Interaction of Polyene Antibiotics with Subcellular Membrane Systems

I. Mitochondria

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SUMMARY

The action of the polyene antibiotics, ascosin, candicidin, amphotericin B, candidin, etruscomycin, filipin, nystatin, and pimaricin, on *Neurospora* and rat liver mitochondrial succinate-cytochrome c reductase was investigated. Only the ascosin and candicidin preparations inhibited this reaction. Various criteria indicated that both these antibiotic preparations were impure. Thin-layer chromatography of an acetone extract of ascosin revealed the presence of antimycin A. The inhibition of the succinate-cytochrome c reductase by ascosin was not due to the polyene per se, but was a consequence of contamination by antimycin A.

Although mitochondrial function (electron transport, as well as oxidative phosphorylation and ion transport) is not affected by these antibiotics, *Neurospora* mitochondria were able to bind filipin. The antibiotic could be recovered from the mitochondria after extraction with dimethyl formamide. Previous studies have shown that the presence of sterol is a *necessary* prerequisite for polyene binding to cell membranes. *Neurospora* mitochondria contain ergosterol although less than the microsomal fraction, which is partly derived from the cell membrane. The above findings indicate that the presence of sterol may not be *sufficient* to confer polyene sensitivity to a membrane system. Mitochondria have a much higher phospholipid:sterol ratio than cell membranes, suggesting that the relative concentration of these lipids may be an important factor in determining polyene sensitivity.

INTRODUCTION

In the past few years convincing evidence has accumulated indicating that the cell membrane of sensitive organisms is the site of action of the polyene antifungal antibiotics. Several laboratories have now shown that the polyene antibiotics, such as nystatin, amphotericin B, and filipin, produce a permeability alteration in molds and yeasts that results in the leakage of essential cytoplasmic constituents from the cell. Subsequent studies have established that the inability of the cell membrane to function as a selective restraining barrier in the presence of the polyenes is a consequence of antibiotic interaction with a component

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present only in the cell membrane of sensitive organisms. The circumstantial evidence suggesting that sterol is this unique component involved in the binding of the polyenes to the cell membrane has been reviewed (1). The recent demonstrations that (a) growth in the presence of sterol confers polyene sensitivity to *Mycoplasma* (2, 3), and that (b) polyene antibiotics preferentially penetrate lipid monomolecular layers containing sterols (4), provide direct evidence for the contention that the presence of sterols in the cell membrane is a *necessary* prerequisite for polyene sensitivity.

Up to now, there have been no detailed studies to determine whether subcellular membrane systems, e.g., those surrounding the nucleus and mitochondrion, are also sensitive to the polyene antibiotics. Ghosh and Chatterjee (5) and Gale (6) have published photomicrographs showing that the nucleus remained intact when protozoa and fungi were incubated with extremely high concentrations of several polyenes sufficient to cause a massive leakage of cytoplasmic material into the medium. These observations suggest that the nuclear membrane may be insensitive to the antibiotics. However, there are conflicting reports in the literature on the polyene sensitivity of mitochondria. Recent studies, reviewed in (7), have suggested that mitochondrial function is performed by enzymes localized in the external and internal membranes. Thus, the finding of Gottlieb and Ramachandran (8) that ascosin, a heptaene polyene antibiotic, inhibited yeast succinate–cytochrome c reductase seemed especially significant, although the same laboratory has also reported that filipin, a pentaene polyene antibiotic, had no effect on oxidation of citric acid cycle substrates (9). Subsequently, Shaw *et al.* published experiments which suggest that the lack of inhibition by filipin may have been due to rapid inactivation of the antibiotic by the mitochondria (10). We have therefore reinvestigated these observations because they are inconsistent with our contention that all the polyene antibiotics act in the same way (1). This paper will present evidence that the polyene preparations which inhibit electron transport are contaminated with antimycin A. None of the pure polyenes inhibited, although the antibiotics were bound by the mitochondria. These results suggest that the presence of sterols may not be a *sufficient* prerequisite for polyene sensitivity. A preliminary report of these experiments has been given (11).

MATERIALS AND METHODS

Chemicals. The polyene antibiotics were generously provided by the following companies: Commercial Solvents Corporation, Terre Haute, Indiana (ascosin); Farmitalia, Milan, Italy (etruscomycin); Lederle Laboratories, Pearl River, New York (pimaricin); Merck & Company, Rahway, New Jersey (candidin); S. B. Penick and Company, New York, New York (candicidin); the Squibb Institute for Medical Research, New Brunswick, New Jersey (nystatin, amphotericin B); the Upjohn Company, Kalamazoo, Michigan (filipin). Antimycin A was purchased from the Wisconsin Alumni Research Foundation, Madison, Wisconsin. Digitonin, ergosterol, and cytochrome c were obtained from the Sigma Chemical Company, St. Louis, Missouri.

