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Dissociation Between the Induction of Potassium Efflux and Cytostatic Activity of Polyene Macrolides in Mammalian Cells

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The paper contains data on the induction of K^+ efflux and viability of baby hamster kidney (BHK-21) cells after their treatment with macrolide antibiotics inducing specific pores in membrane. New water-soluble semisynthetic derivatives of amphotericin B and aureofacin (*N*-glycosyl and trimethylammonium methyl ester derivatives) as well as the parent compounds were used to compare the concentration of antibiotics inducing permeabilizing and cytostatic effects. We found that a two- to eight-times-higher concentration of polyene antibiotic was required to observe a cytostatic effect than for release of 50% of the cellular potassium (K_{50} concentration) from BHK-21 cells. These differences were larger for water-soluble derivatives than for the parent compounds. The amount of intracellular potassium in treated cells incubated under optimal growth conditions was higher than that in cells which had been further washed with K^+-free maintenance medium. The membrane permeability changes induced by low concentrations of specific polyenes were observed to be reversible. BHK-21 cells were able to repair polyene-induced membrane permeability within 3 to 12 h under optimal growth conditions, after cell treatment with K_{50} concentration of specific macrolide antibiotics. The repair phenomenon is postulated as an explanation for the dissociation observed between permeabilizing and cytostatic effect of specific polyenes in BHK-21 cells.

It has been generally recognized that the cytotoxic effect of polyene macrolide antibiotics on eucaryotic cells is the result of lethal membrane permeability changes induced upon the formation of hydrophobic complexes with membrane-located sterols (5, 7, 13, 16, 19).

A diversity of membrane effects and permeability changes have been related to the structure of interacting antibiotics (2, 13, 14). Small macrolide ring neutral polyenes, like filipin, form voluminous aggregates with sterol in the membrane, irreversibly disrupting the cell membrane and thus promoting the leakage of a variety of metabolites (13–15). Nonconductive half pores are formed by pimaricin (a small macrolide ring amphoteric polyene), and conductive channels of 4 to 10 Å (0.4 to 1 nm) in diameter are formed by the large macrolide ring compounds like amphotericin B (7, 13).

The highest specificity of membrane alterations induced by polyenes was recorded for nonaromatic and aromatic heptaens, which form open channels for the free diffusion of small ions, resulting in the discharge of the potassium gradient (1, 2, 6, 13, 14). These specific membrane changes causing potassiumless death (1) can be repaired by the cells so that the lethal action of a specific polyene antibiotic can be reversed (1, 2, 10, 15). The reversibility of the lethal action of specific polyenes (1, 2, 10, 15) seems to be connected with the dissociation observed between the permeabilizing and the toxic effects (4, 14, 17). However, it is not known whether channel formation and the consequences of this event are indeed responsible for the lethal effect of specific polyene antibiotics. Some recent investigations pointed to the possibility that membrane effects other than channel formation and potassium efflux are lethal (4, 14). The finding that sublethal doses of the specific polyenes induce the uptake of large molecules greater than 1.0 nm in diameter also indicates that some membrane alterations, other than pore formation, significantly influence membrane properties (17, 18). Potentiation of the activity of cytostatic agents by polyenes, due to the enhancement of their uptake, also corroborates the broader impact of polyenes on membranes (14, 18).

The problem of the dissociation between the induction of potassium efflux and the growth inhibitory effects of polyene macrolides on mammalian cells has hardly been studied (15). Such effects have been demonstrated for amphotericin B only (15). The purpose of this study was to investigate the effects of some specific poly-

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enes on the induction of K^+ leakage and on viability and growth of baby hamster kidney cells (BHK-21). We used two new semisynthetic derivatives of polyene macrolides: the *N*-methyl glucosamine salt of the *N*-glycosyl (NG) derivative (8) and the methyl ester of the quarternary trimethylammonium (DMS) derivative (9). Both types of derivatives are soluble in water (8, 9), which is more advantageous for experimental and pharmacological use than the parent compounds, which are not soluble in water (13).

We observed that two- to eight-times-higher concentrations of polyene antibiotics were required for the cytostatic effect than for the induction of 50% potassium leakage from cells. These differences were higher for NG and DMS derivatives than for the parent compounds. The repair process observed is postulated as an explanation for the dissociation between permeabilizing and cytostatic effect of polyenes on cells.

