Penetration of Lipid Monolayers by Polyene Antibiotics

CORRELATION WITH SELECTIVE TOXICITY AND MODE OF ACTION*

R. A. DEMEL AND L. L. M. VAN DEENEN

From the Organisch Chemisch Laboratorium, Der Rijksuniversiteit te Utrecht, The Netherlands

STEPHEN C. KINSKY

From the Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri

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Previous studies have shown that polyene antibiotics alter permeability in sensitive fungi and thus lead to the loss of essential cytoplasmic constituents, which ultimately culminates in cell death (1-3). Subsequent investigations have led to the contention that the selective toxicity of the polyene antibiotics is due to interaction with a component present only in the membrane of sensitive organisms. This hypothesis was based on the observation that whole cells, protoplasts, and isolated membrane fractions of polyene-insensitive bacteria do not bind any of the antibiotics (4, 5).

Several lines of evidence support the conclusion that sterol may be this unique component. (a) Sterols can prevent the inhibitory action of polyene antibiotics on fungal growth (6), and spectrophotometric studies have provided evidence for combination between the antibiotics and cholesterol or ergosterol (7). (b) All polyene-sensitive organisms (fungi, protozoa, higher algae, pleuropneumonia-like organisms, flatworms, snails, and mammalian erythrocytes) contain sterols, whereas polyene-insensitive organisms (bacteria, blue-green algae) do not. (c) The ability of membrane fractions from Neurospora crassa to bind nystatin is completely abolished by extraction with organic solvents, and antibiotic-binding capacity can be partially restored by addition of ergosterol (5). (d) A close correlation exists between the ability to bind nystatin and the ergosterol content of various subcellular fractions from Saccharomyces cerevisiae (8). (e) Digitonin, a "sterol-specific" complexing agent that mimics the action of the polyenes on protozoa and fungi, markedly inhibits polyene uptake by intact cells or isolated membrane fractions (9-11).

Recently, Goldfine and Ellis have shown that bacteria in general are not able to synthesize choline derivatives (12). Thus, lecithins also satisfy the requirement of a membrane component present only in polyene-sensitive organisms, and interaction with these lipids may also explain the selective toxicity of the antibiotics. Several reports have, in fact, indicated that polyene activity may be nullified by the addition of "phosphatides" to the culture medium, suggesting a reduction of the effective antibiotic concentration due to complex formation (10, 13, 14). This possibility is also supported by the observation that lecithin prevents antibiotic binding (10).

It therefore seemed desirable to determine whether polyene antibiotics can interact with lipids other than sterols. The use of spectrophotometric methods for determining complex formation is not suitable for a comparison of the abilities of the antibiotics to interact with sterols and phospholipids because not all of the lipids form stable aqueous suspensions. In the present study, we have consequently investigated the ability of polyene antibiotics to penetrate lipid monolayers of varying composition prepared at an air-water interface. The term "monolayer penetration" is used, as in papers cited by Pethica (15), to denote interaction of a soluble surface-active compound with an insoluble material spread at a phase boundary. It was hoped that such an approach might also explain why some polyenes are more potent than others and thus provide a clue to the molecular basis of polyene antibiotic action.

EXPERIMENTAL PROCEDURE

Materials—The polyene antibiotics were obtained from the following sources: the Equibb Institute for Medical Research, New Brunswick, New Jersey (nystatin, amphotericin B); Farmitalia, Milan, Italy (etruscomycin); the Upjohn Company, Kalamazoo, Michigan (filipin); and Lederle Laboratories, Pearl River, New York (pimaricin). Stock solutions (0.4 mg per ml) were made in redistilled dimethylformamide, stored at -20°C, and used within 5 days of preparation.

Cholesterol and ergosterol were purchased from Fluka A. G., Switzerland. The phospholipids were synthesized by methods previously described (16, 17); they were kindly provided by Dr. G. H. de Haas. The lipids were dissolved in chloroform and were determined to be chromatographically pure by thin layer (silica gel) chromatography in the following solvent systems: for phospholipids, diisobutylketone-acetic acid-H₂O (40:25:5) and chloroform-methanol-H₂O (65:35:4); for neutral lipids, diethyl ether-hexane (30:70). Lipid extracts of beef erythrocytes and Staphylococcus aureus were prepared by minor modification of the procedure of Bligh and Dyer (18). Separation of these extracts into neutral lipid and phospholipid fractions was done.
by column chromatography on silica gel according to the procedure of Hanahan (19).