Isolation of mitochondria and microsomes. Neurospora mitochondria were isolated and concentrated by minor modification of Hall and Greenawalt's procedure (12) as indicated below, using the preparation medium (PM) described by these authors (0.25 M sucrose, 0.005 M EDTA, and 0.15% crystalline bovine serum albumin). Minimal medium (500 ml in 2-liter Erlenmeyer flasks) was inoculated with a heavy washed conidial suspension of "wild type" *Neurospora*, strain 5297a, to give a final spore concentration of approximately 6×10^6/ml. Three drops of Dow-Corning Antifoam A were added, and the flasks were incubated for 18–24 hr at 30° in a New Brunswick Gyrotory shaker. The hyphal filaments were harvested by filtration through 2-ply cheesecloth and gently "wrung out" to remove excess water. Approximately 20 g wet weight of hyphae (obtained from one flask) were washed by stirring with 100 ml of PM and collected by filtration through cheesecloth as above.

The hyphae were broken by persistent grinding with an equivalent weight of sea sand in a chilled mortar and diluted to 200 ml with PM. This suspension was centrifuged for 15 min at 1500 *g*. (All centrifugations were carried out at 2–5°.) The supernatant fluid was removed by aspiration and centrifuged again at 1500 *g* for 10 min to ensure maximum removal of intact cells, nuclei, and sand particles. Mitochondria were isolated from this supernatant fluid by centrifugation for 15 min at 8000 *g*. The pellet, which had a distinct reddish color presumably due to the presence of cytochromes, was suspended in sufficient PM (albumin omitted) to give a final protein concentration of 4–8 mg/ml. This particulate fraction, designated fraction II, was used mainly for assay of succinate–cytochrome c reductase activity. Electron photomicrographs confirmed the presence of a high concentration of intact mitochondria although microsomes, vesicular elements, and other unidentified membrane systems were readily detectable. Purer mitochondria were required for studies on polyene binding and lipid analysis. For this purpose mitochondria were isolated from fraction II by the elegant density equilibrium centrifugation method devised by Luck (13). One milliliter of fraction II was layered onto 4 ml of a linear sucrose gradient ranging from 0.59 M sucrose ($d = 1.076$ at 20°) to 1.81 M ($d = 1.23$). After centrifugation for 3–5 hr at 38,000 rpm in a Spinco L-2 (SW 39 rotor), the tubes were punctured at the bottom and the main mitochondrial band at $d = 1.17$–1.21 was collected (fraction II SD). Electron photomicrographs of mitochondrial pellets fixed with glutaraldehyde and stained with osmium tetroxide were virtually identical with those published by Luck (13) and indicated almost complete absence of the contaminating particles observed in fraction II.

For the preparation of *Neurospora* microsomes, the supernatant fluid obtained after sedimentation of fraction II was centrifuged for 15 min at 30,000 *g* to remove any remaining mitochondria. The supernatant fluid from this step was then spun at 105,000 *g* for 2 hr (Spinco No. 40 rotor) and the resulting pellet was suspended in sufficient PM (albumin omitted) to give a final protein concentration of approximately 10 mg/ml. In some experiments, this microsomal fraction, designated fraction V, was further purified by density equilibrium centrifugation using the procedure described previously for mitochondria. In this case, the microsomal band at $d = 1.08$–1.13 was collected (fraction V SD).

Rat liver mitochondria were isolated according to the method of Hunter *et al.* (14). We are indebted to Dr. E. Smith and Professor F. E. Hunter for providing these mitochondria and also for taking the electron photomicrographs used to check the purity of the above fractions.

Assay of succinate–cytochrome c reductase. Cytochrome c reduction with succinate as electron donor was followed in the usual manner by determining the increase in absorbancy at 550 mμ. The following components were added, in order, to cuvettes with a 1-cm light path: 0.01 ml of the antibiotic solution (see below), 0.1 ml of succinate (10^{-2} M), 0.81 ml of buffer (0.025 M Tris–0.33 M sucrose, pH 7.3), 0.05 ml of mitochondrial suspension, 0.01 ml of NaCN (10^{-1} M) and 0.02 ml of cytochrome c (20 mg/ml). The reaction was initiated by the addition of cytochrome c and dilutions of mitochondria were used which gave linear rates for at least 3 min. Both beef and horse heart cytochrome c were equally effective. The rate of cytochrome c reduction was proportional to the concentration of mitochondrial protein under these conditions. The antibiotic solutions were prepared with dimethyl formamide (DMF). When the antibiotic was omitted, 0.01 ml of DMF was added as solvent control. This amount of DMF (final concentration: 1%) had a negligible effect on succinate–cytochrome c reductase.

