ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1976, p. 618-624

Vol. 9, No. 4
Printed in U.S.A.

Effect of Amphotericin B on Growth and Membrane Permeability in *Dictyostelium discoideum*

EDWARD F. ROSSOMANDO,* GERARD CREME, BARBARA MALDONADO, MARY ANN HESLA, AND ELLIS E. GOLUB

Department of Oral Biology, The University of Connecticut Health Center, Farmington, Connecticut 06032

Received for publication 27 January 1976

In this study we have determined the effect of the polyene antibiotic amphotericin B on the growth of the eukaryotic microorganism *Dictyostelium discoideum*. These experiments show that the addition of drug to axenically growing cultures results in an inhibition of growth and cell division. However, with continued incubation, growth is resumed. To determine if the inhibitory effect was due to cell death, the effect of the drug on cell viability was measured. The results showed 10 to 20 times more drug was required to kill cells than to inhibit growth. Since previous studies had indicated that drugs of this type modified cellular permeability, the effect of this drug on osmotic stability of these cells was determined. Results reported in this study show that amphotericin B treatment modifies the cell surface, producing osmotically unstable cells, and that this modification occurs before the onset of cell death and within the same concentration range as used to bring about the inhibition of growth and division. Based on these data it is suggested that the modification in cellular permeability produced by the drug results in the inhibition of growth. This study also reports the results of experiments on the fate of the membrane-damaged cells. These experiments, using radioactive thiourea, showed the restoration of cellular permeability barrier and suggested that the resumption of cell growth occurs after the completion of the repair process.

Polyene macrolide antibiotics, such as amphotericin B, bind to sterols in plasma membranes, causing an increase in membrane permeability with the subsequent loss of essential metabolites (4, 7, 9). Previous work from this laboratory has shown that the eukaryotic microorganism *Dictyostelium discoideum* is sensitive to this drug and that at appropriate drug concentrations the cells could be lysed (6). In addition, during the course of our studies on the response of this organism to the drug, we observed an effect on cell growth. In this paper we report the results of our studies on this effect and by way of explanation suggest that cell growth can be affected by membrane permeability.

MATERIALS AND METHODS

Organisms. The axenic strain of *D. discoideum*, AX-3, a derivative of NC-4 (haploid), was grown in 70 ml of HL-5 growth medium in 500-ml flasks on a gyratory shaker at 22 C as described previously (6). Under these conditions, the cells have a doubling time of 10 to 12 h.

Materials. Amphotericin B (Fungizone) was obtained in powdered form (E. R. Squibb) and solutions were prepared by the addition of 5 ml of a low-phosphate saline buffer (LPS) made with 0.04 M phosphate, pH 6.4, containing KCl, 1.5 g/liter; $MgCl_2 \cdot 6H_2O$, 0.5 g/liter; and streptomycin sulfate, 0.5 g/liter. Amphotericin B solutions were stored at 4 C, in the dark, for up to 5 days. Peptone and yeast extract were from Difco. Deoxycholic acid was obtained as the sodium salt. [^{14}C]thiourea (25.9 mCI/mmol) was obtained from New England Nuclear Corp. and diluted with water before use. Mevalonic acid was from Sigma Chemical Co.

Cell growth conditions. For determining the effect of amphotericin B on the onset of cell growth, cells were grown to stationary phase (1×10^7 to 2×10^7 cells/ml)[5] diluted in fresh HL-5 to a final concentration of 1×10^7 to 2×10^5 cells/ml, the drug was added by dilution from a freshly prepared stock solution, and the incubation was continued. At about 12-h intervals, approximately 0.1- to 0.2-ml aliquots were removed from the cell culture under sterile conditions and the cell concentration was determined by cell count using a hemocytometer (Neubauer Bright Line) under ×200 total magnification.

The effect of amphotericin B on exponentially growing cells was determined by using the incubation procedure described above, except cells were grown to a concentration of 1×10^6 to 2×10^6 cells/ml before the addition of the drug. At this concentration, the drug was added and the cell concentration was determined at intervals as described above.

