buffer, pH 7·5, with 0·11 M glucose. Glucose and NAC were added before 0-min sampling. Where indicated, KCl was added after 60-min sampling to yield an extracellular potassium concentration of 0·01 M.
† Dry weight.
‡ "Equilibrium" intracellular potassium concentration calculated using values for extracellular potassium (after addition of KCl), cell dry weight, and per cent cell water (90%) and assuming attainment of potassium equilibrium = 3·8 mg/g cell dry weight.

Added K^+ was taken up both by untreated and by NAC cells (Table 2). With untreated cells, the resulting increase in cellular K^+ was greater when Na^+ was present. The effect of Na^+ was more pronounced when the cells were pretreated with this ion (Table 2) than when they were exposed to Na^+ and K^+ simultaneously (latter data not included). With NAC cells, pre-exposure to Na^+ also increased the uptake of added K^+. However, simultaneous exposure to Na^+ and K^+ actually produced a decreased cellular K^+ concentration as compared with similar experiments in the absence of Na^+ (data not included). Therefore, Na^+ enhanced both K^+ retention and accumulation in normal yeast cells. With NAC-treated cells, Na^+ increased K^+ loss, reduced K^+ accumulation when added with the K^+, and increased accumulation when added prior to K^+.

A satisfactory explanation of the effects of sodium might be as follows. In the absence of extracellular K^+, Na^+ enters the yeast cell on the potassium carrier.[13]

Once inside the cell, Na^+ competes favorably with K^+ for the sodium carrier,[14] and the net effect is a decreased loss of K^+. After the addition of KCl, K^+ is taken up on the potassium carrier. The resulting increase in total cellular K^+ is more pronounced for cells exposed to Na^+ prior to K^+ addition, because the presence of intracellular Na^+ reduces K^+ efflux via the sodium carrier. The enhancing effect is less when Na^+ is added coincident with the K^+, since the K^+ reduces the uptake of Na^+ on the potassium carrier and thus delays the accumulation of sufficient intracellular Na^+ to retard K^+ efflux.

Pre-exposure to Na^+ increased K^+ uptake by NAC-treated cells; this is in agreement with the above explanation. In contrast, however, NAC-treated cells showed an increased K^+ loss or reduced K^+ accumulation when Na^+ was added with the K^+. Thus NAC produced an alteration in the usual transport balance between these ions that may result from a decreased efficiency of the K^+ transport mechanism or an altered carrier specificity.

Potassium loss-uptake

Although NAC-treated cells did lose K^+ to the medium (Table 1), it was clear that the ion transport systems were still partially effective. On the addition of KCl to cells depleted by incubation with 20 μg NAC/mg, the cellular K^+ levels rose beyond the values calculated for K^+ equilibrium between intracellular and extracellular water (Table 2), although they did not reach those attained with untreated cells. At the upper level of NAC, K^+ uptake occurred but "equilibrium" levels were not attained.

The apparent uptake against a concentration gradient by the antibiotic-treated cells was also investigated by anaerobic experiments performed in Tris buffer at 30°