Lipid extraction and analysis. Mitochondria and microsomes were extracted by a modification of the procedure of Bligh and Dyer (15). Two milliliters of chloroform and 4 ml of methanol were added to 1.6 ml of the particulate suspension, containing 2–8 mg protein. Any insoluble material was

removed by centrifugation for 20 min at 20,000 *g*. The supernatant fluid was quantitatively transferred to conical glass tubes, and 2 ml of chloroform and 2 ml of water were added. After thorough mixing, the two phases were separated by centrifugation for 20 min at 2000 *g*. The upper aqueous layer was removed by aspiration and the lower chloroform layer, including any material present at the interface, was diluted to 10 ml with methanol. If necessary, this solution was clarified by centrifugation.

Ergosterol was determined by the Liebermann-Burchard method after precipitation of the sterol as the digitonide. Appropriate aliquots (2–4 ml) of the lipid extract were taken to dryness at 80° under a stream of N_2 and redissolved in 1 ml of ethanol:acetone (1:1). Digitonin (0.5 ml of a solution containing 10 mg per milliliter of 50% ethanol) was added and the tubes were kept at 4° overnight. The precipitate was collected by centrifugation and washed once with 2 ml of acetone. The precipitate was dried *in vacuo* and redissolved in 1 ml of glacial acetic acid and 1.5 ml of chloroform. One milliliter of acetic anhydride: sulfuric acid (4:1) was added and the resulting blue-green color was measured in a Klett-Summerson colorimeter (No. 66 filter) exactly 5 min after addition of the acid reagent. The amount of ergosterol present was determined from a calibration curve obtained by carrying known amounts of ergosterol through the above procedure.

Lipid phosphate was determined by mod-

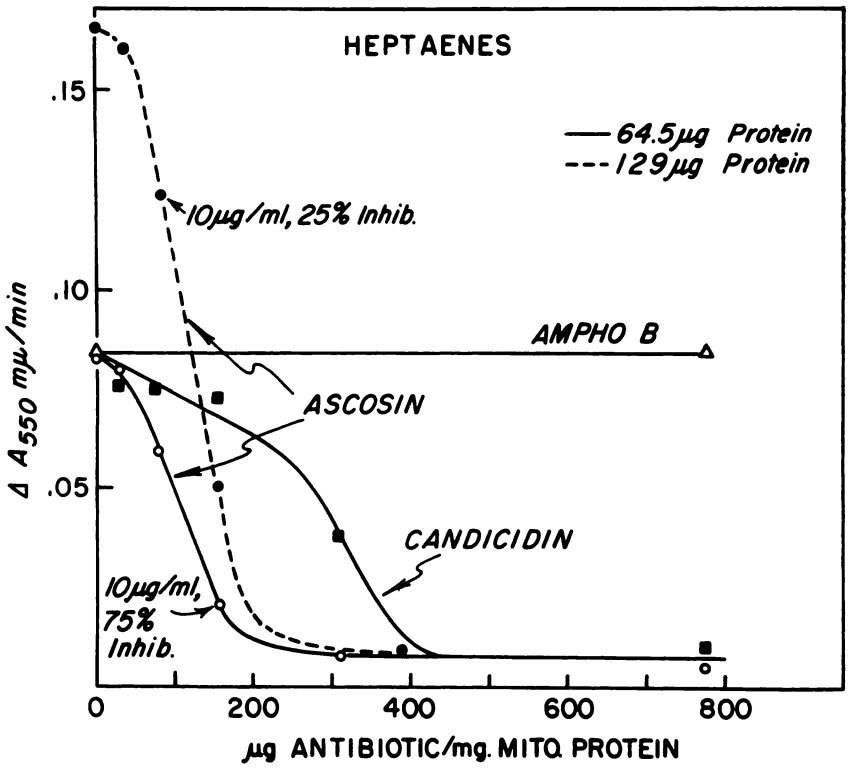

FIG. 1. *Effect of polyene antibiotic (heptaene) preparations on succinate–cytochrome c reductase*

Neurospora mitochondria (fraction II) were assayed for succinate–cytochrome c reductase in the presence and absence of the antibiotics by the procedure described in the text. In the case of ascosin, activity was determined using two concentrations of mitochondrial protein, 64.5 and 129 µg/ml. Appropriate amounts of the antibiotics (in 0.01 ml DMF) were added to give the antibiotic:protein ratios shown on the abscissa.

ification of the procedure of Lowry (16) after digestion with sulfuric and perchloric acid.

Miscellaneous methods. All spectra were recorded with a model 14 Cary recording spectrophotometer. Protein was measured by the method of Lowry *et al.* (17). The effect of filipin on erythrocyte hemolysis was determined by procedures previously employed (18, 19). Additional experimental methods are described in the following section.