Determination of cell viability. The effect of amphotericin B on *D. discoideum* viability was deter-

mined on cells harvested from stationary phase. The cells, resuspended in 2 or 3 ml of LPS at a cell density of 1×10^7 cells/ml, were incubated with amphotericin B at 22 C on a gyratory shaker. At appropriate intervals, the drug effect was terminated by a 1:100 dilution of an aliquot of incubation solution into SM broth (peptone [Difco], 10 g/liter; $MgSO_4 \cdot 7H_2O$, 1 g/liter; KH_2PO_4, pH 6.4 to 6.5, 2.2 g/liter; and yeast extract 1 g/liter). The number of viable cells present in the culture was determined, on aliquots appropriately diluted, and spread on an agar surface (SM both broth containing 2% agar), together with *Aerobacter aerogenes* as a food source. Clones, indicating growth of *D. discoideum* from single cells, were observed after incubation of plates for 4 days at 22 C. The number of viable cells was determined by counting the clones.

Determination of membrane permeability with thiourea. In the thiourea experiments, [^{14}C]thiourea (2×10^5 counts/min) was added to cell cultures prepared as described above. Amphotericin B was added and the culture was incubated at 22 C. At intervals, 0.2 ml of the incubation mixture was removed, diluted with 2 ml of water, and shaken vigorously for 1 min on a rotary shaker. The amount of radioactive thiourea retained by the cells was determined by filtering on a glass fiber circle (Whatman GF/C), drying the filter circle for 10 min under heat lamps, and counting by liquid scintillation.

Determination of osmotically unstable cells. The number of osmotically unstable cells was determined on cells harvested from stationary phase. Cells were incubated in LPS with the drug as described above. At appropriate intervals, the reaction was terminated by a 1:100 dilution of incubation culture into distilled water. Cells surviving this procedure were considered osmotically stable. The number of cells resistant to this procedure was determined by a clonal analysis as described above.

RESULTS

Effect of drug on the onset of cell growth and division. The growth of the *D. discoideum* axenic strain Ax-3 is usually achieved by the dilution of cells from stationary growth phase culture approximately 1:100 into fresh growth medium. After this dilution, the cells divide in about 10 to 12 h and continue to do so until a cell concentration of 1×10^7 to 2×10^7 cells/ml is reached at which point no further increase in cell concentration is observed. The concentration of cells present during a typical growth experiment is plotted in Fig. 1 as a function of incubation time at 22 C. No lag is observed in the onset of growth after the dilution into fresh growth medium. In contrast, Fig. 1 also shows that when cells are diluted into fresh medium containing amphotericin B, the cell concentration does not increase as rapidly as observed in the control culture. In fact, a delay is observed in the onset of exponential growth phase. Whereas the length of this delay does increase with drug concentration, the cell concentration eventually begins to increase at a rate which approximates the untreated controls. However, when this culture reaches the stationary phase of growth, there is about a 10% reduction in cell concentration.

The drug preparation used in this study contains approximately equal parts of amphoteri-