MATERIALS AND METHODS

Cells and media. BHK-21 cells adapted to grow in shaker culture using serum-free medium (12) were employed. Cells were incubated in shaker culture at 37°C and subcultured every 48 to 72 h. The growth medium consisted of Waymouth 752/1 medium, supplemented with 0.2% fatty acid-free bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.), streptomycin (100 μg/ml), and penicillin (100 U/ml). The medium was buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (12) at pH 7.4. Maintenance medium used for cell washing contained: 5 mM D-glucose; 140 mM NaCl; 0.08 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $NaH_2PO_4 \cdot H_2O$, and 0.4 mM $Na_2HPO_4 \cdot 2H_2O$ (pH 7.4). The medium was kept at 4°C before use.

Polyene antibiotics. Amphotericin B (3) was kindly provided by E. R. Squibb and Sons, Inc. (New Brunswisk, N.J.). Aureofacin (T. Ziminsky, J. Zielinski, J. Golik, J. Gumieniak, E. Borowski, and K. L. Rinehard, Jr., Abstr. Proc. Int. Symp. Antibiot., Weimar, 1979) was isolated and purified in the Department of Pharmaceutical Technology and Biochemistry at the Technical University of Gdansk (Poland). NG and DMS derivatives of amphotericin B and aureofacin were prepared according to the procedures of Falkowski et al. (8, 9). Purity of substances was determined by $E^{1\%}_{1cm}$ measurements at 382 nm. All substances were 75 to 96% pure. All concentrations given in the text are for 100% pure compounds. Solutions of polyene macrolides were prepared just before use. Stock solutions of compounds (1 to 20 μg/ml) were prepared in dimethyl sulfoxide. These solutions were further diluted with medium to obtain the required concentration of antibiotic, and 0.5 ml of the proper dilution was added to 50 ml of the cell suspension. The amount of dimethyl sulfoxide in the cell suspension was never higher than 0.5% (vol/vol), a concentration which did not influence BHK-21 cell permeability or cell growth.

Cell growth inhibition. Cells were suspended at a density of 5×10^5 cells per ml in growth medium. The cell suspension was divided into 50-ml samples, and 0.5 ml of the proper dilution of antibiotic was added to each cell sample. Two samples of cell suspension were prepared for each concentration of antibiotic. Cells were incubated at 37°C for 72 h in a gyratory shaker (110 rpm). During the incubation time, duplicate 0.5-ml samples were taken from each cell suspension, and the numbers of viable and nonviable cells were determined by trypan blue dye exclusion test using a Cytograph type 6300 (Bio/Physics System, Inc., Mahopac, N.Y.). The same procedure was used for untreated cell samples and the samples supplemented with 0.5% dimethyl sulfoxide.

Determination of the intracellular potassium. After cell incubation with polyenes for 30 min as described above, six 3-ml samples were removed from each cell suspension, and the amount of intracellular potassium was measured under two conditions (15). The first set of three samples was centrifuged for 5 min at $500 \times g$ at 25 to 37°C. The supernatant fluid was carefully removed as completely as possible using a Pasteur pipette, and the cells were washed twice with 3 ml of maintenance medium. After the second washing, cells were suspended in 3 ml of distilled water, and K^+ was determined by atomic absorption technique (using atomic absorption spectrophotometer AA-575, Varian Techtron Pty, Ltd., Springvale, Austria). The second set of three samples was treated as above, except the cells were not washed but suspended in water after growth medium was removed. The difference between K^+ content of the untreated cell samples for the two sets was the amount of potassium originating from the growth medium residue when cells were not washed (15). This value was subtracted from all results of the second set of samples.

Cell ability to repair polyene-induced membrane alterations. Cells suspended in growth medium were treated with polyene antibiotic and incubated as described above. After incubation for 30 min, 4 h, 8 h, 12 h, 20 h, and 24 h post-treatment, duplicate samples were removed from each cell suspension. Intracellular potassium was determined after washing the cells twice with K^+-free maintenance medium (as described above).