RESULTS

Inhibition of Cytochrome c Reduction by Polyene Antibiotics

We were able to confirm the observations of Gottlieb and Ramachandran (8) that preparations of ascosin inhibited succinate–cytochrome c reductase. Candicidin also inhibited electron transport. However, amphotericin B, filipin, etruscomycin, nystatin, candidin, and pimaricin were without any effect at concentrations of 50 μg/ml, equivalent to approximately 800 μg of antibiotic per milligram of mitochondrial protein in the assay mixture. One feature of the inhibition by ascosin seemed especially significant. The extent of inhibition was markedly dependent on the amount of mitochondria added. Thus, as indicated in Fig. 1, ascosin, 10 μg/ml, inhibited cytochrome c reduction 75% when 64.5 μg of mitochondrial protein was used in the assay but only 25% when the protein concentration was doubled. Antimycin A also manifests an inverse relationship between the extent of inhibition and mitochondrial concentration (20). These results suggest that the inhibition by ascosin and candicidin may be due to contamination with antimycin A, particularly since the other polyenes did not have any effect on electron transport. The likelihood of this possibility was strengthened when the relative potencies of ascosin, candicidin, and antimycin A were compared. Table 1 gives the minimum concentration of each antibiotic per milligram mitochondrial protein that was required for complete inhibition of succinate–cytochrome c reductase using *Neurospora* and rat liver mitochondria as the source of enzyme. The data in the last 2 columns indicate that as little as 0.13% and 0.05% contamination of the ascosin and candicidin preparations, respectively, by antimycin A would be sufficient to account for the observed inhibition.

The actual presence of antimycin A in the ascosin preparation was demonstrated in the following way. Antimycin A is very soluble in acetone whereas the polyene antibiotics are relatively insoluble in this solvent. Accordingly, 4.86 mg of ascosin was extracted 5 times with small volumes of acetone (approximately 2 ml). The acetone extracts were combined, concentrated under a stream of N_2 to about 0.2 ml, and chromatographed on silica-gel thin-layer plates with chloroform:acetone (90:10) as the developing system. When viewed under UV light, a material with the mobility of pure antimycin A was readily detectable (Fig. 2). This spot was eluted with acetone,

TABLE 1

Comparative potencies of antimycin A, ascosin, and candicidin

The minimum amount of each antibiotic, per milligram mitochondrial protein, required to give "complete" inhibition (95–100%) of succinate–cytochrome c reductase activity, was determined graphically by experiments analogous to those described in Fig. 1. As indicated for ascosin in Fig. 1, this value is obviously independent of protein concentration.

Mitochondrial source	μg/mg protein required for 100% inhibition			Ratio (× 100)	
	Antimycin	Ascosin	Candicidin	Antimycin: Ascosin	Antimycin: Candicidin
Rat liver	0.054	35.3	102	0.15	0.053
N. crassa	0.2	189	390	0.11	0.051

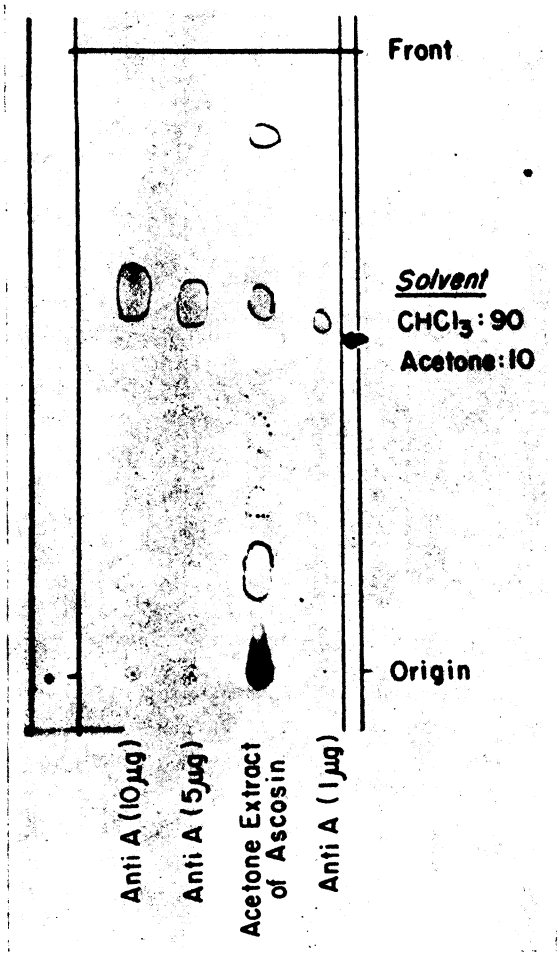

FIG. 2. *Presence of antimycin A in ascosin preparation*

An acetone extract of ascosin, prepared as described in the text, was spotted on thin-layer plates of Adsorbosil (Applied Science Labs, State College, Pennsylvania) along with reference spots of pure antimycin A. After development, the plate was photographed under UV light (maximum emission: 360 mμ) with a Polaroid camera using type 42 film.

