Killer toxin from *Saccharomyces cerevisiae* inhibited the pumping of protons into the medium by metabolically active sensitive cells. Such inhibition coincided with that of the uptake of potassium ions which are thought to be accumulated by yeast cells in order to neutralize the membrane potential created because of the extrusion of protons. The consumption of glucose, however, was identical in killer-treated and untreated cells. These alterations can be explained by the ability of the toxin to reduce the chemical proton gradient across the plasma membrane as measured by the accumulation of the weak permeable $[^{14}C]$propionic acid. With this method, an internal pH of 6.42 was calculated from normal cells (the external pH was 4.6) while that of toxin-treated cells was decreased as a function of time. The proton concentration gradient was reduced from 66- to 17-fold. It is shown that the toxin-induced alteration of the proton gradient is due to an enhanced proton permeability of the yeast plasma membrane upon binding of the toxin. It is suggested that killer toxin acts as a macromolecular proton conductor similar in some respects to the known proton conductors 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone, since all the described effects are also observed with these substances.

We provide evidence that the toxin increases the proton permeability of yeast plasma membrane, thus partially disrupting the chemical gradient of protons across it. The results are discussed in the light of previously observed effects of the toxin, and with regard to the mechanism by which the toxin could act by disrupting an energized membrane state.

**Experimental Procedures**

**Materials**

**Yeast Strains**—All the strains of *Saccharomyces cerevisiae* used were from the laboratory of Dr. G. Fink (Cornell University, Ithaca, NY), and were a generous gift of Dr. J. Conde (Cervezas Cruzcampo, Sevilla, Spain). Strain X 17/17 (a, his 1) was used as sensitive strain. Strain A 8207 B was used as killer producer and the nonkiller strain derived from it, A 8207 B-NK 1 (both a, his 4) (7), was used as control.

**Chemicals**—$[^{14}C]$Propionic acid was obtained from The Radiochemical Centre, Amersham. $[^{14}C]$H$_2$O and $[^{14}C]$methoxy inulin were from New England Nuclear. Yeast extract and peptone were from Difco. DNP, DCCD, and glucose were obtained from Merck. CCCP was from Calbiochem, and 2-deoxyglucose, antimycin A, and EDC from Sigma. CCCP, DNP, DCCD, and antimycin A were dissolved in 96% ethanol.

**Methods**

**Preparation of Toxin**—Partially purified killer toxin was obtained from cultures of strain A 8207 B grown at 22 °C in a medium containing 1% yeast extract, 2% peptone, and 2% glucose supplemented with 400 mg/liter of adenine, 30 mg/liter of histidine, and 30 mg/liter of leucine which had been previously filtered through a Difflco PM 30 membrane. The medium was buffered with 0.25 mM citrate phosphate, pH 4.7. Culture supernatants were filtered as above followed by precipitation with 80% ammonium sulfate. The precipitate was resuspended in 5 mM citrate phosphate, pH 4.7, or 10 mM $\epsilon$-aminocaproic-HCl, pH 4.6, as indicated. Identical preparations from culture supernatants of the nonkiller strain A 8207 B-NK 1 were used throughout the work. When necessary, killer toxin activity was assayed using the well test method of Woods and Bevan (8) as previously described (6).

**Determination of Glucose and ATP**—Glucose was determined by a glucose oxidase-peroxidase coupled assay. To 50-ml samples were added 2 ml of 0.1 M phosphate buffer, pH 7.5, containing 100 mg/ml of glucose oxidase, 5 mg/ml of peroxidase, and 300 mg/ml of o-dianisidine and incubated at 37 °C for 15 min. The reaction was stopped with 2 ml of 6 N HCl and the absorbance at 540 nm was measured.

ATP was determined by a luciferin-luciferase assay. The enzyme was activated according to Kimmich et al. (9). To 0.9 ml of 5 mM arsenate buffer, pH 8.0, containing 4 mM MgSO$_4$ and 20 mM glycylglycine, were added 50 ml of sample and 50 ml of activated luciferase and the mixture counted in a Beckman scintillation counter after 20 s for at least 30 s.

**Separation of Plasma Membranes**—Plasma membranes of strain X 17/17 were obtained according to Serrano (10). In a typical experiment the abbreviations used are DNP, 2,4-dinitrophenol; $\Delta$$\psi$H, the proton electrochemical gradient; $\Delta$$\psi$, pH difference across the membrane; $\Delta$$\psi$K*, the concentration gradient of potassium; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; DCCD, N,N'-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.