RESULTS

Cell growth inhibition. The data on the effect of polyene macrolides on BHK-21 cell growth are presented in Fig. 1 and 2. Results indicate that aromatic heptaens (aureofacin and its NG and DMS derivatives) were more potent inhibitors of cell growth than nonaromatic ones (amphotericin B and its NG and DMS derivatives). The amount of nonviable cells for all samples illustrated by Fig. 1 and 2 did not exceed 1%. The amount of 0.5% dimethyl sulfoxide used for solubilizing polyenes in the medium did not affect the growth of BHK-21 cells (data not shown). When higher concentrations of polyene antibiotics than those shown in Fig. 1 and 2, were used, toxic effects on cells were observed.

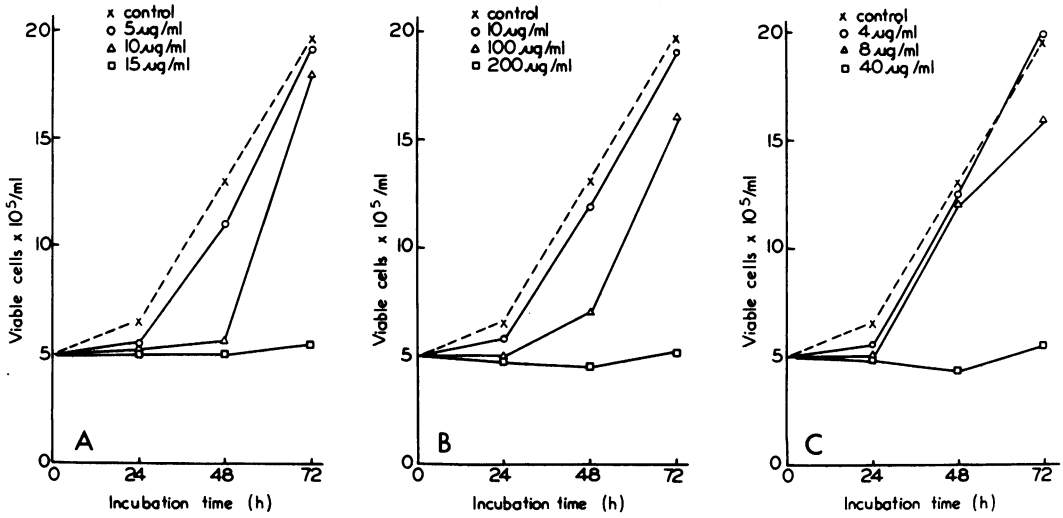

FIG. 1. *The effect of amphotericin B (A) and its NG (B) and DMS (C) derivatives on growth of BHK-21 cells. Cells were suspended at a density of 5 × 10⁵/ml in growth medium containing polyene antibiotic and were cultivated in shaker culture at 37°C. For cell enumeration, duplicate 0.5-ml samples were taken every 24 h, and viable cells were determined by trypan blue exclusion dye test. Each point on the curves represents the mean obtained for six measurements. The standard deviation for all measurements did not exceed ±5%.*

