ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1979, p. 750-756
0066-4804/79/12-0750/07$02.00/0

Vol. 16, No. 6

Compounds Affecting Membranes that Inhibit Protein Synthesis in Yeast

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Received for publication 5 July 1979

The regulation of translation has been investigated in yeast cells by means of ionophores and other compounds affecting the ionic concentration inside the cell. Treatment of a variety of cells with these compounds produces a drastic inhibition in the protein-synthesizing activity of the cell. Protein synthesis in yeast is strongly inhibited by amphotericin B and nystatin. Mammalian cells are blocked in their translation capacity by gramicidin D, nigericin, monensin, nystatin, A23187, and bromolasalocid. The effects of these compounds on protein synthesis in *Escherichia coli* and *Staphylococcus aureus* were also investigated. Amphotericin B is a powerful inhibitor of both protein and ribonucleic acid syntheses in yeast cells at concentrations that do not affect the transport of the labeled amino acid or nucleoside precursor. The analysis of the polysomal profiles in yeast spheroplasts could indicate that initiation is the target of amphotericin B action on translation. Studies on the reversion of the protein synthesis blockade by amphotericin B by increasing the potassium concentration in the medium suggest that changes in the potassium concentration in cellular cytoplasm might be responsible, at least in part, for the inhibition of protein synthesis.

The molecular mechanism by which the translation machinery is regulated in the cell is not yet well understood. It is obvious that some mechanisms must operate in the cell to regulate protein synthesis under physiological conditions (8, 11, 12, 16), and the target of such regulation seems to be at the initiation of messenger ribonucleic acid (RNA) translation (11). Different lines of evidence indicate that in this regulation the structure of the ribosome binding site of the messenger RNA plays an important role (16). On the other hand, overwhelming evidence from in vitro systems indicates that the phosphorylation of several initiation factors and ribosomal proteins (11) influences the capacity of cell-free systems to synthesize proteins (16). However, there is no in vivo evidence as yet to support such a phosphorylation mechanism in intact cells under different physiological conditions. It has also been observed that monovalent ions can specifically affect the in vitro translation of several messenger RNAs in such a way that the translation of some of them is strongly inhibited by those ions, whereas the translation of other messenger RNAs is stimulated (4). These findings correlate with the changes in the permeability of the membrane to ions observed in vivo when changes in protein synthesis occur (3, 4).

These observations have led us to investigate the possible relationship between changes in plasma membrane activity distorting the ionic concentration in the cytoplasm membrane, modifying the gradient of ions and protons maintained by cellular membranes (3, 15). By using these compounds we sought to answer the following questions. (i) Is there a direct relationship between plasma membrane integrity and the translation capacity of the cells? (ii) What step in translation is affected by modification of the ionic composition of cellular cytoplasm? (iii) What is the actual mediator between the changes in membrane integrity and the protein-synthesizing apparatus?

MATERIALS AND METHODS

Saccharomyces cerevisiae Y166 was used throughout this work. It is the haploid mating type α, auxotrophic for histidine and tryptophan, and unable to ferment maltose. The cells were grown in YEP medium (6) and subcultivated once a day by adding 0.2 ml of stationary cultures to 10 ml of fresh medium and incubated with shaking at 30°C. Krebs II ascites cells were taken from mice previously injected with ascitic tumor liquid. *Escherichia coli* and *Staphylococcus aureus* cells were employed as described in Table 1, footnotes *a* and *b*.

Protein synthesis was estimated by incubating yeast cultures with an initial optical density of 0.5 at 660 nm in minimal medium (6) supplemented with 0.1 mM [^{35}S]methionine (Amersham, 15 mCi/mmol) at 30°C. After incubation, the samples were heated for 10 min

at 90°C with 1 ml of 10% trichloroacetic acid, precipitates were collected on GF/C glass fiber filters (Whatman), and radioactivity was determined.

RNA synthesis was estimated in a similar way by adding 0.01 mM [^{14}C]uridine (Amersham, 533 mCi/mmol) to yeast cultures. The methionine pool was measured by estimating soluble radioactivity in 10% trichloroacetic acid after incubation of yeast cultures with 0.1 mM [^{35}S]methionine (15 mCi/mmol) for 5 min at 30°C.