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† To whom all correspondence should be sent.

‡ Bevan, E. A., and Mackower, M. (1963) Proc. Int. Congr. Genet. 1, 293.
Effect of Yeast Killer Toxin

imint, to 40 ml of a cell suspension (8 × 10^6 cells/ml) in 25 mM Tris-HCl buffer, pH 7.5, 2 mM β-mercaptoethanol, 0.2 mM EDTA was added 80 ml of glass beads (0.5 mm in diameter). The mixture was shaken for 4 min in a Vibrocent cell mill and the homogenate filtered through a sintered glass funnel which was washed subsequently with an additional 40 ml of Tris buffer. The extract thus obtained was centrifuged at 400 × g for 10 min to discard the cell debris. The supernatant was then centrifuged at 35,000 rpm for 30 min in a 75 T2 rotor (Beckman). The pellet of this centrifugation was resuspended with 4 ml of Tris buffer and homogenized by 6 strokes in a Dounce homogenizer. The homogenate was placed at the top of a 20-60% sucrose gradient which was centrifuged for 10 h in a SW 25 rotor. Plasma membrane fractions were identified by the sodium azide-resistant ATPase activity, pooled, and used for the experiments.

**ATPase Assay—**ATPase was assayed at pH 4.6 (pH at which the enzyme has about 50% of maximal activity) as follows. To 800 ml of 30 mM ε-aminoacproic-HCl buffer, pH 4.6, containing 10 mM MgCl_2 (with and without sodium azide) were added 100 ml of membranes (about 50 μg of protein) and 50 ml containing the inhibitors or the appropriate amount of killer toxin in 50 μl of 10 mM ε-aminoacproic-HCl buffer, pH 4.6. The reaction was started by addition of 50 μl of 20 mM ATP and incubated for 30 min at room temperature. The reaction was stopped by addition of 2 ml of a mixture containing 2% sulfuric acid, 0.5% SDS, and ammonium molybdate (the first reagent for the determination of inorganic phosphate). After 5 min, 20 ml of 10% ascorbic acid were added and after 10 min further, the absorbance at 750 nm was determined. When required, sodium azide was added to a final concentration of 0.9 mM.

**Determination of Cellular Volume—**The cellular volume was determined according to Rottenberg (11). Typically, a cell suspension (2 × 10^5 cells/ml) in 10 mM ε-aminoacproic-HCl buffer, pH 4.6, was incubated for 30 min with 0.1 mM glucose. After the preincubation, to 0.5 ml of the cell suspension was added 1 μl of [3H]H_2O (1 nCi/ml), 5 μl of [3H]methoxy inulin (0.1 μCi/ml, 9 mg/ml), and 0.5 ml of ε-aminoacproic-HCl buffer, pH 4.6. After 10 min of incubation at room temperature, two 400-μl aliquots were withdrawn and centrifuged for 5 min in a Beckman microfuge. To 20 μl of the supernatant and to the pellets (after the supernatants have been removed by aspiration) was added 1 ml of 1% SDS and incubated overnight at room temperature. After this incubation 800 μl of each sample were counted for [3H] and [1^4C] in a Beckman liquid scintillation counter. The internal water volume was determined as

\[ V_1 = V_0 \times \left[ \frac{[3H]}{[1^4C]} \right] \]

(1)

where \( p \) stands for pellet, \( s \) for supernatant, and \( V \) is the volume of the sample used. An internal volume of 1.36 μl/10^6 cells was obtained.

**Determination of \( [H^+]_0 \)—**[H^+] was calculated as the extrusion of protons. Both processes can be visualized with selective electrodes immediately after glucose is added to a cell suspension. When a preparation of yeast killer toxin was added to a suspension of sensitive cells an immediate inhibition of both proton efflux and potassium influx was observed (Fig. 1A). Interestingly, when the rate of glucose consumption was measured at early stages of toxin action in which no dead cells can be detected (6), no difference was observed between the control and killer-treated cells (Fig. 1B). The utilization of the sugar was clearly diminished in toxin-treated cells (not shown). When the protonophore CCCP was added to identical cell suspensions two different effects were observed depending on the relative concentration of the ionophore. At low concentrations (12.5 mM/10^6 cells) both the proton efflux and the potassium uptake were inhibited (Fig. 2A) while at higher concentrations (500 mM/10^6 cells) both ion fluxes were reversed (Fig. 2B). In the former conditions glucose consumption was unaffected, while in the latter it was