as were the reference spots on either side. Titration with *Neurospora* mitochondrial succinate–cytochrome c reductase confirmed the presence of 1.76 μg of antimycin A. According to the data of Table 1, 6.32 μg of antimycin A should have been obtained from 4.86 mg of ascosin. Subsequent enzymic assay indicated that the acetone-extracted residue still contained 3.45 μg of antimycin A, suggesting very tight binding of the antibiotic to some component in the ascosin preparation.

It has previously been reported that the ascosin preparation, in common with all the other polyene antibiotics, caused the lysis of mammalian erythrocytes and *Neurospora* protoplasts (19). This property is consistent with the spectrum of the ascosin preparation showing the characteristic absorption maxima of heptaenes at 330–345, 350–365, 370–385, and 395–405 mμ (Fig. 3). The spectra of ascosin and candicidin also suggest that these antibiotics are relatively impure since the extinction at each of the maxima is significantly less than the corresponding peaks for amphotericin B, which did not inhibit succinate–cytochrome c reductase (Fig. 1). Thus, on the basis of the above experiments, it may be concluded that all the polyenes act on the cell membrane and that the unique ability of the ascosin preparation to inhibit electron transport is due to contamination with antimycin A. It should be noted that both ascosin and antimycin A are produced by a *Streptomyces* species.

Binding of Polyene Antibiotics by Mitochondria

Although electron transport is not inhibited by the polyenes, *Neurospora* mitochondria can bind the antibiotics. As indicated in the Discussion, these observations may have considerable bearing on current theories of the molecular basis by which the polyene antibiotics affect membrane function. The following experiments were carried out primarily with filipin since Shaw *et al.* (10) reported that, when this antibiotic was mixed with either a crude extract or a mitochondrial fraction of yeast, it no longer was able to inhibit fungal growth. On the basis of these results, they concluded that the inability to demonstrate an effect of filipin on electron transport was due to mitochondrial inactivation of the antibiotic. However, we observed in preliminary experiments that the addition of high concentrations of filipin (50 μg/ml, equivalent to approximately 800 μg per milligram mitochondrial protein) during

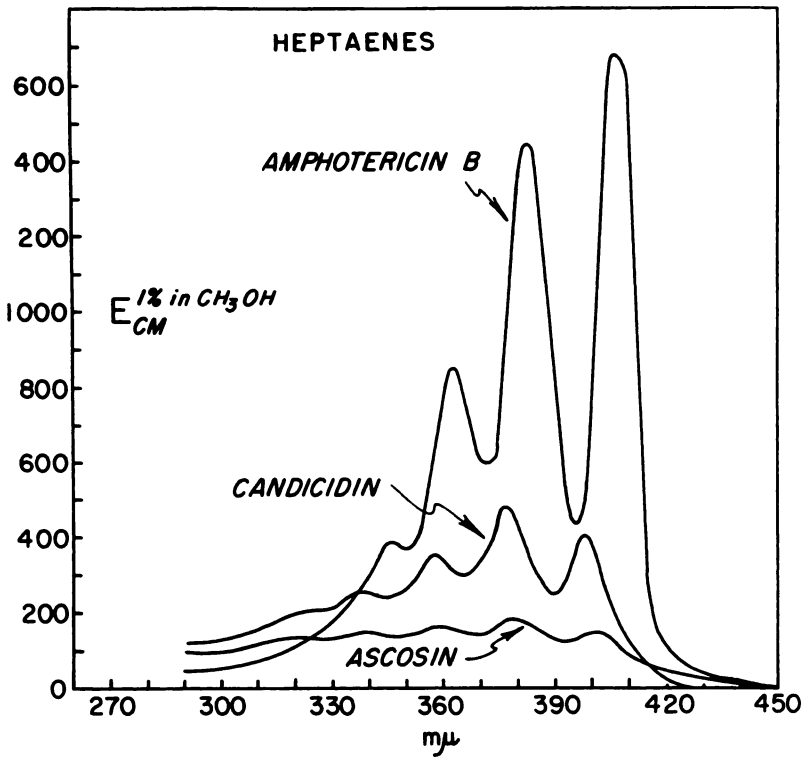

FIG. 3. *Spectra of polyene antibiotic (heptaene) preparations*

The antibiotics were dissolved in methanol to give the following concentrations: amphotericin B, 4.9 μg/ml; candicidin, 9.8 μg/ml; ascosin, 39.2 μg/ml. From the spectra determined in cuvettes with a 1-cm light path, the extinction of a 1% solution was calculated.