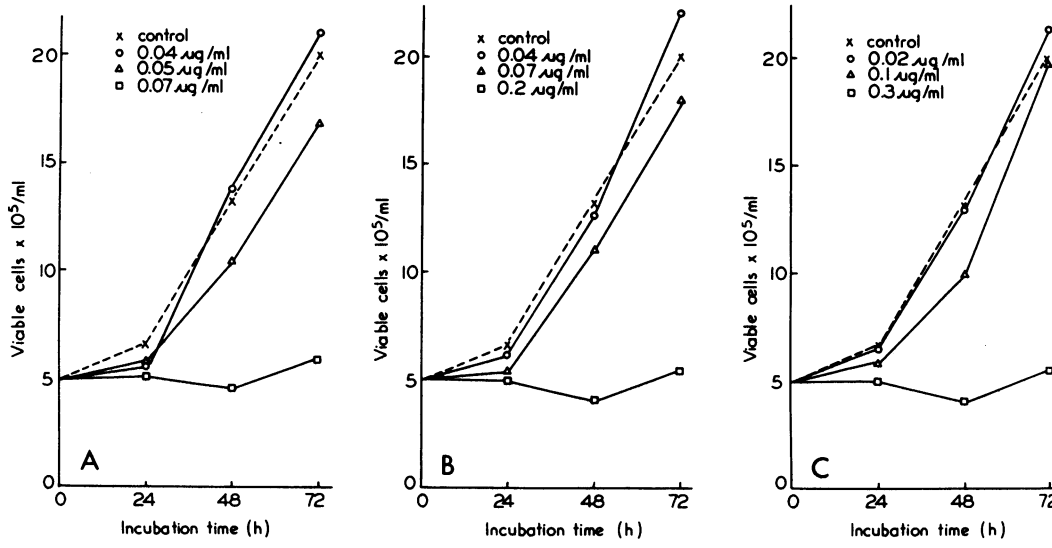

FIG. 2. *The effect of aureofacin (A) and its NG (B) and DMS (C) derivatives on growth of BHK-21 cells. For experimental conditions see legend to Fig. 1.*

NG and DMS derivatives of heptaens were less toxic than the parent compounds.

Intracellular potassium under polyene treatment. The effect of increasing antibiotic concentration on the level of intracellular potassium (before and after washing the cells) is illustrated in Fig. 3 and 4. The experimental conditions (media, temperature, and sedimentation rate) were optimized such that untreated BHK-21 cells did not release any potassium until they were suspended in water. Dimethyl sulfoxide at a concentration of 0.5% had no effect on intracellular potassium in BHK-21 cells; therefore, all K^+ leakage to the growth or maintenance medium was caused by polyene macrolide antibiotic treatment. Potassium concentration in the growth medium and in the untreated BHK-21 cells was 2.5 and 100 mM, respectively. The

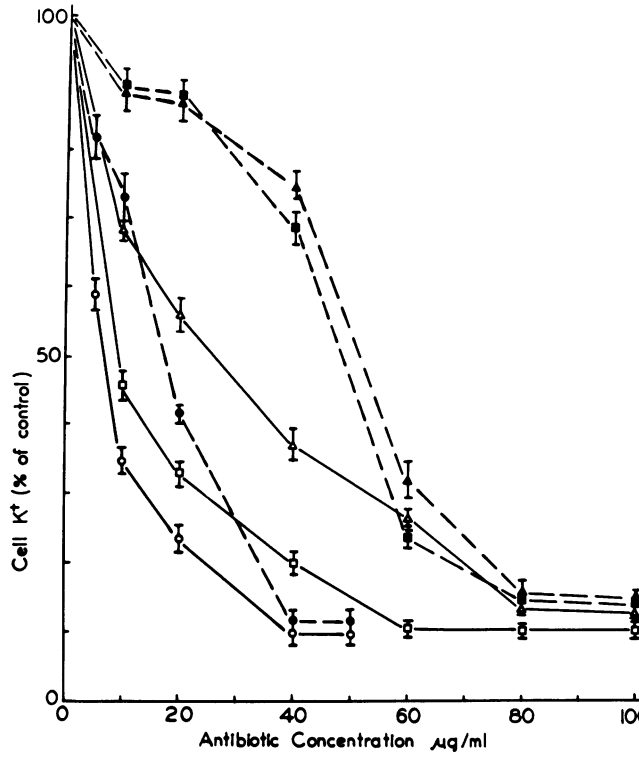

FIG. 3. *The potassium efflux from cells treated with amphotericin B and its derivatives. Cells at a density of 5 × 10^5 cells per ml were incubated with polyene antibiotic in growth medium. Then six 3-ml samples were removed from each cell suspension, and intracellular potassium was measured under two conditions: without washing (closed symbols), and after washing with K^+-free maintenance medium (open symbols). Each point represents the mean of three independent measurements for : (○) amphotericin B; (△) NG—amphotericin B; (□) DMS-amphotericin B. Bars represent standard deviations. For other experimental conditions see the text.*

latter number was obtained after measuring K^+ in a known cell volume with the assumption that K^+ concentration in the cell is homogeneous.

The concentrations of polyene macrolides causing the cytostatic effect (IC concentration) (Fig. 1 and 2) and the concentrations inducing 50% K^+ leakage (K_{50} concentrations) are compared and summarized in Table 1.

The ratio of both activities (IC/K_{50}) remained within one order of magnitude for all compounds tested; however, it was higher for NG and DMS derivatives than for parent compounds. The highest IC/K_{50} ratio was observed for NG-amphotericin B-treated cells.