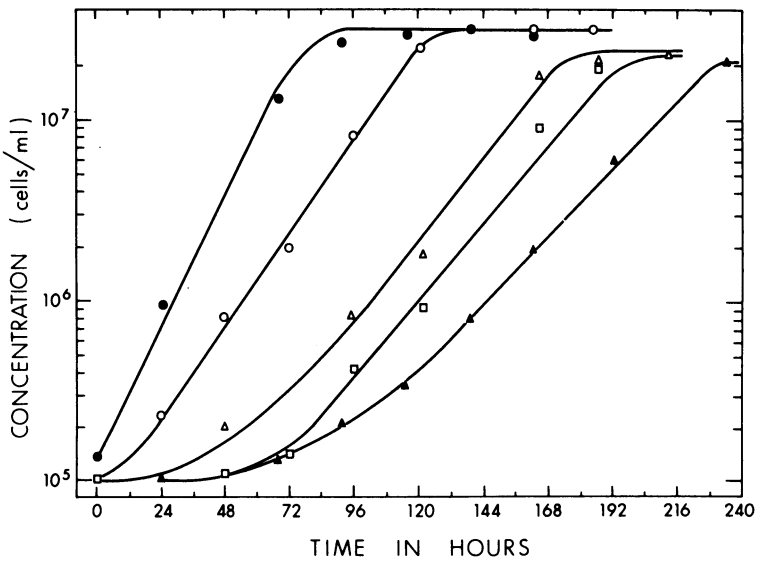

FIG. 1. *The effect of amphotericin B on the onset of growth of D. discoideum. Cells, previously grown to stationary phase, were diluted 1:100 into fresh growth medium. Drug was added and cells were incubated with shaking at 22 C. At intervals, aliquots were removed and cell concentrations were determined with a hemocytometer. Symbols: (●) Control; (○) 1 μg/ml; (△) 2 μg/ml; (□) 4 μg/ml; (▲), 5 μg/ml.*

cin B and deoxycholic acid (sodium salt) added to facilitate solubilization. Control experiments showed no effect of deoxycholic acid on the cell growth and division process, on cell viability, or on thiourea incorporation (see below).

Effect of drug on exponentially growing cells. The results described above were obtained on cells taken from stationary-phase growth. To determine if the effect observed might be limited to cells from this growth stage, the effect of amphotericin B on cells from the exponential stage of growth was determined.

For these experiments, cells were grown, under axenic conditions, to the mid-log stage (1×10^6 to 2×10^6 cells/ml) and the drug was added. The effect of several drug concentrations is shown in Fig. 2. It is clear that, even at concentrations of 1 μg/ml, there is a reduction in the growth rate. In addition, the rate decreases with increasing drug concentration such that at 4 μg/ml the rate is zero. However, even at this concentration, an increase in cell titer is observed after 96 h.

This effect of the drug on exponentially growing cells is qualitatively similar to that obtained for cells from stationary phase. In addition, the toxicity of amphotericin B depends (other conditions being equal) on the cell concentration; these results, when compared to those presented in Fig. 1, might suggest that exponentially growing cells are more sensitive to the drug. However, as a 10-fold difference in cell concentration existed between experiments, the results cannot strictly be compared.

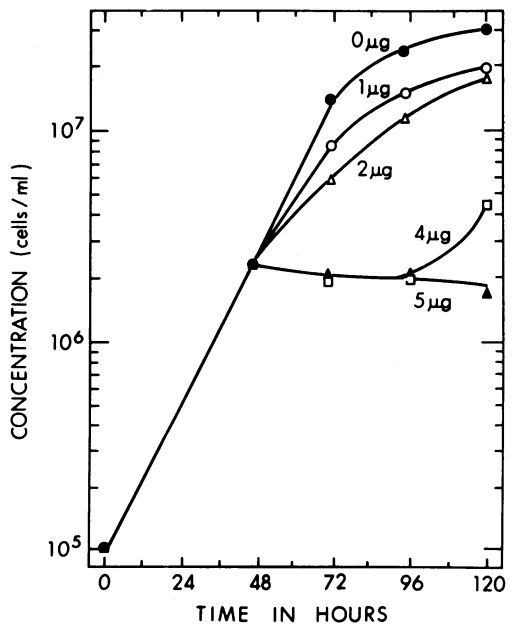

FIG. 2. *The effect of amphotericin B on cells at mid-exponential growth. Cells cultures were prepared as described except drug was added at concentrations of 2 × 10^6 cells/ml. Growth was continued and cell titers were determined at intervals. Concentration of drug as indicated.*

Effect of drug on cell viability. Because the observed decrease in growth rate could be accounted for by a reduction in the titer of viable cells, the effect of the drug on cell viability was measured. The fraction of the original population remaining viable is plotted in Fig. 3 as a function of time of exposure to the drug at 22 C. Below about 10 μg/ml there is no detectable decrease in the viability of the cell population even after a 6 h of incubation. In contrast, above about 50 μg/ml, there is a measurable decrease in the concentration of viable cells even after 30 min. In addition, the rate of inactivation remains constant for at least 3 h with no decrease observed. These results suggest that the interaction of the drug with cells can be characterized by a first-order reaction with one reagent, in this case, the drug in large excess.