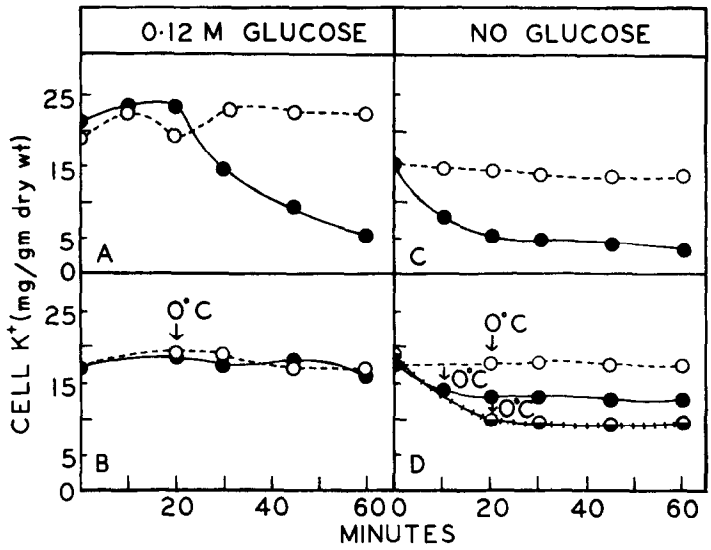

FIG. 1. Effect of temperature and glucose on anaerobic potassium loss from NAC-treated cells. The glucose and NAC (20 μg/mg, dry wt, of cells in A and C; 30 μg/mg in B and D) were added before 0-min sampling; 0·6 M Tris buffer, pH 7·5. All incubation mixtures were initially at 30°; at (↓) the indicated flasks were transferred to a 0° water bath; ○ - - - - ○, cells not treated with NAC; ●——● or ◒ +++ ◒, cells treated with NAC.

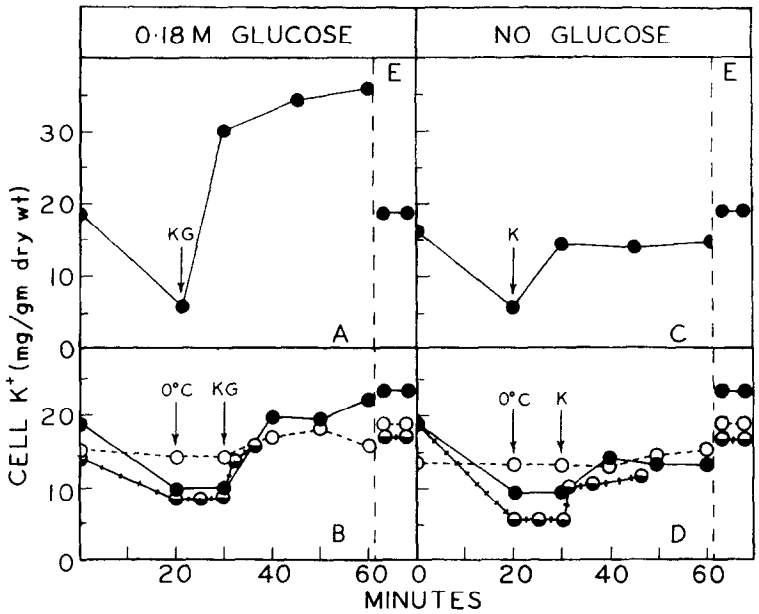

FIG. 2. Potassium uptake by NAC-treated cells as a function of temperature and of glucose. All incubation mixtures (anaerobic) were initially at 30° in 0·06 M Tris buffer, pH 7·5. At 0 min, 20 μg NAC was added per mg cell dry weight. At times marked (↓) the indicated flasks were either transferred to a 0° water bath (0°) or were supplemented with KCl to give approximately 0·06 M concentration (K) or glucose to yield 0·18 M (G). ○ - - - - ○, no NAC added; ●——● or ◒ +++ ◒, 20 μg NAC. In NAC series marked ◒ +++ ◒, the K^+ content of the cells was determined at 25 min. The concentration at 20 and 30 min was assumed to be identical, since the cell K^+ level is stable with time at 0°. E + calculated "equilibrium" intracellular K^+ concentration (cf. Table 2 for explanation).

and 0° with and without glucose. In this way, competition by Na^+ ions was avoided, and the energy supply for cellular activity was controlled. At 30° without glucose, K^+ loss was much more pronounced for NAC cells than for control cells (Fig. 1 C). In the presence of glucose, K^+ loss occurred only with NAC-treated cells and then only after a 20-minute lag period (Fig. 1 A).

Cellular K^+ was retained by untreated cells at 0°, either with or without glucose (Fig. 1 B, D), and loss of K^+ from NAC cells halted abruptly when the temperature was dropped from 30° to 0° (Fig. 1 D). This showed that NAC did not increase ion permeability, and ion transport presumably ceased at the lower temperature.

Cells whose K^+ content had been reduced by NAC treatment and untreated cells both showed similar responses to added KCl. Downhill transport* of K^+ occurred at 0° in the presence or absence of glucose as well as at 30° without glucose (Fig. 2 B, C, D). Uptake of K^+ against a gradient was observed only at 30° in the presence of glucose (Fig. 2 A). A shown by others, an energy source was necessary in untreated cells, even under aerobic conditions, for the uptake of K^+ against a gradient (unpublished experiments). It should be emphasized that NAC treatment (20 μg/mg cells) did not prevent the cells from concentrating added K^+, although the efficiency of transport was obviously reduced since these cells lost K^+ to a low-K^+ medium.