Cell ability to repair polyene-induced membrane alterations. Results presented in Fig. 5 indicated that BHK-21 cells treated with DMS-aureofacin were able to regain their normal ability to control K^+ membrane transport. The K^+ efflux, observed immediately after antibiotic treatment, had been stopped by the cell defense mechanism called the repair process (1). After cell treatment with K_{50} concentrations of DMS-aureofacin, the repair process was completed within 8 h. Subsequently, BHK-21 cells did not lose any potassium during the washing procedure and multiplied as well as untreated cells. At higher than K_{50} concentration, the repair process was slower or was not observed under these experimental conditions. The same experiment was repeated for other polyene antibiotics. BHK-21 cells were treated with K_{50} concentrations of amphotericin B (6.8 µg/ml), aureofacin (0.025 µg/ml), NG-amphotericin B (22.8 µg/ml), NG-aureofacin (0.55 µg/ml), and DMS-amphotericin B (8.8 µg/ml). The results were similar to those obtained for cells treated with DMS-aureofacin (Fig. 5). BHK-21 cells treated with K_{50} concentrations of antibiotics were able to repair polyene-induced membrane permeability changes. This process was completed within 3, 6, 8, 8, and 12 h of cell incubation at optimal growth conditions after treatment with NG-aureofacin, NG-amphotericin B, DMS-amphotericin B, aureofacin, and amphotericin B, respectively. After the repair process was completed,

the potassium concentration in treated cells was 100 ± 2.6 mM.

DISCUSSION

One of the crucial and as yet unsolved problems in the understanding of polyene macrolide antibiotic action on eucaryotic cells is which of the primary effects of polyenes on membranes is directly responsible for the cytotoxic activity of these compounds. A variety of membrane phenomena occur upon the interaction of various structural types of polyene macrolides with the membrane-located sterol. Two extreme effects include: (i) the nonspecific severe disruption of the membrane by neutral polyenes containing a small macrolide ring (filipin); (ii) the formation of specific channels, open for the free diffusion of only small inorganic ions, by large macrolide ring heptaens (amphotericin B) (2, 13, 15). A whole spectrum of intermediate effects are induced by other structural groups of polyene macrolides (2, 13). The most interesting, from a theoretical as well as a practical standpoint, are polyenes which induce specific membrane changes (13, 15). The question of what is the role of channel formation and potassium gradient discharge induced by specific polyenes in the lethal action of these compounds has not been answered. We tested six specific polyene macrolides (2): amphotericin B, NG-amphotericin B, DMS-amphotericin B, aureofacin, NG-aureofacin, and DMS-aureofacin, with the emphasis on the new water-soluble derivatives. BHK-21 cells were used as model mammalian cells. The cytostatic concentrations of polyenes inhibiting BHK-21 cell growth were two to eight times higher than those required for the induction of 50% potassium release from the cells (Table 1). Thus, our results are similar to those obtained for *Candida albicans* (7) and *Saccharomyces cerevisiae* (14).

What can be the explanation for finding that much higher concentrations of polyene macrolides are required for the growth inhibitory effect than for K^+ efflux induction? It has been suggested that membrane effects other than the specific pore formation are responsible for the lethal effect in *C. albicans* (4) and *S. cerevisiae* (14) when treated with higher than K_{50} concentrations of large macrolide ring polyenes. Results presented in this paper allowed us to postulate that the most reasonable explanation for the dissociation observed between the concentration of polyene required for K^+ efflux induction and that required for growth inhibition can be based on a repair phenomenon (1, 2). BHK-21, used as model mammalian cells, when treated with lower than IC concentrations of specific polyenes (Table 1) were able to repair the polyene-induced membrane permeability changes (Fig. 5). When cells were treated with toxic concentrations of polyene, no detectable repair was ob-

TABLE 1. *Dissociation between induction of K^+ efflux and growth inhibition by polyene macrolides in BHK-21 cells*

Antibiotic	K_{50}[a] (μg/ml)	IC[b] (μg/ml)	IC/K_{50}
Amphotericin B	6.8	15	2.2
NG-amphotericin B	22.8	200	8.8
DMS-amphotericin B	8.8	40	4.5
Aureofacin	0.025	0.07	2.8
NG-aureofacin	0.055	0.2	3.6
DMS-aureofacin	0.046	0.3	6.2

[a] The concentration of antibiotic that caused the leakage of cellular K^+ by 50% to K^+-free medium. Numbers are calculated from results shown in Fig. 3 and 4.