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**RESULTS**

**Killer Effect on Proton Efflux and Potassium Influx—**Yeast cells acidify the culture medium as they metabolize glucose. Simultaneously, the cells take up and accumulate potassium, possibly as a way of neutralizing the electrical potential generated because of the extrusion of protons. Both processes can be visualized with selective electrodes immediately after glucose is added to a cell suspension. When a preparation of yeast killer toxin was added to a suspension of sensitive cells an immediate inhibition of both proton efflux and potassium influx was observed (Fig. 1A). Interestingly, when the rate of glucose consumption was measured at early stages of toxin action in which no dead cells can be detected (6), no difference was observed between the control and killer-treated cells (Fig. 1B). At late stages of killing, however, the utilization of the sugar was clearly diminished in toxin-treated cells (not shown). When the protonophore CCCP was added to identical cell suspensions two different effects were observed depending on the relative concentration of the ionophore. At low concentrations (12.5 mM/10^6 cells) both the proton efflux and the potassium uptake were inhibited (Fig. 2A) while at higher concentrations (500 mM/10^6 cells) both ion fluxes were reversed (Fig. 2B). In the former conditions glucose consumption was unaffected, while in the latter it was

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**FIG. 1.** Killer toxin effect on ion movements and glucose consumption in sensitive cells of *S. cerevisiae*. Cell suspensions of strain X 17/17 containing 8 × 10^6 cells in 3.6 ml of 5 mM citrate phosphate buffer, pH 4.7, plus 5 mM KCl. The cell suspensions were placed in a water-jacketed vessel at 25 °C, pH was measured with an electrode GK 2401 C (Radiometer) and K+ with an electrode F2002 (Radiometer). Both were coupled to a pH meter PHEM 64 from Radiometer and the results recorded in a two-channel LKB recorder.

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**Measurement of Proton Permeability—**Cell suspensions in 10 mM ε-aminoacproic-HCl buffer, pH 4.6, were treated as follows. To 3 ml of cell suspension (2 × 10^5 cells/ml) was added killer toxin or proton conductors, which was then incubated for 15 min at room temperature. After the incubation the cells were washed twice with distilled water and resuspended in 3 ml of 30 mM pHM containing 60 μg/ml of antimycin A. After 2 min of incubation at room temperature, 2-deoxoglycose was added to a final concentration of 50 mM and the incubation continued. At intervals, 10 mM HCl was added and the pH monitored as indicated above.

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**Effect of Yeast Killer Toxin—**The enzyme has about 20-60% of maximal activity) as follows. To 800 ml of 30 mM ε-aminoacproic-HCl buffer, pH 4.6, containing 10 mM MgCl_2 (with and without sodium azide) were added 100 ml of membranes (about 50 μg of protein) and 50 ml containing the inhibitors or the appropriate amount of killer toxin in 50 μl of 10 mM ε-aminoacproic-HCl buffer, pH 4.6. The reaction was started by addition of 50 μl of 20 mM ATP and incubated for 30 min at room temperature. The reaction was stopped by addition of 2 ml of a mixture containing 2% sulfuric acid, 0.5% SDS, and ammonium molybdate (the first reagent for the determination of inorganic phosphate). After 5 min, 20 ml of 10% ascorbic acid were added and after 10 min further, the absorbance at 750 nm was determined. When required, sodium azide was added to a final concentration of 0.9 mM.

**Determination of Cellular Volume—**The cellular volume was determined according to Rottenberg (11). Typically, a cell suspension (2 × 10^5 cells/ml) in 10 mM ε-aminoacproic-HCl buffer, pH 4.6, was incubated for 30 min with 0.1 mM glucose. After the preincubation, to 0.5 ml of the cell suspension was added 1 μl of [3H]H_2O (1 nCi/ml), 5 μl of [3H]methoxy inulin (0.1 μCi/ml, 9 mg/ml), and 0.5 ml of ε-aminoacproic-HCl buffer, pH 4.6. After 10 min of incubation at room temperature, two 400-μl aliquots were withdrawn and centrifuged for 5 min in a Beckman microfuge. To 20 μl of the supernatant and to the pellets (after the supernatants have been removed by aspiration) was added 1 ml of 1% SDS and incubated overnight at room temperature. After this incubation 800 μl of each sample were counted for [3H] and [1^4C] in a Beckman liquid scintillation counter. The internal water volume was determined as

\[ V_1 = V_0 \times \left[ \frac{[3H]}{[1^4C]} \right] \]

(1)

where \( p \) stands for pellet, \( s \) for supernatant, and \( V \) is the volume of the sample used. An internal volume of 1.36 μl/10^6 cells was obtained.