To prepare spheroplasts, a yeast culture was grown in 40 ml of YM-1 medium (6) to an optical density of 0.15 to 0.20 at 660 nm. The culture was collected and suspended in 4 ml of 1 M sorbitol with 1% commercial Glusulase (Endo Laboratories, Inc.), and the mixture was incubated for 1 h at 30°C. The spheroplasts were further incubated in 20 ml of YM-5 medium (6) for 3 h at 30°C. Aliquots of 2 ml were withdrawn for each experimental point. To "freeze" polysomes, cycloheximide was added to a final concentration of 200 μg/ml. The spheroplasts were collected by centrifugation, suspended in 250 μl of buffer P [50 mM tris(hydroxymethyl)aminomethane, 100 mM KCl, 30 mM $MgCl_2$], and lysed by adding 25 μl of 5% sodium deoxycholate (Sigma) and 40 μl of 5% Brij 35 (Sigma); 100-μl volumes of the lysates were layered on top of linear gradients (15 to 30% sucrose) over a cushion of 1 ml of 40% sucrose, containing 50 mM tris(hydroxymethyl)aminomethane, 100 mM KCl, and 30 mM $MgCl_2$. The gradients were centrifuged at 45,000 rpm for 45 min in an OTD-2 Sorvall ultracentrifuge employing an AH-650 Sorvall rotor, and they were finally analyzed in an ISCO gradient fractionator.

Sources of the inhibitors used were as follows: amphotericin B, GIBCO; A23187, monensin, and nigericin, Lilly Laboratories; bromolasalocid and polymyxin B_1, F. Hoffman-La Roche and Co.; cycloheximide, Calbiochem; gramicidin D, nystatin, ouabain, and valinomycin, Sigma.

RESULTS

Effect of ionophores on protein synthesis in a variety of cells. A number of ionophores and other compounds affecting the potassium concentration inside the cell were tested for their effects on protein synthesis in the yeast *S. cerevisiae*. The compounds tested included the gramicidin D complex, nigericin, polymyxin B_1, amphotericin B, valinomycin, monensin, nystatin, A23187, bromolasalocid, and the cardioglycoside compound ouabain. The action of these compounds on protein synthesis was studied with time using different concentrations of the inhibitors (Fig. 1). The most powerful in preventing translation were nystatin and amphotericin B; these results are in agreement with previous observations by other workers (15). It is noteworthy that neither ouabain, monensin, A23187, or bromolasalocid had significant inhibitory effects on protein synthesis in this system, although all four compounds were very effective in preventing translation in the tumor Krebs II ascites cells (Table 1). The action of these compounds on translation was also tested in both gram-positive and gram-negative bacteria (Table 1). *S. aureus* was very susceptible to gramicidin D, nigericin, polymyxin B_1, ouabain, valinomycin, monensin, A23187, and bromolasalocid. On the other hand, only polymyxin B_1 had significant inhibitory activity on protein synthesis in *E. coli*.

Action of amphotericin B and nystatin on macromolecular synthesis in the yeast *S. cerevisiae*. From the results illustrated in Fig. 1 and Table 1 we selected nystatin and ampho-