However, when the rate of cell death is plotted as a function of drug concentration, as shown by the solid line in Fig. 4, it is apparent that at low concentrations (below about 20 μg/ml) and at a cell concentration of 1×10^7 cells/ml there is no detectable cell death. This lag in the onset of a finite rate of inactivation suggests that a finite number of drug molecules is

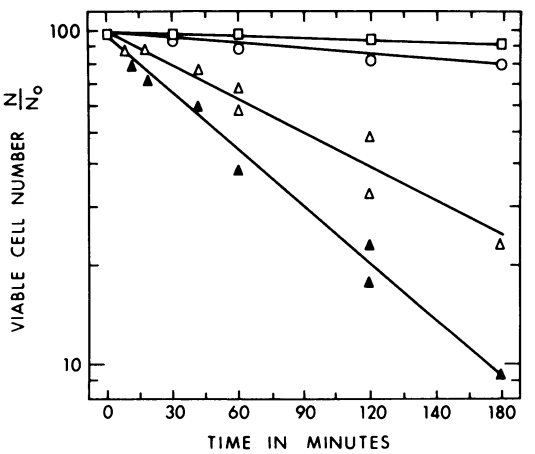

FIG. 3. *Effect of amphotericin B on cell viability. Cells (1×10^7 cells/ml) were incubated with drug at 22 C with shaking in LPS buffer. At intervals, aliquots were removed and diluted 1:100 into SM broth, and the number of viable cells was determined after spreading aliquots of diluted samples together with A. aerogenes on 2% agar plates. Clones were counted after 4 days at 22 C. Symbols: (□) 10 μg/ml; (○) 25 μg/ml; (△) 50 μg/ml; (▲) 100 μg/ml.*

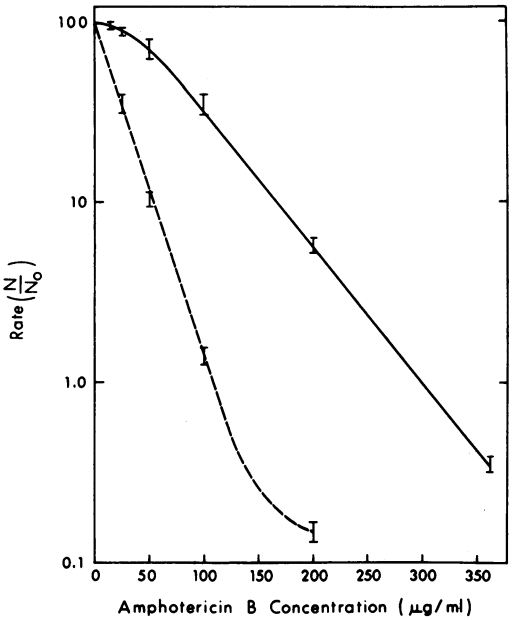

FIG. 4. *Comparison of rate formation of osmotically unstable cells and rate of cell death. Rates were determined after 30 min of incubation with drug. Number of viable cells was determined as described in the legend to Fig. 3. Number of osmotically unstable cells was determined after dilution of an aliquot of incubation mixture 1:100 into distilled water. Symbols: (——) Rate of cell death; (- - -) rate of formation of osmotically unstable cells.*

required to bring about cell death. This finding is in agreement with conclusions derived from previous work which suggested a threshold value as a characteristic feature of polyene antibiotics (3).

Effect of drug on cellular permeability: Formation of osmotically unstable cells. Previous studies on intact cells have indicated that drugs of this type modify cellular permeability (9). As a consequence of these changes, cells might be expected to accumulate water and swell. This possibility suggested that one effect of the drug on *D. discoideum* would be the formation of an osmotically fragile cell.

To determine if osmotically unstable cells were formed during the course of an incubation with amphotericin B, drug-treated cells were diluted into distilled water and the number of cells remaining viable after this procedure was determined. Osmotically unstable cells were detected and the rate of formation of these cells was measured over a range of drug concentrations. These data, shown in Fig. 4, indicate a greater rate for the formation of osmotically fragile cells than for the rate of cell death. Further, a comparison of the rates shown in Fig. 4 indicates that at drug concentrations as low as 5 μg/ml a significant fraction of cells are osmotically unstable. These data, by showing that osmotically fragile cells are formed faster than dead cells, suggest that their formation preceeds cell death. In addition, as osmotically fragile cells are formed at drug concentrations below 20 μg/ml, the formation of these cells could occur when the growth and cell division process was being inhibited.