**Determination of \( [H^+]_0 \)—**[H^+] was calculated as the extrusion of protons. Both processes can be visualized with selective electrodes immediately after glucose is added to a cell suspension. When a preparation of yeast killer toxin was added to a suspension of sensitive cells an immediate inhibition of both proton efflux and potassium influx was observed (Fig. 1A). Interestingly, when the rate of glucose consumption was measured at early stages of toxin action in which no dead cells can be detected (6), no difference was observed between the control and killer-treated cells (Fig. 1B). At late stages of killing, however, the utilization of the sugar was clearly diminished in toxin-treated cells (not shown). When the protonophore CCCP was added to identical cell suspensions two different effects were observed depending on the relative concentration of the ionophore. At low concentrations (12.5 mM/10^6 cells) both the proton efflux and the potassium uptake were inhibited (Fig. 2A) while at higher concentrations (500 mM/10^6 cells) both ion fluxes were reversed (Fig. 2B). In the former conditions glucose consumption was unaffected, while in the latter it was
inhibited (not shown). Killer toxin and CCCP were thus acting in a similar way; both were causing the uncoupling between the oxidation of glucose and the establishment of a proton gradient (via ATP) across the cell membrane. This action was observed at low concentrations of CCCP and at early stages of killer action.

Determination of Internal pH and Effect of Killer Toxin on $\Delta pH$—The determination of internal pH by studying ion distribution is based upon the use of acids or amines in which the neutral species diffuses across the membrane while the ion is impermeable. When a permeable acid such as propionic acid has reached an equilibrium distribution, $[\text{AH}]_\text{in} = [\text{AH}]_\text{out}$. Since the acid dissociates on both sides, assuming the dissociation constant is not changed, it follows that:

$$K_d = \frac{[\text{H}^+]_\text{in} [\text{A}^-]}{[\text{AH}]_\text{in}} = \frac{[\text{H}^+]_\text{out} [\text{A}^-]}{[\text{AH}]_\text{out}}$$

from where $[\text{H}^+]_\text{in}$ can be calculated according to Equation 2. It should be demonstrated, however, that the accumulation of the acid does not collapse $\Delta pH$ (that is, the $C_{\text{C}}/C_{\text{S}}$ should be the same over a wide range of concentrations), that the internalized product is not metabolized, and that it can be extruded with proton conductors which collapse $\Delta pH$. Fig. 3A shows that when either DNP or CCCP was added to cells which were accumulating $[^{14}\text{C}]$propionic acid, the acid was immediately lost from the cells. The same effect was observed when unlabeled propionic acid was added to a concentration of 20 mM. It was demonstrated by silica gel chromatography that the internal propionic acid was not modified and that the same $C_{\text{C}}/C_{\text{S}}$ was obtained with concentration of propionic acid ranging from 3 $\mu\text{M}$ to 10 mM (not shown). Considering an internal volume of 1.36 $\mu\text{l}/10^8$ cells, a $C_{\text{C}}/C_{\text{S}}$ of approximately 24 was obtained by filtration, which gives an internal pH of 6.42 for $S.\text{cerevisiae}$ (external pH 4.6). Values of $C_{\text{C}}/C_{\text{S}}$ of around 20 were obtained by centrifugation which gave a very similar internal pH.

When $S.\text{cerevisiae}$ X 17/17 was incubated with killer toxin and the distribution of $[^{14}\text{C}]$propionic acid monitored by filtration, it was observed that the accumulation of the acid was reduced by about 50% after 10 min of treatment (Fig. 3B), when DNP was present no accumulation was observed. Given the partial purity of the killer preparation used and to insure that the observed effects were killer specific, an identical amount of material from the nonkiller isogenic strain A 8207 B-NK1 was added to a separate series. In this case the accumulation of $[^{14}\text{C}]$propionic acid ran parallel to that of the control series. Table I shows the values of internal pH for control cells, killer toxin-treated cells, and proton conductor-treated cells as calculated from the steady state distribution of $[^{14}\text{C}]$propionic acid in experiments such as those in Fig. 3. It is shown that killer toxin reduced significantly the concentration gradient of protons. It should be emphasized that the described effects are observed 10 min after addition of the toxin, when dead cells are still not detected, although the concentration gradient of protons was reduced further when longer incubations with the toxin were performed. At this