* The direction of this transport—i.e. from C_o > C_i or from C_i > C_o—depended on relative intracellular (C_i) and extracellular (C_o) concentrations, but was with the gradient in either case (i.e. downhill).

The initial uptake of K^+ by NAC cells (Fig. 2 B, D) was extremely rapid (within 1 min). This appears analogous to the rapid uptake of sugars observed by Cirillo,[15] and is probably associated with external binding sites.[13, 15, 16] It is possible that external K^+-binding sites of NAC-treated cells have become denuded of K^+ or that NAC treatment has exposed more sites.

Warburg experiments

Anaerobic CO_2 production from glucose was markedly reduced by 20 μg NAC alone (Fig. 3) and was almost eliminated by both NAC and Na^+. Sodium, on the other hand, regularly enhanced glycolysis of untreated cells by 10 to 15 per cent. The presence of K^+ from 0 time increased CO_2 production, particularly for the NAC-treated cells; the addition of KCl at 70 min produced a slow CO_2 increase in the control cells and a reversal of inhibition in NAC cells.

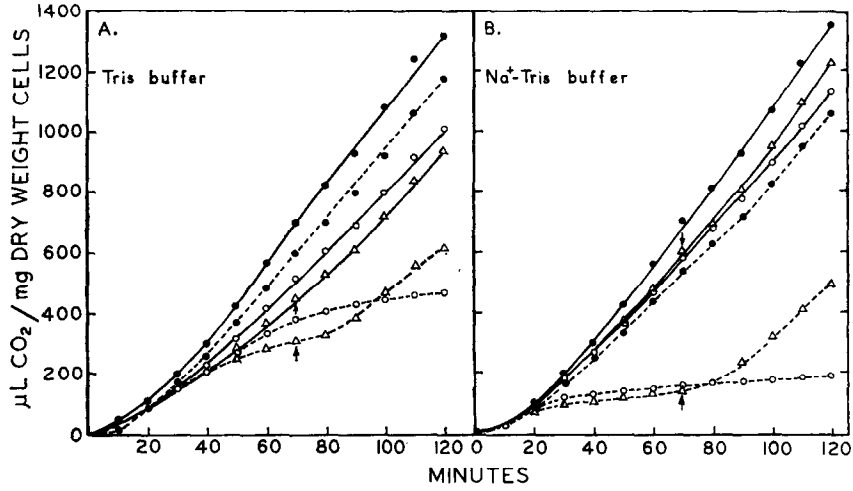

FIG. 3. The effect of N-acetylcandidin (NAC) on anaerobic glycolysis of yeast at 30° in Tris buffer (A) and Na^+–Tris buffer (B) at pH 7·5; ——, without NAC; - - - -, with NAC (20 μg/mg); (○), no KCl; (●), KCl added at 0 min; (△), KCl added at 70 min (↓).

Smaller differences were noted in aerobic experiments (not shown), probably because of the less stringent requirements for K^+ under these conditions.[17] Addition of N_4^+ or K^+ stimulated slightly CO_2 production and O_2 uptake by untreated cells. NAC produced only a partial inhibition of respiration and glycolysis even in Na^+–Tris buffer. Addition of K^+ at 0 min was protective but at 90 min was without effect.

A comparison of the anaerobic CO_2 production with the cellular K^+ levels under similar conditions (Tables 1 and 2; Figs. 1 and 2; Marini *et al.*[4]), revealed that the changes in glycolytic rate were a reflection of the variations in K^+ levels. For instance, Na^+ increased both glycolysis and K^+ retention in the absence of NAC; Na^+ inhibited the two processes in the presence of NAC. It should be emphasized that the inhibition of glycolysis by the polyene antibiotics under the present experimental conditions has previously been shown[4] to be a result of the lowered cellular K^+ level rather than the

reverse—i.e. glycolytic damage causing K^+ loss. Proof of this was the observation that NH_4^+ ions (which can substitute for K^+ in glycolysis) protected glycolysis completely against inhibition by the polyene, nystatin but did not prevent K^+ loss.