To determine if the formation of osmotically unstable cells could account for the delay in the onset of growth, the number of these cells was determined in a culture over a 5-h incubation period. The results, shown in Fig. 5, indicate that by the end of the 1 h, about 99% of the cells are osmotically unstable. However, this experiment also shows that during the next 3 h there is a gradual decrease in the number of these osmotically unstable cells. The rate of cell death was also followed during this experiment. This result, also presented in Fig. 5, shows that after about 4 h about 90% of the cells have been killed. However, as 99% of the cells in the culture were osmotically unstable by the end of 1 h, a comparison of the rates suggests that those cells eventually killed were originally osmotically unstable. It should also be noted that about 10% of the osmotically unstable cell population is not killed. This finding suggests that these cells have recovered os-

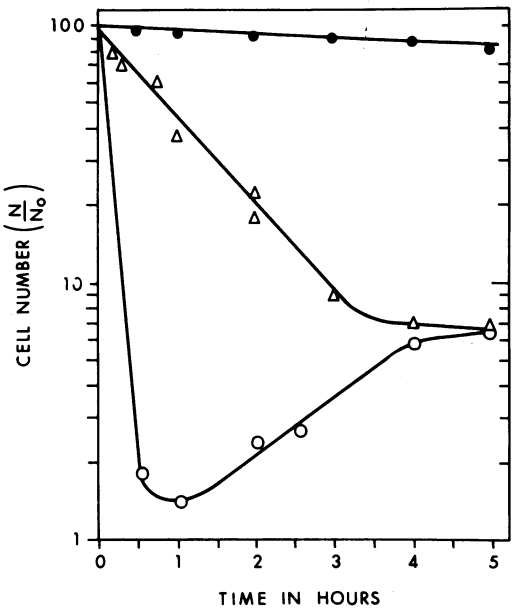

FIG. 5. *Kinetics of formation of osmotically stable cells and lysed cells. Experimental procedure was similar to that described in the legends to Fig. 3 and 4. Drug concentration was 100 μg/ml. Symbols: (△) Viable cell number; (○) number of osmotically stable cells; (●) viable cell number in absence of drug.*

motic stability by the end of the 5-h incubation and that they are now intact.

Effect of osmotically unstable cell concentration on recovery. To investigate this recovery further, the number of osmotically unstable cells formed at several drug concentrations was determined. A representative experiment, presented in Fig. 6, shows that increasing drug concentrations increases both the rate of formation of osmotically unstable cells and the fraction of the cell population converted to the unstable form. These results also indicate an apparent effect of drug concentration on the rate of recovery. To better analyze this effect, the number of osmotically unstable cells present at any time during the recovery period, i.e., after the first hour, has been normalized to the number present at the end of the 1 h (that time when the maximum number are present). These data, plotted in Fig. 7 as a function of recovery time while showing that the rate of recovery is increasing with drug concentration, suggests that this effect is the result of increasing the concentration of osmotically unstable cells present in the culture.

Retention of thiourea during recovery of osmotic stability. We have found that the concentration of osmotically unstable cells could be determined by using radioactive thiourea. It has been shown that amphotericin B may cause changes in cell membrane permeability, allowing penetration of second agents into viable cells (5). In Table 1 we show that thiourea enters *D. discoideum* only in the presence of amphotericin B. The amount of radioactivity associated with osmotically unstable cells was determined by comparing the amount of radioactivity associated with cells in both isotonic and hypotonic media. The results, also shown in Table 1, indicate that about 80% of the radioactivity is associated with the osmotically unstable cells. However, 3 h later when these osmotically unstable cells have either been killed or have recovered osmotic stability, as determined in the viability assay, the total amount of radioactivity has decreased. This latter re-