![Fig. 2. Effects of CCCP on ion movements in S. cerevisiae.](image)

**Fig. 2.** Effects of CCCP on ion movements in $S.\text{cerevisiae}$. Cell suspensions of $S.\text{cerevisiae}$ strain X 17/17 were incubated as in Fig. 1 and proton and potassium movements monitored. A, the cell suspension contained $8 \times 10^6$ cells and CCCP was added to a final concentration of 33 $\mu$M. B, the cell suspension contained $2 \times 10^6$ cells and CCCP was added to a final concentration of 0.33 $\mu$M. Glucose concentration was 6 $\mu$M.

**TABLE I**

| Condition of treatment | $[^{14}\text{C}]$Propionic acid | $[\text{H}^+]_\text{in}$ | $[\text{H}^+]_\text{in}$ | Nmol/10$^9$ cells accumulated | $[^{14}\text{C}]$Propionic acid |
|------------------------|-------------------------------|-----------------|-----------------|-------------------------------|-------------------------------|
| Control                | 23.9 $\pm$ 1.1                | 1.00            | 3.8             | 6.42                          | 66.5                          |
| Material from nonkiller strain | 24.2 $\pm$ 3.2 | 3.7             | 6.40            | 67.4                          |
| Killer toxin (10 min)   | 15.2 $\pm$ 1.7                | 0.68            | 6.0             | 62.2                          | 41.5                          |
| Killer toxin (3 h)      | 6.8 $\pm$ 0.4                 | 0.30            | 14.2            | 5.84                          | 17.6                          |
| 3.3 mM DNP             | 2.1 $\pm$ 0.1                 | 0.10            | 60.6            | 5.22                          | 4.1                           |
| 0.33 mM CCCP           | 3.8 $\pm$ 0.2                 | 0.17            | 27.9            | 5.55                          | 9.0                           |

**Fig. 3.** $[^{14}\text{C}]$Propionic acid uptake by cells of $S.\text{cerevisiae}$ X 17/17. A, time course of $[^{14}\text{C}]$propionic acid uptake. A cell suspension (2 $\times$ 10$^6$ cells/ml) in 10 mM $\alpha$-aminopropionic acid-HCl buffer, pH 4.6, was incubated for 30 min with 0.1 mM glucose. After the incubation the uptake of $[^{14}\text{C}]$propionic acid was measured as described under "Experimental Procedures." Four identical series were prepared and the following additions were made to three of them at the time indicated by the arrows: A, DNP to a final concentration of 1 mM. O, unlabeled propionic acid to 20 mM. C, CCCP to 0.1 mM. To the fourth (O) no addition was made (control). Prior to the addition all series presented the same uptake of $[^{14}\text{C}]$propionic acid as represented here by (O). B, effect of killer toxin and DNP on the accumulation of $[^{14}\text{C}]$propionic acid by sensitive cells of $S.\text{cerevisiae}$ strain X 17/17. X 17/17 Glucose was present no accumulation was observed. Given the partial purity of the killer preparation used and to insure that the observed effects were killer specific, an identical amount of material from the nonkiller isogenic strain A 8207 B-NK1 was added to a separate series. In this case the accumulation of $[^{14}\text{C}]$propionic acid ran parallel to that of the control series. Table I shows the values of internal pH for control cells, killer toxin-treated cells, and proton conductor-treated cells as calculated from the steady state distribution of $[^{14}\text{C}]$propionic acid in experiments such as those in Fig. 3. It is shown that killer toxin reduced significantly the concentration gradient of protons. It should be emphasized that the described effects are observed 10 min after addition of the toxin, when dead cells are still not detected, although the concentration gradient of protons was reduced further when longer incubations with the toxin were performed. At this
latter stage a high percentage of cells were not viable (6). Fig. 4 shows the time course of the effect of the toxin and DNP on the proton concentration gradient. It can be observed that both toxin and the ionophore collapse the gradient. However, while DNP require a short period of time to complete its effect, the toxin takes longer.