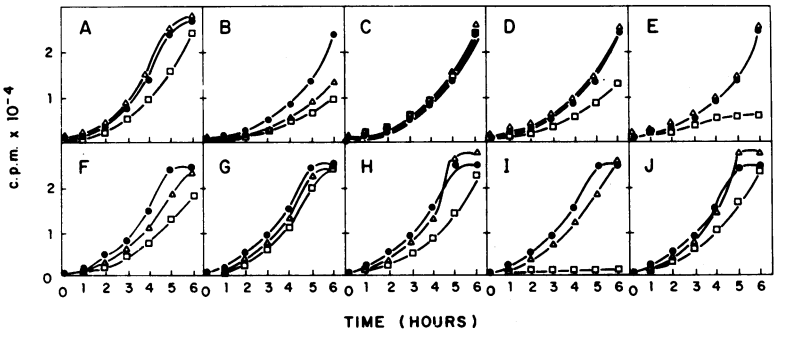

FIG. 1. *Effects of a number of membrane-active compounds on protein synthesis by yeast. Protein synthesis was measured as indicated in the text. Yeast cells were placed in 0.4 ml of minimal medium plus 0.6 μCi of [^{35}S]methionine (15 mCi/mmol) and the compounds indicated. A 0.025-ml aliquot was withdrawn at the indicated times and processed to estimate protein synthesis. (A) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M gramicidin D complex. (B) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M nigericin. (C) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M ouabain. (D) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M polymyxin B_1. (E) ●, Control; △, 2.7×10^{-7} M and □, 8.1×10^{-7} M amphotericin B. (F) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M valinomycin. (G) ●, Control; △, 10^{-4} M and □, 4×10^{-4} M monensin. (H) ●, Control; △, 10^{-5} M and □, 4×10^{-5} M A23187. (I) ●, Control; △, 2×10^{-7} M and □, 4×10^{-6} M nystatin. (J) ●, Control; △, 10^{-5} M and □, 4×10^{-5} M bromolasalocid.*

TABLE 1. *Effects on protein synthesis of a number of compounds affecting the membrane*

Drug	*E. coli*[a]		*S. aureus*[b]		*S. cerevisiae*[c]		Ascitic Krebs II cells[d]	
	Concn (M)	% of control	Concn (M)	% of control	Concn (M)	% of control	Concn (M)	% of control
Gramicidin D complex	10^{-4}	100	10^{-4}	<1	10^{-4}	100	10^{-5}	<1
	4×10^{-4}	96	4×10^{-4}	<1	4×10^{-4}	87	5×10^{-5}	<1
Nigericin	10^{-4}	88	10^{-4}	9	10^{-4}	57	10^{-5}	<1
	4×10^{-4}	76	4×10^{-4}	<1	4×10^{-4}	40	5×10^{-5}	<1
Polymyxin B_1	10^{-5}	<1	10^{-5}	<1	10^{-4}	96	10^{-5}	87
	4×10^{-5}	<1	4×10^{-5}	<1	4×10^{-4}	50	5×10^{-5}	21
Ouabain	10^{-5}	80	10^{-5}	6	10^{-4}	100	2×10^{-4}	88
	4×10^{-5}	80	4×10^{-5}	<1	4×10^{-4}	100	5×10^{-4}	4
Amphotericin B	2.7×10^{-7}	100	2.7×10^{-5}	100	2.7×10^{-7}	92	2.7×10^{-7}	41
	8.1×10^{-7}	100	8.1×10^{-7}	100	8.1×10^{-7}	22	8.1×10^{-7}	46
Valinomycin	10^{-4}	100	10^{-4}	12	10^{-4}	100	10^{-4}	43
	4×10^{-4}	100	4×10^{-4}	<1	4×10^{-4}	70	2×10^{-4}	24
Monensin	10^{-4}	85	10^{-4}	7	10^{-4}	90	10^{-5}	<1
	4×10^{-4}	80	4×10^{-4}	<1	4×10^{-4}	90	5×10^{-5}	<1
Nystatin	10^{-6}	100	10^{-6}	100	2×10^{-7}	100	5×10^{-5}	<1
	2×10^{-6}	100	2×10^{-6}	100	10^{-6}	<1	10^{-4}	<1
A23187	10^{-5}	100	10^{-5}	<1	10^{-5}	100	5×10^{-6}	<1
	4×10^{-5}	83	4×10^{-5}	<1	4×10^{-5}	83	10^{-5}	<1
Bromolasalocid	10^{-5}	100	4×10^{-5}	<1	5×10^{-5}	100	10^{-6}	23
	4×10^{-5}	100	4×10^{-5}	<1	5×10^{-6}	90	5×10^{-6}	<1

[a] Protein synthesis was estimated in incubation mixtures containing 10 μl of a culture of *E. coli* in the stationary phase, 1 μl of 10 mM [^{35}S]methionine (specific activity, 15 mCi/mmol), the required inhibitor, and the defined medium to complete 100-μl volumes. The incubation time was 6 h at 37°C. Incorporation in the control without antibiotic was 33,340 cpm.