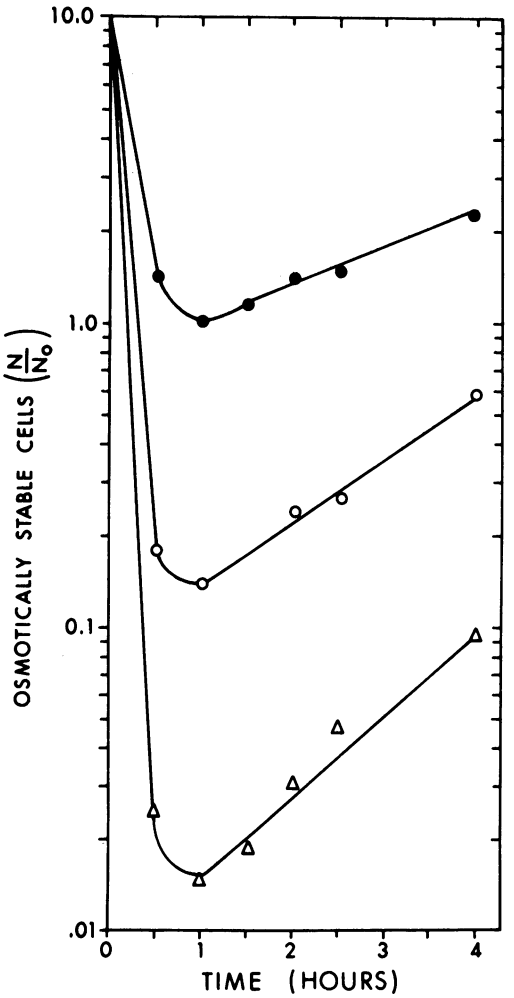

FIG. 6. *Effect of amphotericin B on fraction of cell population converted to osmotically unstable cells. Cells were incubated with the drug, and the reaction was terminated by dilution (1:100) into distilled water. Cells surviving this procedure was determined by plating with A. aerogenes. Symbols: (●) 50 μg/ml; (○) 100 μg/ml; (△) 200 μg/ml.*

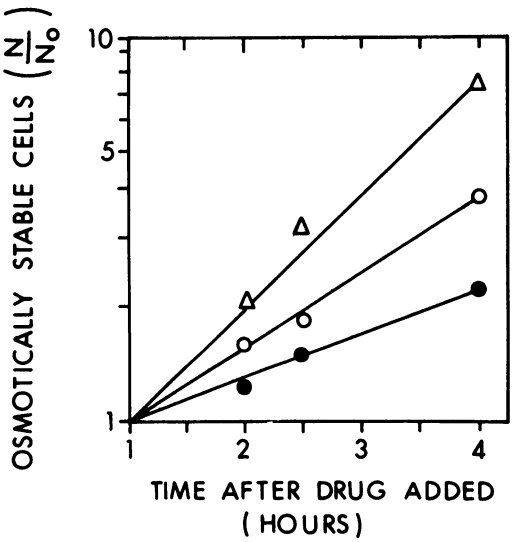

FIG. 7. *Effect of osmotically unstable cell concentration on rate of recovery. Data were taken from Fig. 6. Rates of recovery were determined by normalizing the cell number at each time to a value obtained at 1 h. Symbols: (●) 50 μg/ml; (○) 100 μg/ml; (△) 200 μg/ml.*

TABLE 1. *Retention of thiourea after addition of amphotericin B*

Additions	Thiourea retained (counts/min per 2 ml)			
	1 h[a]		4 h[a]	
	Total[b]	Hypo	Total	Hypo
None	22	20	21	25
Amphotericin B (100 μg/ml)	750	130	400	410

[a] Refers to time after addition of amphotericin B.
[b] Conditions refer to termination of the drug reaction by a 1:100 dilution of an aliquot of reaction mixture into either distilled water (Hypo) or SM broth (Total).

sult reflects the release of thiourea during cell death (lysis). However, no additional loss of radioactivity occurs upon exposing cells at this time to hypotonic medium. These findings suggest that those cells which recover osmotic stability retain thiourea.