the course of cytochrome c reduction had no effect on the rate. (In the usual assay procedure, the polyenes were "preincubated" approximately 5 min with the mitochondria before the addition of cytochrome c to start the reaction.) Therefore, antibiotic inactivation would have to occur almost instantaneously. A likely alternative explanation for the results obtained by Shaw *et al.* is that filipin was bound to the mitochondria. To test this possibility, the effect of filipin on erythrocyte lysis was examined after preincubation of the antibiotic in the presence and absence of *Neurospora* mitochondria. Previous studies have shown that filipin is an extremely potent hemolytic agent (18, 19) and, if the mitochondria do bind the antibiotic, we would expect that a higher concentration of filipin would be required to initiate erythrocyte lysis. Figure 4 shows that the minimum threshold concentration of filipin is increased significantly when the antibiotic is preincubated in the presence of mitochondria, indicating a reduction of the effective free antibiotic concentration due to mitochondrial binding. Additional experiments (not illustrated) have shown that the extent of binding is dependent on the concentration of mitochondria and temperature, being greater at 37° than at 22°.

The experiment described in Fig. 5 further suggests that the preceding results were a consequence of filipin binding to the mitochondria rather than of inactivation. Mitochondria which had been incubated with the antibiotic, at a ratio of approximately 75 μg mitochondrial protein per microgram of filipin, were extracted with dimethyl formamide. The spectrum of the supernatant, obtained after removal of the mitochondria by centrifugation, was identi-

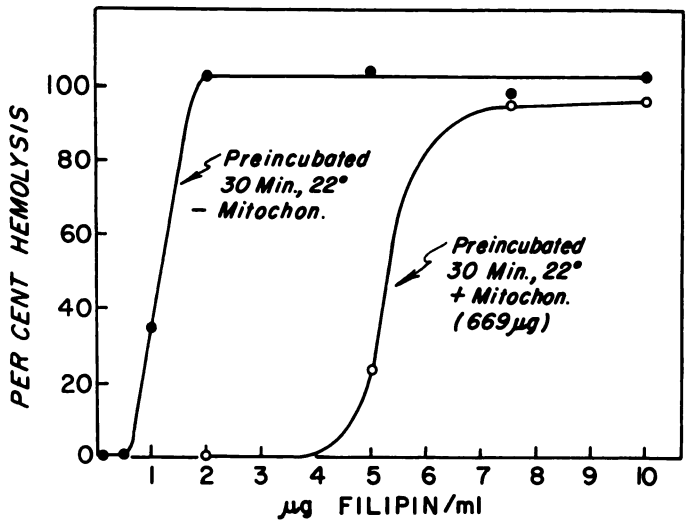

FIG. 4. *Effect of preincubation with mitochondria on the concentration of filipin required for erythrocyte lysis*

Different amounts of filipin were preincubated with and without *Neurospora* mitochondria (fraction II SD, 669 μg) in 0.5 ml of 0.05 M potassium phosphate–0.154 M NaCl, pH 6.8 (saline-buffer) for 30 min at 22°. More saline-buffer (0.42 ml) was then added, followed by 0.08 ml of washed human erythrocytes. After 5 min incubation at 38°, the tubes were centrifuged for 10 min at 10,000 *g* to remove mitochondria and intact erythrocytes, and the absorbancy of the supernatant fluid was determined at 550 mμ. The final concentration of filipin is shown on the abscissa. An amount of erythrocytes was added which gave an absorbancy (550 mμ) = 0.618 upon complete lysis with 1 ml of H_2O. Control experiments indicated that the mitochondria had no effect on the extent of hemolysis in the absence of any preincubation period, i.e., when filipin was added after the erythrocytes.

cal with the spectrum of the antibiotic incubated in the absence of mitochondria. From the absorption maxima at 326, 342, and 361 mμ, it was calculated that at least 90% of the filipin had been recovered.

Lipid Composition of Neurospora Mitochondria

Several studies have now clearly established that only membrane systems which contain sterols are able to bind the polyene antibiotics. Therefore, we should expect to find ergosterol (the principal sterol in fungi) in *Neurospora* mitochondria. Table 2 shows that ergosterol is present in the mitochondria although, on a protein basis, the concentration is only ⅙ of the ergosterol found in microsomes which had also been isolated in a sucrose density gradient. Microsomes were included for comparison since previous studies have indicated that at least 80% of the *Neurospora* mycelial binding capacity for nystatin can be recovered in this fraction (21). Experiments analogous to that described in Fig. 4 have also shown that microsomes rapidly bind filipin and thereby inhibit hemolysis. Shatkin and Tatum (22) have published electron photomicrographs of *Neurospora* hyphae which indicate that the endoplasmic reticulum is contiguous with the cell membrane, and current evidence is consistent with the view that the microsomes are derived from the endoplasmic reticulum. Thus, the lipid composition of the microsomes may approximate that of the *Neurospora* cell membrane and comparison with mitochondria may provide an explanation for the insensitivity of these structures to the polyene antibiotics. Table 2 shows that the microsomes have a much lower phospholipid:sterol ratio than mitochondria. This ratio did not change appreciably when the crude microsomal fraction (100,000 *g* pellet) was further purified by equilibrium centrifugation in a sucrose gradient.