The Mechanism by Which Killer Toxin Collapses ΔpH—Two mechanisms could account for the observed reduction of ΔpH. (i) the inhibition of a proton-translocating ATPase, and (ii) the enhancement of membrane permeability to protons which would result in the masking, or even the reversal, of the proton movements due to the hydrolysis of ATP. Table II shows that no difference was observed in the ATP content of killer toxin-treated cells compared to control cells. Moreover, when plasma membrane ATPase was measured in the presence and in the absence of the toxin no difference was observed, although proton pumping by intact cells was inhibited by 70% in the presence of the toxin. Table II also shows that

![Diagram](image-url)

**FIG. 4.** Time course of the effect of killer toxin and DNP on the proton concentration gradient of *S. cerevisiae strain X 17/17*. Cell suspensions (2 x 10^8 cells/ml) in 10 mM e-aminocaproic-HCl buffer, pH 4.6, were incubated for 30 min with 0.1 mM glucose. After incubation, 50-μl aliquots of cell suspension were incubated with 10 units of killer toxin (C), 1 mM DNP (A), or buffer (B) in a final volume of 0.3 ml. At indicated times the accumulation of [14C]protonic acid was calculated at the steady state. At 60 min and 120 min of incubation, glucose was added to a concentration of 30 mM.

**TABLE II**

| Addition                     | ATP  | ATPase | H⁺ pumping |
|------------------------------|------|--------|------------|
|                              | nmol/10⁶ cells | milliunits/mg protein | nmol H⁺/min |
| Control                      | 0.26 | 25     | 41         |
| Killer toxin (10 units)      | 0.25 | 26     | 12         |
| Inactivated toxin            | 0.26 | 25     | 42         |
| Material from nonkiller strain | 0.27 | 26     | 43         |
| DCCD (1.25 mM)               | 0.28 | 13     | 8          |
| EDC (50 mM)                  | ND²  | 26     | 25         |
| DNP (1 mM)                   | 0.28 | 27     | 6          |

² ND, not determined.

when cells were treated with the protonophore DNP, no inhibition of membrane ATPase was observed, even though proton pumping by intact cells was inhibited by 80%. On the other hand, upon treatment with DCCD both membrane ATPase (about 50% inhibition) and proton pumping (80% inhibition) were affected. The fact that in the presence of the carbodiimide EDC plasma membrane ATPase was not inhibited although inhibition of proton pumping was found will be discussed below. Although these results do not definitively prove that the proton ATPase is not affected by killer toxin, they strongly suggest it, especially when considered together with the data on changes in proton permeability (see below).

Proton permeability can be measured by looking at the equilibration of the ion when acid is added to a cell suspension which contains the appropriate number of cells. In order to be able to observe the proton influx due to the change in membrane permeability it is necessary that this be the only proton movement across the cell membrane. This condition is fulfilled when antimycin A and 2-deoxyglucose are added, depleting cells of ATP and therefore suppressing any energy-dependent proton movement (12). Fig. 5A shows that when HCl was added to a control cell suspension in which the pH was continuously monitored, a drop in the pH was observed which subsequently remained unchanged. On the other hand, if the
cells had been previously treated with the proton conductor CCCP the exogenously added protons quickly equilibrated across the cell membrane (Fig. 5C). When cells from the killer-sensitive strain X 17/17 were treated with the toxin for 15 min previous to the experiment the pattern was similar to that obtained with CCCP (Fig. 5B); that is, the cell membrane was now permeable to protons. When the preparation from the nonkiller isogenic strain was used (not shown) the pattern was identical with that of Fig. 3A.

Interestingly, when potassium was monitored simultaneously in these experiments it was observed that as the protons moved to the cell interior to equilibrate, an efflux of potassium was detected when both CCCP and killer toxin were added. This, along with the experiments depicted in Figs. 1 and 2, proves that the movement of both ions are closely connected.

**DISCUSSION**

The results presented in this paper strongly suggest that killer toxin acts on sensitive cells by disrupting the normal state of electrochemical ion gradients. It is known that yeast cells depleted of ATP spontaneously lose potassium and that this loss is accompanied by the gain of an equivalent amount of protons (13). It seems therefore logical that if proton pumping is inhibited when killer toxin is added to cells, a consequent reduction in the uptake of potassium is also observed. Even though the inhibition of the proton ATPase cannot be ruled out completely, the most likely explanation of the observed inhibition of ion movements is that as the toxin enhances the permeability of the membrane to protons, a flux to the cell interior begins which, as a function of the amount of toxin, neutralizes partially or totally the observable efflux due to the ATPase (6). This mechanism could explain the efflux of potassium ions which occurs in the late phase of killer action. If a massive inflow of protons occurs in this phase, it should be accompanied by a potassium efflux. Moreover, when the cell is finally de-energized, any metabolite or ion which is accumulated by the cell against its concentration gradient will tend to equilibrate and an efflux would be observed. This could be the case for potassium or ATP, both of which have been reported to leak out of killer toxin-treated cells (3, 4).