Effects of lipid precursors on recovery of osmotic stability and retention of thiourea. The effects of acetate and mevalonate, precursors of lipid biosynthesis in *D. discoideum* (2), on both the recovery of osmotic stability and thiourea retention were determined. The result of a representative osmotic stability experiment is shown in Fig. 8A. In the absence of any additions there are the previously observed increases in the number of osmotically stable cells when compared to the number present after 30 min. In contrast, after about 3 h, in the presence of either acetate or mevalonate, there are twice as many osmotically stable cells present in the incubation mixture. Presented in Fig. 8B are experiments which show that the presence of either acetate or mevalonate in the incubation mixture also results in an increase in the concentration of cells containing thiourea. Taken together, these results suggest that these compounds increase the process of recovery of osmotic stability.

DISCUSSION

In this study we have presented results which show that the polyene antibiotic amphotericin B can bring about the reversible cessation of growth in the cellular slime mold *D. discoideum*. A similar result has been obtained with filipin on the growth of *Mycoplasma laidlawii* (8). In that case, the results were explained as being due to killing of cells and subsequent growth of a filipin-resistant population (8). As previous studies have shown that concentrations of 200 to 300 μg of amphotericin B per ml can kill *D. discoideum* (6), the

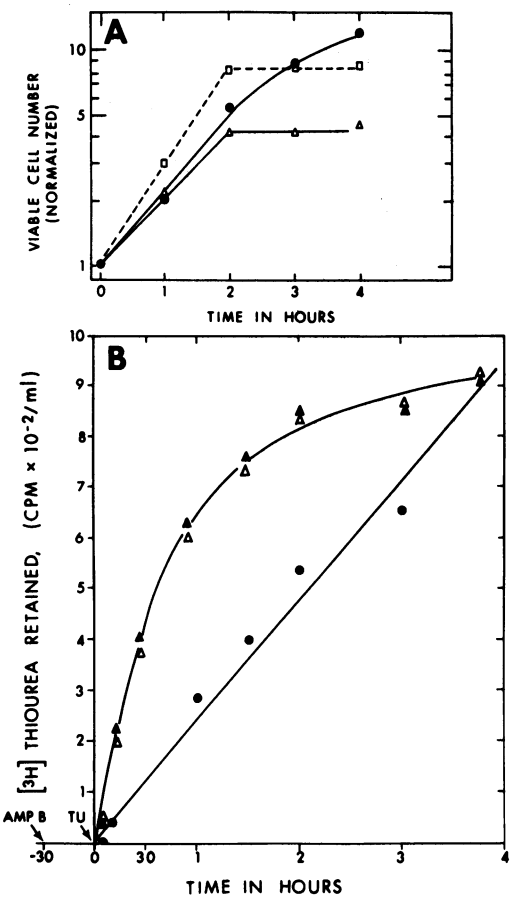

FIG. 8. *Effect of acetate and mevalonate on recovery of D. discoideum as measured by an increase in viability (A) or retention of thiourea (B) after addition of 100 μg of amphotericin B per ml. Procedures for (A) were similar to those described in the legend to Fig. 3 using distilled water to terminate the reaction. Viable cell number at each experimental time was normalized to viable cell number observed 1 h after addition of amphotericin B. Retention of thiourea was measured after incubation of cells (1 × 10⁷ cells/ml) with amphotericin B. After a 30-min incubation period, radioactive thiourea (2 × 10⁵ counts/min) was added and incubation was continued. At intervals, aliquots were removed and the cellular content of radioactivity was determined as described in Materials and Methods. (A) △, Control; ●, mevalonate (17 μg/ml); □, acetate (1 μg/ml). (B) ●, Control; △, mevalonate (17 μg/ml); ▲, acetate (1 μg/ml).*

reversible inhibition of growth observed in the present study might be explained in a similar manner. However, results described here show that at drug concentrations below about 10 μg/ml, cell death is not detectable. This finding suggests that the inhibition of cell growth observed with *D. discoideum* occurs in the ab-

sence of cellular death. However, these results do show that, below 10 μg/ml, the drug induces osmotic instability and that a significant fraction of the cell population is so affected within 1 h. The formation of this population of cells could provide an explanation for the lag in the onset of growth. Thus, the inhibition of growth could be associated with the formation of osmotically unstable cells.