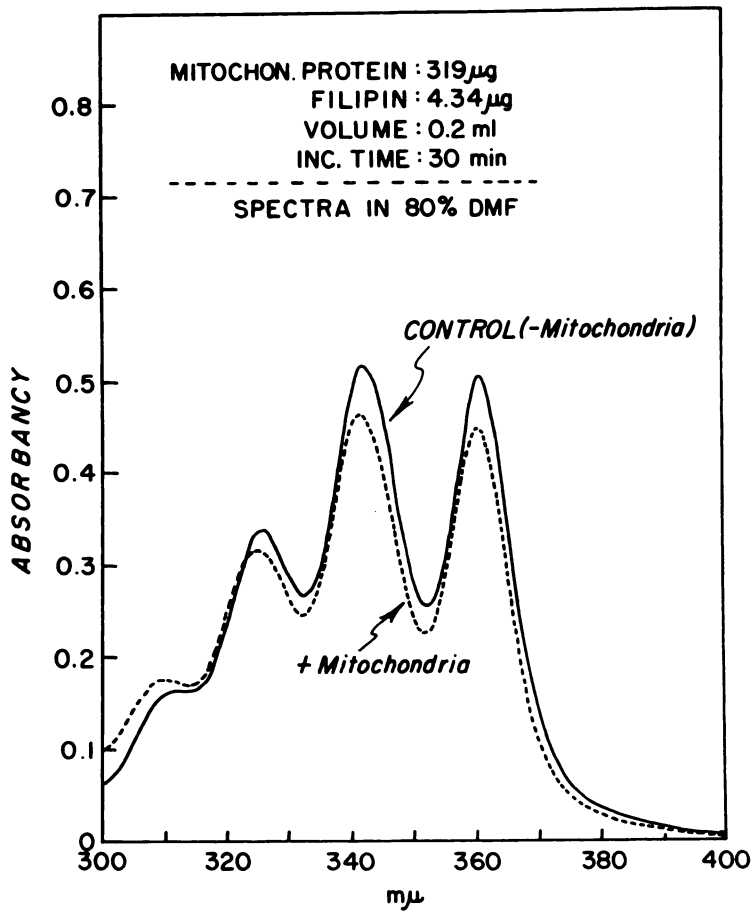

FIG. 5. *Recovery of filipin after incubation with Neurospora mitochondria*

Filipin was incubated with mitochondria at 22° as indicated in the figure. After 30 min, sufficient DMF was added to give a final concentration of 80%. The mitochondria were then removed by centrifugation, and the spectrum of the supernatant fluid was determined. A control tube contained an equivalent amount of filipin incubated in the absence of mitochondria.

TABLE 2

Phospholipid and ergosterol content of Neurospora mitochondria and microsomes

Fraction[a]	mµmoles lipid-P: mg protein	mµmoles ergosterol: mg protein	mµmoles lipid-P: mµmoles ergosterol
Mitochondria (II SD)	431	12.5	34.5
Mitochondria (II SD)	356	7.26	49.1
Microsomes (V)	228	24.8	9.2
Microsomes (V SD)	432	57.6	7.5

[a] Analyses were carried out on different mitochondrial and microsomal fractions.

DISCUSSION

The present investigation was undertaken to determine whether mitochondrial function is affected by the polyene antibiotics. Electron transport, specifically succinate–cytochrome c reductase, was examined in detail because of the report (8) that ascosin inhibited this reaction. We could find no inhibition by high concentrations of any pure polyene, and the experiments described above show that the inhibitory properties of ascosin, and probably candicidin, are due to contamination by antimycin A.[1] Polyene antibiotics are known to form complexes with sterols. Therefore, the lack of inhibition does not seem surprising since sterols have so far not been implicated as participants in any oxidative pathway.[2] It may be argued that electron transport is not a valid indicator of mitochondrial function and that other parameters, such as oxidative phosphorylation or ion accumulation, may be sensitive to the polyenes. However, Lardy *et al.* (23) observed that several polyenes (nystatin, filipin, amphotericin B, candidin) had no effect on P:O ratios of rat liver mitochondria with glutamate as substrate and, recently, Pressman (24) reported that amphotericin B did not influence ion transport in these mitochondria. These studies therefore demonstrate that there is no direct effect of the polyenes on isolated mitochondria *in vitro*.