**Determination of Surface Pressure**—Force-area measurements and experiments on monolayer penetration were performed at the air-water interface in a paraffin-coated glass trough, 58 cm long \times 14 cm wide. The total capacity of the trough was 700 ml. The trough was filled with unbuffered water that had been distilled from alkaline permanganate and then redistilled in a quartz still. The aqueous surface was swept clean with a Teflon bar. Surface pressures were determined with a conventional Langmuir-Adam surface balance. All experiments were performed at room temperature (ca. 22°C).

**Force-Area Curves of Antibiotics**—Known amounts of the polyene antibiotics were carefully released onto the surface from an Arla micrometer syringe. The initial surface area was 560 cm². This area was then compressed, and the change in surface pressure was determined after 3 minutes. The measurement of an entire force-area curve took about 30 minutes. The area occupied by each molecule was calculated with the use of the following molecular weights (information supplied by manufacturer): filipin, 571; nystatin, 932; amphotericin B, 960; etruscomycin, 700; and pimaricin, 851. It was estimated that the antibiotics were 90% pure. The possibility that chemical modification, particularly oxidation, occurred during the course of these experiments could not be excluded. Thus, these values should be regarded only as approximations that may have to be revised when purer antibiotics and an “anaerobic” trough become available.

**Monolayer Penetration at Constant Area**—Monolayers were prepared by spreading either individual lipids or lipid mixtures of varying composition, as described below, on a surface area of 560 cm². This area was then compressed to approximately 140 cm² to give initial surface pressures between 2 and 30 dynes per cm. The polyene antibiotics were carefully injected beneath the monolayer. A stable pressure change was obtained within 2 minutes.

**Monolayer Penetration at Constant Pressure**—The procedure was essentially the same as above except that, after injection of the antibiotics, the increase in area necessary to maintain the initial surface pressure was determined.

**RESULTS**

**Force-Area Curves of Polyene Antibiotics (Fig. 1)**—At the concentrations employed in these experiments, the polyene antibiotics are water "soluble."" Insofar as these substances are oriented at the air-water interface, they all have collapse pressures below 24 dynes per cm. In a control experiment, a volume of dimethylformamide equal to that used for dissolving the antibiotics gave no change in surface pressure. Previous studies have indicated that filipin is the most potent, and nystatin the least potent, of the more common polyene antibiotics and that etruscomycin, amphotericin B, and pimaricin occupy intermediate positions (20-22). In the present experiment, there was no apparent correlation between the spreading properties of the various polyenes and their biological potency. Because of this difference in biological activity, detailed experiments on monolayer penetration were performed with filipin and nystatin.

**Penetration of Pure Lipid Monolayers by Filipin (Fig. 2)**—Figs. 2 and 3). This result is particularly significant since the pressure change was less at higher initial pressures but was still significant at pressures much greater than the collapse pressure of the antibiotic, 14 dynes per cm (Fig. 1, Curve 3). The magnitude of these pressure changes was dependent upon the initial antibiotic concentration (compare Curves 1 and 2). Filipin also interacted with ergosterol monolayers although the effect was much less when compared with cholesterol, especially at low initial pressures (Curve 3). Except at low pressures, there was essentially no interaction of filipin with a variety of phospholipids (Curves 4 to 7).

**Penetration of Pure Lipid Monolayers by Nystatin (Fig. 3)**—Nystatin also was able to penetrate monolayers of cholesterol and ergosterol at initial pressures greater than the collapse pressure of the antibiotic, 23.6 dynes per cm (Fig. 1, Curve 2). Nystatin, like filipin, gave only a slight interaction with a phospholipid at low initial pressures. Nystatin produced a much smaller pressure change in monolayers of either cholesterol or ergosterol than did equimolar quantities of filipin (compare Figs. 2 and 3). This result is particularly significant since previous studies have shown that filipin is a much stronger