[b] The minimum concentration of antibiotic that inhibited 100% increase of viable cells without any cytocidal effect (cytostatic concentration) after incubation in growth medium at 37°C for 72 h (Fig. 1 and 2).

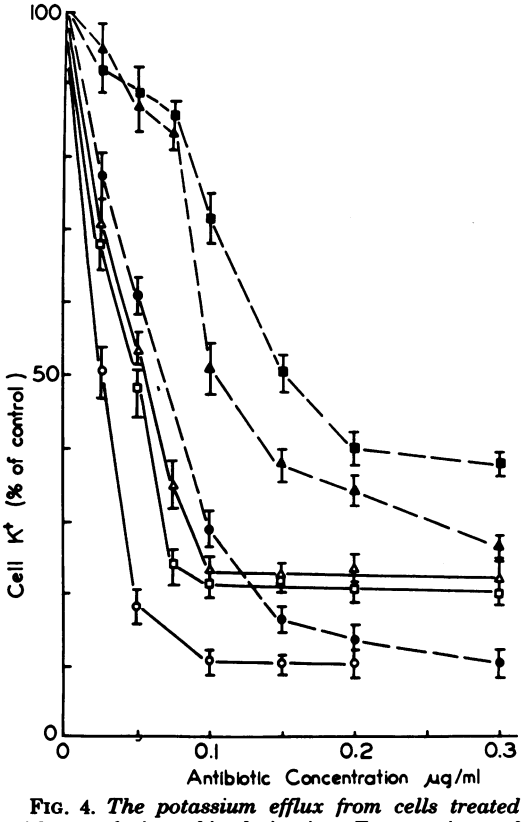

FIG. 4. *The potassium efflux from cells treated with aureofacin and its derivatives. For experimental conditions, see the legend to Fig. 3. Each point represents the mean of three independent measurements for: (○) aureofacin; (△) NG-aureofacin; (□) DMS-aureofacin.*