The possibility of an *in vivo* effect of filipin on mitochondria has been suggested by Shaw *et al.* (10). They observed that mitochondria isolated from filipin-treated yeast did not have the full oxidative capabilities of control mitochondria obtained from cells which had not been treated with the antibiotic. Filipin is the most potent polyene known and, under their incubation conditions (6 hr shaking at 25°; filipin concentration: 135 μg/ml or 27 μg/mg dry weight of yeast), the ability of the cell membrane to act as a selective restraining barrier would be abolished. This would result in a situation analogous to dilution of the mitochondria with hypotonic medium and, therefore, irreversible damage to some components of the electron transfer chain would be anticipated. In an effort to reconcile an *in vivo* effect of filipin with lack of an *in vitro* effect it was suggested that mitochondria rapidly inactivate the antibiotic. The present experiments do not support this conclusion. Furthermore, on this basis, one would have to assume that mitochondria within the yeast cell do not inactivate filipin. The foregoing remarks emphasize that the secondary indirect effects of a polyene-induced permeability alteration must be clearly distinguished from the direct action of the antibiotics on the cell membrane.

Although the cell membrane is the site of polyene antibiotic action, the present studies with mitochondria may provide additional information on the factors which render a membrane polyene sensitive. In the following, we shall accept the current belief that the mitochondrion is a prototype membrane system, albeit with rather specialized functions (7). The demonstration

[1] Dr. C. P. Schaffner (Institute of Microbiology, Rutgers University) has also found an antimycin in the preparation of ascosin used by Gottlieb *et al.* (8). (Personal communication.)

[2] Detwiler *et al.* (25) have reported that low concentrations of digitonin stimulate bovine heart muscle succinate–cytochrome c reductase, whereas high concentrations inhibit this reaction. However, it seems unlikely that the effects of digitonin were due to combination with sterol since they were reversible by addition of tocopherol. Kusel and Weber (unpublished experiments) have found that low concentrations of digitonin and filipin may occasionally stimulate cytochrome c reductase activity in crude extracts of the protozoan *Crithidia fasciculata*, whereas high concentrations were inhibitory. In some preparations of *Neurospora* mitochondria, we have observed that filipin (50 μg/ml, ca. 800 μg per milligram mitochondrial protein) also had a slight stimulatory effect on cytochrome c reduction, but never more than 15% of the control rate. Other polyenes, when tested with these preparations, neither stimulated nor inhibited. Filipin could conceivably produce a stimulation by altering the permeability of the mitochondria thus making the substrates (succinate or cytochrome c) more accessible to the enzymic sites. We would predict therefore a greater effect of the antibiotic with lower amounts of either succinate or cytochrome c, i.e., when the substrate concentration became rate limiting. This was never observed.

that ergosterol is present in *Neurospora* mitochondria is consistent with the finding that they are able to bind filipin. These experiments confirm previous studies which indicate that the presence of sterols is necessary for polyene interaction with (i.e., binding to) a membrane (1–4). However, in view of the fact that mitochondrial function is not affected by any of the antibiotics, it seems probable that the presence of sterols per se is not sufficient to confer polyene sensitivity. We would like to suggest on the basis of these experiments that the phospholipid:sterol ratio (P:S) is the factor which determines whether or not a membrane is sensitive to the polyenes. Membranes with a high phospholipid:sterol ratio—e.g., bacteria ($P:S = \infty$) or mitochondria ($P:S$ = ca. 40)—are not affected by the antibiotics,[3] whereas erythrocytes from various sources [$P:S$ = ca. 1 (26)] are extremely sensitive to the polyenes. Experiments on the penetration of lipid monomolecular layers by the polyenes have suggested that these antibiotics may inhibit the ability of membranes to act as selective restraining barriers by producing a change in membrane lipid structure, specifically a spatial reorientation of the sterol molecules (4). It has been observed [see Fig. 4 in ref. (4)] that the surface pressure increase, which results when filipin is injected under cholesterol monolayers, was markedly diminished by the presence of phosphatidyl choline. This finding constitutes additional preliminary evidence that the phospholipid:sterol ratio may be very important in determining polyene sensitivity.

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[3] Ergosterol constitutes 2–3% of the total lipid content of *Neurospora* mitochondria (Table 2). This figure agrees well with the sterol content of various mammalian mitochondria (7). As has been pointed out previously (11), these preliminary lipid analyses are consistent with the conclusion of Luck (13) that *Neurospora* mitochondria probably do not arise from other intracellular membranes.

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