[b] Protein synthesis was estimated in incubation mixtures containing 10 μl of a culture of *S. aureus* (5×10^7 cells per ml), 1 μl of [^{35}S]methionine (1,020 Ci/mmol, 3 mCi/ml), the required inhibitor, and the defined medium to complete 100-μl volumes. Incubation time was 6 h at 37°C. Incorporation in the control without antibiotic was 7,568 cpm.

[c] Protein synthesis was estimated in 100-μl volumes in minimal medium of incubation mixtures of *S. cerevisiae* (0.5 units of absorbance at 660 nm) containing [^{35}S]methionine and the required inhibitor. The incubation time was 6 h at 30°C. Incorporation in the control without antibiotic was 91,930 cpm.

[d] Protein synthesis was estimated in incubation mixtures containing 10 μl of Ehrlich ascites tumor liquid grown in mice, 1 μl of [^{35}S]methionine (1,020 Ci/mmol, 0.3 mCi/ml), the required inhibitor, and Earle medium to complete 100-μl volumes. Incubation time was 6 h at 37°C. Incorporation in the control without antibiotic was 17,1[illegible] cpm.

tericin B to look further into their effects on protein synthesis, RNA synthesis, and methionine transport in *S. cerevisiae*. Figure 2 shows the effects of different concentrations of amphotericin B and nystatin on protein synthesis under different incubation conditions. Concentrations of amphotericin B and nystatin as low as 10^{-5} M blocked translation in yeast cells completely. Figure 3 illustrates the dependence of cell concentration on amphotericin B inhibition. Under the conditions used, 50% inhibition is achieved at an average of 5×10^8 molecules of amphotericin B per cell. The time course of amphotericin B and nystatin action is shown in Fig. 4. It should be noted that although nystatin exerted its action almost immediately after its addition to the medium (Fig. 4B), a lag of about 1 h was observed with amphotericin B; however, after that time the blockade on translation by 10^{-5} M amphotericin B was complete (Fig. 4B). Treatment of cells with 1.3×10^{-5} M amphotericin B for 1 h and removal of the compound from the medium by repeated washings renders the cells unable to grow and synthesize proteins when placed in new, fresh medium. However, similar treatment with 5×10^{-6} M amphotericin B is completely reversible.

Amphotericin B is also effective in blocking RNA synthesis in yeast cells, as measured by uridine incorporation into acid-precipitable material (Table 2).

It is well known that amphotericin B disturbs the gradient of ions maintained by the plasma membrane (5, 15). In addition, it interferes with the transport through the membrane of several metabolites. It was therefore of interest to determine whether the inhibition of translation observed was due to a direct effect on the protein-synthesizing machinery, or whether inhibition in the transport of methionine was the

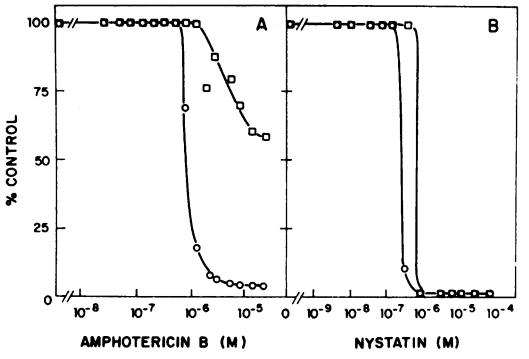

FIG. 2. *Effect of different concentrations of amphotericin B and nystatin on protein synthesis by yeast. Cultures of yeast cells (0.1 ml) in minimal essential medium (initial absorbance of 0.5 at 660 nm) were incubated at 30°C. The indicated concentration of the compound and 0.15 μCi of [^{35}S]methionine (15 mCi/mmol) were added at time zero (○) or after 5 h of incubation (□). Time incorporation of [^{35}S]methionine into 5% trichloroacetic acid was determined at 6 h. (A) 100% of control incorporation represents 86,372 cpm (○) and 15,202 cpm (□), respectively. (B) 100% of control incorporation represents 83,639 cpm (○) and 15,748 cpm (□), respectively.*