Endogenous aerobic glycolysis was negligible (data not shown); thus the log-phase cells did not utilize the buffer components nor did they contain significant quantities of available endogenous substrates.

The pH did not fluctuate beyond the range of 6·5 to 7·5, except in the Na^+ buffer which has relatively low buffering capacity in this pH range. Here a maximum decrease of 2 pH units during glycolysis was observed.

DISCUSSION

In common with the other antifungals, N-acetylcandidin has a pronounced effect on the K^+ content of yeast and indirectly on glycolysis. Although Marini *et al.*[4] found that Na^+ ions were not essential for the inhibition of glycolysis by polyenes, it is clear from the present detailed studies that Na^+ increased both the rate of K^+ loss by treated cells and the inhibition of glycolysis. The effects produced by Na^+ probably are associated with the ion transport phenomenon,[18] as discussed under Results, and will not be considered further.

Log-phase cells of yeast strain LK2G12 lost K^+ gradually in the absence of a readily available energy source. NAC, at 20 μg/mg, accelerated this net loss (even in the presence of glucose) without eliminating the capability of the organism to concentrate K^+. The membrane permeability to small ions was not increased (as had previously been proposed[1, 4]), since K^+ loss stopped when the cells were chilled to 0°. This conclusion is supported by Cirillo's[19] observation that the characteristics of sugar transport in these cells were not significantly altered.

The initial rapid uptake of K^+ by NAC cells at 0° may represent the binding of K^+ to external sites exposed as a result of membrane alteration by NAC. This suggestion is consistent with the rapid inhibition of glycolysis by NAC and with Rothstein's[20] finding that glycolysis by yeast was more sensitive to changes in external K^+ than to intracellular K^+ levels.

Alteration of yeast membrane structure by deoxycorticosterone has been proposed by Conway and Hingerty[21] to explain the reduction in K^+ uptake against a gradient and the reduction in Na^+ loss with a gradient which this steroid produces. Binding of NAC to the cell membrane may similarly cause an alteration which reduces the efficiency or specificity of K^+ transport. Of special interest is the fact that NAC and other polyenes appear to bind with the sterol of the cell membrane,[1-3] indicating a possible parallelism with the action of the steroid.[21]

The higher level of NAC (100 μg/mg) not only produced an increased rate of K^+ loss (Table 2), but prevented concentration of K^+ by the cells. Uptake with the gradient was still observed (Table 2). Neither 20 nor 100 μg of NAC/mg exposed the alkaline phosphatase (ATPase) activity of log-phase cells (unpublished). This enzyme is not demonstrable in untreated cells but is readily detected after cell lysis. Thus membrane alteration, even by 100 μg NAC, was probably associated with a change in, or damage to, the ion transport system, rather than with a direct increase in membrane permeability. The loss of ability to concentrate K^+ at 100 μg NAC was probably a result of the "leakage" of an increasing variety of cell constituents (in this instance

NH_4^+) which occurs at higher polyene levels.[5] Under the conditions used in Table 2, the addition of K^+ did not reverse the inhibition of glycolysis or oxidation whereas NH_4^+ was rapidly effective. Since energy is required for K^+ concentration, the process was thus effectively blocked.

Alteration of the yeast cell membrane by NAC may approximate the simplest form of polyene action. All polyene antifungals that we have tested initiate K^+ loss at low concentrations; however, other described effects are not necessarily general. For example, even at 100 μg/mg cells, NAC does not initiate the loss of inorganic phosphate or cause ATPase to become "available" to external substrate; nystatin produces these effects but only at high concentrations, whereas filipin initiates phosphate loss and ATPase activity essentially in parallel with K^+ loss (unpublished data). Also, Cirillo has informed us that filipin causes a rapid diffusional leakage of sorbose from log-phase cells. Thus filipin, in contrast to NAC, appears to produce true holes. Continued investigation of the effect of polyenes on K^+ transport may, therefore, provide an insight into those events characteristic of the action of the entire class of antifungal agents.

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