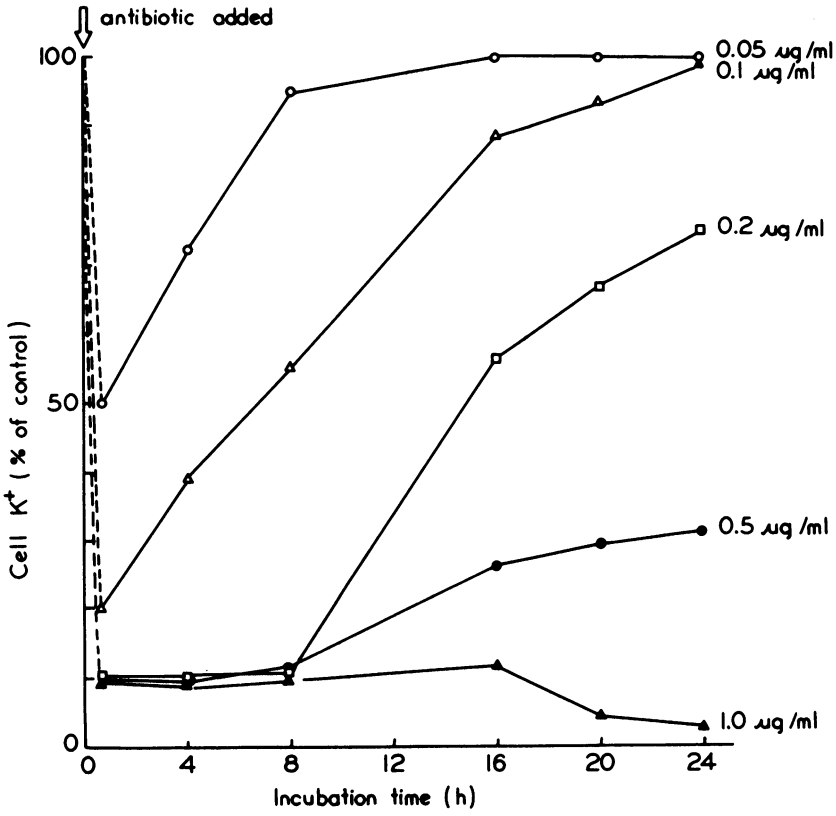

FIG. 5. *Ability of BHK-21 cells to repair the membrane alterations induced by DMS-aureofacin. Cells (2.5 × 10⁷) were suspended in 50 ml of growth medium, and 0.5 ml of DMS-aureofacin solution was added to obtain the desired final concentration. During incubation at 37°C in a gyratory shaker, two 3-ml samples were taken, and cells were washed twice in 3 ml of maintenance (K^+-free) medium. The potassium content in cells was measured by atomic absorption spectrophotometry. Each point represents the mean value from three independent measurements. The standard deviations for all results did not exceed 5%.*

served. Cells treated with K_{50} concentrations of specific polyene were able to complete the repair process within 3 to 12 h of incubation, i.e., during the log phase of cell growth (Fig. 2). Comparing the data on K^+ efflux with measurements of cell replication after the treatment with polyene macrolides (Fig. 1 to 4) showed for all polyenes tested that K_{50} concentration had negligible effect on BHK-21 cell growth and multiplication. These results showed that after the repair of membrane alterations, cells were able to grow and multiply as well as BHK-21 cells that were not treated.

The above results confirmed our previous work with other eucaryotic cell models (1, 2) and the results obtained for mammalian cells by Fisher et al. (10) and Kotler-Brajtburg et al. (15). After treatment with specific (2) polyene macrolide antibiotics, cells were able to repair membrane damage. The decomposition of polyene antibiotics under experimental conditions helps to monitor the repair process. However, it is not known whether the repair of polyene-induced membrane alterations begins after antibiotic decomposition or whether both processes occur simultaneously. Not all parameters influencing the repair process as well as its mechanism have been elucidated.

Our conclusion is also supported by the correlation between the presence or absence of dissociation between K^+ efflux induction and lethal concentrations of polyenes, and the ability of impaired cells to repair damage (1, 2, 4, 6, 14). The dissociation between these two parameters appears with a large ring compound, whereas for small ring polyenes both effects occur at the same concentration (4, 14). The first group of compounds induce repairable membrane alterations, and the second group, induce nonrepairable membrane damage (1, 2, 15).

The amount of potassium in the cells after treatment with polyene in growth medium was

higher than that after additional cell washing with K^+-free maintenance medium (Fig. 3 and 4). The values were also higher than expected for free K^+ diffusion from cells (100 mM) to growth medium (2.5 mM). These differences suggested that under experimental conditions polyene-treated BHK-21 cells were able to compensate to some degree for K^+ loss by free diffusion through membrane channels. That compensation was most likely due to the stimulation of the potassium active transport (2). The degree of the compensation depended upon the concentration of antibiotic used and was better observed at lower, rather than cytostatic concentrations of polyenes. Our results are in agreement with those reported by Kotler-Brajtburg and co-workers (15), who found that the higher concentration of amphotericin B (heptaen), but not filipin (pentane), was required to decrease potassium content by 50% in unwashed L cells than in the cells that were washed three times with K^+-free buffer (15).

The dissociation observed between permeabilizing and toxic effects of specific polyene macrolides (4, 14, 15) is believed to be connected with the potentiation by these antibiotics of the activity of other agents (10, 13, 15, 17). The bigger the difference between the concentrations of polyene inducing both these effects, the higher the chance for the pharmaceutical use as potentiating agent (15). In regard to the clinical application, our results showed that NG and DMS derivatives are more promising as potentiating agents than their parent compounds. Taking the maximum value of IC/K_{50} as a criterion of potentiating properties (15), NG-amphotericin B, with $IC/K_{50} = 8.8$, seemed to be the most promising compound for that purpose among the heptaens tested.

It is difficult to conclude that the higher specificity observed for NG and DMS derivatives, when compared with their parent compounds, is somehow associated with their solubility in water. However, Fisher and co-workers (11) observed that the methyl ester of amphotericin B is less toxic for mammalian cells than its parent compound. They believe that this is connected with the differences in solubility of these compounds in water (11).

In conclusion, we postulate that repair of specific polyene-induced membrane alterations in BHK-21 cells can explain the dissociation observed for these polyenes between their permeabilizing and cytostatic effects on mammalian cells.

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