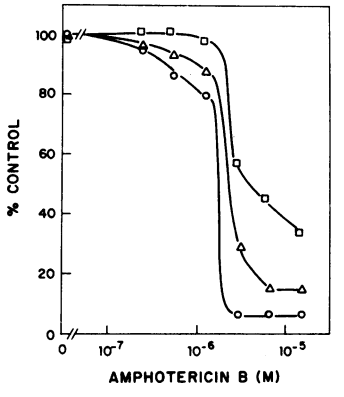

FIG. 3. *Effect of different concentrations of amphotericin B on protein synthesis with different concentrations of yeast cells. Protein synthesis by yeast cells was estimated as indicated in the text. The concentrations of cells tested were (○) 0.2, (△) 0.5, and (□) 1.25 absorbance units at 660 nm.*

target for translation blocking. Hence, the methionine pool was determined (Table 3). No effect whatsoever was seen, even when a concentration of amphotericin B as high as 1.3×10^{-5} was used. Under similar conditions the inhibition of protein synthesis was complete (see Fig. 1 to 4). We therefore conclude that the inhibitory effect on translation is not due to inhibition of transport of the radioactive precursor.

Step in translation blocked by amphotericin B. The next question was, which step in protein synthesis is blocked by amphotericin B treatment? The approach used was to analyze the polysomes at different times after the treatment of yeast spheroplasts with amphotericin B. An aliquot was taken in parallel to determine protein synthesis. Polysomal profiles (Fig. 5) indicate that an increase in monosomes, with a concomitant decrease in polysomes, occurs at the time when inhibition of protein synthesis is observed. The "freezing" of polysomal profiles indicates an inhibition in the elongation or termination steps in protein synthesis, whereas polysome run-off suggests inhibition at the level of initiation (17). In the light of these considerations, it seems that amphotericin B treatment acts by interfering with some step in the initiation process of translation. Evidence for this indication cannot be obtained from cell-free systems, because all the compounds used in this work are absolutely inactive when tested in cell-free protein-synthesizing systems (unpublished data).

These results are in contrast with previous work by Herzberg et al. (7), who suggest that

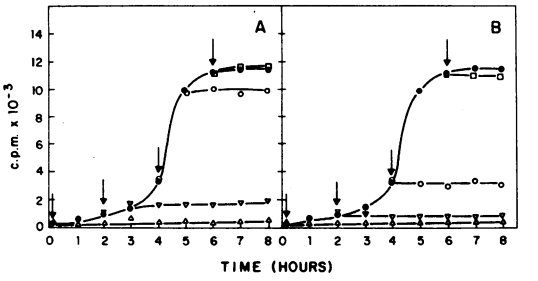

FIG. 4. *Effects of amphotericin B and nystatin on protein synthesis by yeast cells at different times of incubation. The incubation conditions and estimation of protein synthesis were as described in the text. The arrows indicate the times when 10^{-5} M amphotericin B (A) or 8×10^{-6} M nystatin (B) was added.*

TABLE 2. *Inhibition of RNA synthesis in yeast cells by different concentrations of amphotericin B*[a]

Concn added (M)	Amphotericin B added at: 3 h		4 h	
	cpm incorporated	% of control	cpm incorporated	% of control
None	20,712	100	21,166	100
2.7×10^{-6}	17,557	87	21,898	103
5.4×10^{-6}	4,657	23	21,484	101
1.3×10^{-5}	463	2	7,780	37

[a] Conditions were as described in the text. The labeling interval was from 4 to 5 h after the beginning of the incubation period. cpm, Counts per minute of [^{14}C]uridine.

TABLE 3. *Estimation of methionine pool in yeast cells at different times after amphotericin B treatment*[a]

Amphotericin B concn (M)	Time (h)							
	0.5		1		1.5		2	
	cpm incorporated	% of control	cpm incorporated	% of control	cpm incorporated	% of control	cpm incorporated	% of control
None	4,621	100	4,219	100	3,073	100	3,865	100
1.3×10^{-5}	4,072	87	4,257	100	3,841	115	3,911	102

[a] Conditions were as described in the text. cpm, Counts per minute of [^{35}S]methionine.