In addition, while the results show that the majority of the unstable cells are eventually killed, they also show that some of these cells do become osmotically stable. This finding suggests that at any given drug concentration some of the cells damaged by the drug can restore the osmotic balance, a conclusion which implies that the reaction by which the osmotically unstable cells is formed is reversible.

However, since the design of the growth experiments did not include a limited exposure of cells to the drug, the question of how cells which did recover could grow in the presence of amphotericin B remains unanswered. One explanation would be that there is no excess drug present while another would be that the drug is unstable. In fact, a recent study has shown that amphotericin B is unstable in media used for cell culturing and that the drug exhibits a half-life of about 18 h (1). This result would suggest that the osmotically stable cells are able to grow due to degradation of the drug during the incubation.

A mechanism for the recovery of osmotic stability is indicated by the results of the thiourea experiments. Thus, after the demonstration that thiourea entered only osmotically unstable cells, the amount of cell-associated radioactivity increased with the recovery of osmotic stability. This result can be understood if the recovery of osmotic stability involves membrane repair, i.e., resealing of the membrane to thiourea, resulting in the trapping of the radioactive label in the cells.

Evidence that membrane repair involves biosynthetic events is suggested by the results of studies using acetate and mevalonate, previously shown to be precursors in membrane lipid biosynthesis in *D. discoideum* (2). Thus, with the addition of either compound an increase in the number of cells which recovered osmotic stability is observed. Further, these compounds also increased the cellular thiourea retention rate. If, as suggested above, thiourea retention is a consequence of membrane repair, these results indicate a direct effect of these compounds on the repair processes.

Although further experiments will be required to determine the mechanism of membrane repair, these data indicate that even at drug concentrations which induce cell death, a finite fraction of the cell population may survive through the functioning of this repair mechanism.

ACKNOWLEDGMENTS

This research was supported in part by grants from the National Science Foundation (GB-38017) and the American Cancer Society (BC-157).

LITERATURE CITED

1. Cheung, S. C., G. Medoff, D. Schlessinger, and G. S. Kobayashi. 1975. Stability of amphotericin B in fungal culture media. Antimicrob. Agents Chemother. 8:426–428.
2. Johnson, D. F., B. E. Wright, and E. Heftman. 1962. Biogenesis of Δ^{22}-stigmasten-3β-ol in *Dictyostelium discoideum*. Arch. Biochem. Biophys. 97:232–235.
3. Kinsky, S. 1963. Comparative responses of mammalian erythrocytes and microbial protoplasts to polyene antibiotics and vitamine A. Arch. Biochem. Biophys. 102:180–188.
4. Lampen, J. O. 1966. Interference by polyenic antibiotics (especially nystatin and filipin) with specific membrane functions. Symp. Soc. Gen. Microbiol. 16:111–130.
5. Medoff, G., G. S. Kobayashi, C. N. Kwan, D. Schlessinger, and P. Venkov. 1972. Potentiation of rifampicin and 5-fluorocytosine as antifungal antibiotics by amphotericin B. Proc. Natl. Acad. Sci. U.S.A. 69:196–199.
6. Rossomando, E. F., and L. S. Cutler. 1975. Localization of adenylate cyclase in *Dictyostelium discoideum*. I. Preparation and biochemical characterizations of cell fraction and isolated membrane vesicles. Exp. Cell Res. 95:67–68.
7. Sutton, D. D., P. M. Arnow, and J. O. Lampen. 1961. Effect of high concentrations of nystatin upon glycolysis and cellular permeability in yeast. Proc. Soc. Exp. Biol. Med. 108:170–175.
8. Weber, M. M., and S. C. Kinsky. 1965. Effect of cholesterol on the sensitivity of *Mycoplasma laidlawii* to the polyene antibiotic filipin. J. Bacteriol. 89:306–312.
9. Zygmunt, W. A. 1966. Intracellular loss of potassium in *Candida albicans* after exposure to polyene antifungal antibiotics. Appl. Microbiol. 14:953–956.