It is of interest to compare the action of killer toxin and that of CCCP (or DNP). While the toxin can just inhibit proton efflux, with CCCP a reversion of proton flux can also be obtained provided that the concentration of the ionophore is sufficiently high. Moreover, while CCCP or DNP collapses ΔpH in a few minutes, killer toxin apparently requires more than 3 h to do it. These differences can be explained because of the greater mobility and the lack of specificity of the ionophores so that even intracellular membranes would become permeable to protons. It is interesting that glucose consumption was not inhibited by the toxin at early stages of toxin action, or by CCCP at low concentrations, while proton efflux was inhibited (because of the influx of protons from the medium). This can be interpreted as glucose being futilely used by cells in their attempt to establish a proton gradient across the cell membrane. Under these circumstances the decrease of internal pH would not be enough to inhibit glycolytic enzymes. At late stages of killer action or with high concentrations of CCCP the drop of internal pH could explain by itself the inhibition of glycolysis.

According to the hypothesis of Mitchell (14, 15), oxidation of electron donors by a membrane-bound respiratory chain and a (Ca2+ and Mg2+) ATPase are responsible for generating a ΔpH+ that is the immediate driving force for active transport even though no measurements of this parameter are still available in yeast. Since killer toxin acts by reducing ΔpH, it is to be expected that as the driving force is reduced, active transport is affected in the same way. Indeed, it has been shown that killer toxin inhibits the uptake of leucine and histidine, as well as that of protons which are cotransported with these amino acids (6). Fig. 6 depicts the changes which occur after a sensitive cell has bound the toxin. In normal conditions the hydrolysis of ATP by the proton ATPase is accompanied by the extrusion of protons into the external medium, originating a ΔpH. Two consequences are that ΔpK+ (more concentrated inside) can be created and that certain amino acids will be accumulated against their concentration gradient. When killer toxin has affected the cell, independently of whether the ATPase is active or not, the ΔpH is partially collapsed since protons can now cross the membrane. As a consequence, the ΔpK+ is also collapsed and the cotransport of protons and amino acids inhibited. Interestingly, all these effects can also be observed when DNP or CCCP are added to the cells. As shown in Table 1, killer toxin does not completely collapse ΔpH. Whether this partial collapse is enough to de-energize the cell completely remains to be determined, but the possibility that the toxin exerts some other as yet unknown effects should be considered. In this regard the mechanism by which killer toxin de-energizes sensitive cells merits discussion. The experiments shown in Fig. 5 clearly demonstrate that the proton permeability has been enhanced in toxin-treated cells. The question remains as to whether the proton ATPase is affected. The results in Table II suggest that the enzyme is

![Fig. 6. Proposed mechanisms of action of killer toxin from S. cerevisiae on sensitive cells.](image-url)
not affected by the toxin. Thus, ATP levels were not increased in toxin-treated cells. Moreover, when compared to DCCD and DNP the toxin behaved like the proton conductor, since both proton pumping and ATPase activity were inhibited by DCCD while only proton pumping, but not ATPase, was inhibited by DNP and killer toxin. However, ATP levels can be maintained by other homeostatic mechanisms even if an ATPase is inhibited. In addition, even when inhibition of proton pumping is observed in intact cells, changes may not be detected in membrane ATPase activity (note that EDC, a more hydrophilic carbodiimide, inhibited proton pumping but did not affect ATPase activity of the membranes). Therefore, a definitive answer about the action of killer toxin on the proton ATPase will not be obtained until purified preparations of the enzyme are available. Regardless of some possible additional effects, the results presented here indicate that killer toxin acts by disrupting an energized membrane state in a way which could be comparable to that of colicins. Thus, it has been shown that colicin Ia, colicin K, and colicin E1 can alter the permeability of Escherichia coli membranes to different ions (23–25). Although much remains to be done it is now possible to speculate on whether killer toxin is to yeast what colicins are to bacteria.

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