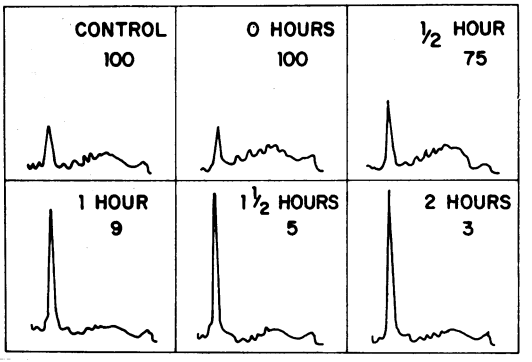

FIG. 5. *Analysis of polysomes from yeast spheroplasts treated with amphotericin B for different times. The obtainment and analysis of yeast polysomes were as described in the text. Aliquots of 2 ml of spheroplasts were incubated at 30°C in the presence of 1.3 × 10^{-5} M amphotericin B. At different times of incubation with that compound, the polysomes were analyzed as indicated. A 200-μl aliquot was withdrawn from each experimental point to estimate protein synthesis by incubation with 1 μl of [^{35}S]methionine (1,020 Ci/mmol, 3 mCi/ml) for 5 min. These values are given at the top of each polysomal profile as the percentage of the control; 100% of the control represents 31,989 cpm of [^{35}S]methionine incorporated.*

valinomycin inhibits translation in reticulocytes by affecting the elongation or termination steps.

Reversibility by potassium ions of the amphotericin B-induced inhibition of translation. The third question concerns the actual mechanism by which amphotericin B modification of the membrane was able to cause such strong inhibition of protein synthesis. It is assumed that a mediator between the plasma membrane and the protein-synthesizing apparatus is responsible for such an effect. Some workers have considered the possibility that a protein bound to the membrane could detach and bind to ribosomes blocking their activity (2, 7). However, this hypothesis does not explain the variety of cell functions influenced by amphotericin B treatment, mainly protein synthesis (Fig. 1 to 4), RNA synthesis (Table 2), respiration, transport of several metabolites, oxidative phosphorylation, etc. As a likely candidate we considered that the loss of potassium ions occurring after amphotericin B binding to the cells could be, at least in part, responsible for the observed blockade of protein synthesis initiation in yeast cells. To test this possibility we tried to reverse the inhibition of protein synthesis caused by amphotericin B by addition of potassium chloride to the medium. This treatment was indeed able to reverse the inhibition of translation (Fig. 6). Figure 6A shows that treatment of the cells with amphotericin B for 90 min caused a 75% inhibition of protein synthesis, whereas no inhibition was observed at this time if 100 mM KCl was present. However, a gradual inhibition of protein synthesis was observed after that time even in the presence of KCl, probably indicating that other cellular functions affected by amphotericin B treatment, such as oxidative phosphorylation, adenosine triphosphate depletion, and metabolite leakage, begin to influence translation at later times. Figure 7 shows the specificity of this reversion. Neither NaCl nor $MgCl_2$ was able to reverse the inhibition, whereas KCl was very effective. The partial reversion seen in the presence of $CaCl_2$ is most probably due to its ability to complex amphotericin B, forming a complex that is unable to interact with the cell (5).

DISCUSSION

The membrane-active compounds used in the present work all produce changes in the gradient of ions maintained by the cell membrane (15). We have illustrated here that as a result of such changes the translation capacity of the cell is strongly inhibited. The inhibition of protein synthesis by these compounds occurs in all types of cells tested: yeast, mammalian, and bacterial cells. It seems plausible to consider that a common mechanism of action operates in the inhibition of translation in all these cell systems, although direct evidence for this assertion is not yet available.

One of the conclusions we can draw from the present results is that amphotericin B and nystatin drastically block protein synthesis in the cytoplasm of yeast cells. In addition, RNA syn-

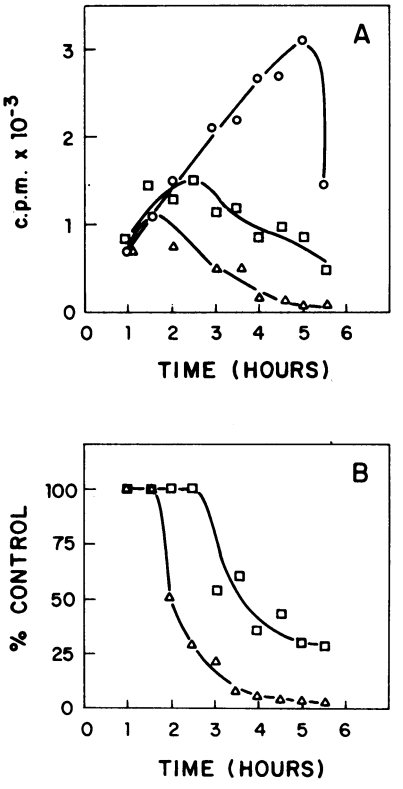

FIG. 6. *Reversal by potassium ions of the inhibition on protein synthesis in yeast cells by amphotericin B. Protein synthesis by yeast cells was analyzed by giving 30-min pulses with 0.15 μCi of [^{35}S]methionine. Amphotericin B and potassium chloride were added after 1 h of incubation:* (○) *control;* (△) *2.7 × 10^{-6} M amphotericin B;* (□) *2.7 × 10^{-6} M amphotericin B and 100 mM potassium chloride. (A) Protein synthesis as counts per minute of [^{35}S]methionine incorporated. (B) Percentage of the control.*

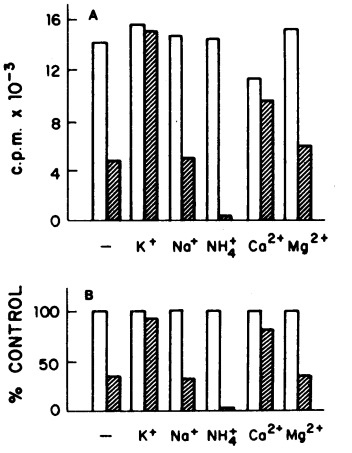

FIG. 7. *Reversal by different ions of the inhibition caused by amphotericin B on protein synthesis in yeast cells. Cultures of yeast cells (0.1 ml) were incubated in minimal medium at 30°C in the absence* (□) *or in the presence* (▨) *of 5.4 × 10^{-6} M amphotericin B. After 1 h of incubation, amphotericin B and the indicated ions were added. Protein synthesis was estimated from 2.5 to 3 h as indicated in the text. The concentrations of ions used were as follows: 100 mM each KCl and NH_4Cl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$. (A) Incorporation of [^{35}S]methionine in counts per minute (cpm); (B) percentage of the control.*

thesis and other cellular processes are also affected by treatment of cells with these compounds. Are all these effects mediated by the distortion produced in the gradients of protons and ions? This question still remains unanswered. However, at least in the case of protein synthesis, it seems clear that the strong inhibition first observed after amphotericin B treatment can be almost totally prevented by the presence of potassium ions in the medium. We interpret these results to mean that amphotericin B causes a leakage of potassium from the cell that results in an inhibition of the initiation of translation. Such inhibition does not occur if we prevent the leakage of this ion by adding extra potassium to the medium, thereby probably increasing its concentration inside the cell. This restoration of the required concentration of potassium ions in the cell cytoplasm allows translation to continue. However, after longer incubation times, the leakage of other compounds essential to the cell would also influence protein synthesis.

We believe that our results showing the inhibition of translation by the presence of ionophores are better explained by ionic changes that occur in the cytoplasm (12), rather than by any modification of the protein-synthesizing apparatus such as phosphorylation of factors or ribosomes, or the generation of stable inhibitors of translation (11, 16).

ACKNOWLEDGMENTS

We acknowledge Fundación Eugenio Rodríguez-Pascual and Antibióticos, S.A. for financial support.

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