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1. By "soluble," we do not mean to imply that each antibiotic molecule is surrounded by solvent molecules. It seems quite probable that the polyenes exist as micelles in solution. This is suggested by the observation that the molar extinction coefficient of all the polyenes at their characteristic absorption maxima is much less in water than in organic solvents such as methanol or dimethylformamide. Furthermore, the spectra of the antibiotics in water is unstable and gradually approaches that in organic solvents. Earlier evidence for the existence of micelles has been presented by Lampen et al. (4).
lytic agent for both mammalian erythrocytes and Neurospora protoplasts, which contain cholesterol and ergosterol, respectively, as the principle sterol in membranes (20, 21, 23). These experiments, as well as the following, argue against the possibility that the more extensive "cell membrane damage" due to filipin indicates that this antibiotic is less specific than nystatin and that it can interact with other lipid constituents of membranes in addition to sterols.

Penetration of Mixed Lipid Monolayers (Fig. 4)—Bacterial protoplasts are completely resistant to the lytic action of the polyene antibiotics. This observation is in perfect accord with the present finding that filipin cannot penetrate a monolayer prepared from a lipid extract of bacteria (Curve 7). Filipin, however, gave a strong interaction with a monolayer prepared from a lipid extract of beef erythrocytes (Curve 4). This pene...
the above experiments, interaction between lipids and polyene antibiotics was examined by measuring the increase in surface pressure that occurred when the monolayer was kept at constant area. The change in area necessary to maintain the initial surface pressure has been determined for the interaction of nystatin and filipin with ($\beta,\gamma$-didecanoyl)-l-$\alpha$-phosphatidylcholine and ergosterol. There was only a very small increase in the area of the phospholipid film with either of the polyenes at initial pressures above 12 dynes per cm (Curves 2 and 3). This finding was not unexpected since previous experiments have shown that negligible penetration of the antibiotics occurs at these pressures. However, filipin and nystatin, which interact strongly with ergosterol at high pressures, also did not produce an appreciable increase in the area (less than 5%) of the steroid monolayer (Curves 1 and 4).

Analogous results were obtained with filipin and cholesterol examined under the conditions used in the preceding experiment (i.e. final concentration of antibiotic, $0.85 \times 10^{-8}$ M). A greater increase in area occurred with higher concentrations of antibiotic. Thus, increases of 20% and 49% were observed after filipin had been injected to final concentrations of $0.85 \times 10^{-7}$ and $1.7 \times 10^{-7}$ M, respectively, beneath a cholesterol monolayer. For a pressure of 10 dynes per cm. Since the experiments were performed at a constant pressure, and since it may be assumed that area occupied by the antibiotic molecules does not change during expansion of the film, it can be concluded that the increase in area was proportional to the number of penetrating molecules. The increase in area per cholesterol molecule in the monolayer at 10 dynes per cm is approximately 1 A$^2$ at a final concentration of filipin of $0.85 \times 10^{-8}$ M. From Stuart models based on the filipin structure recently proposed by Cedar and Ryhage (24), it was calculated that the area occupied by a filipin molecule in the interface was between 100 and 250 A$^2$ (the exact value depending on the orientation chosen). A reasonable estimate of the ratio of cholesterol to filipin in the monolayer therefore ranges between 100:1 and 250:1.

**DISCUSSION**

From the results of this investigation, we conclude that the selective toxicity of the polyene antibiotics is probably due exclusively to interaction with the sterol present in the cell membrane of sensitive organisms. This conclusion is in agreement with, and supplements, recent experiments with the pleuropneumonia-like organism, *Mycoplasma laidlawii* (25). Filipin was found to inhibit growth, and to cause lysis, only of cells that had incorporated cholesterol after cultivation in media containing the sterol.

The polyenic compound, vitamin A, has been shown by Dingle and Lucy to be a powerful hemolytic agent (26). Bangham et al. have demonstrated in monolayer studies that vitamin A interacts more strongly with lecithin than with cholesterol (27). In a previous investigation it was concluded that sterols probably were not required for binding of the vitamin to the cell membrane (21). This conclusion was based on the observation that vitamin A caused a rapid lysis of *Bacillus megaterium* protoplasts, which were unaffected by high concentrations of any polyene antibiotic. The contention that the polyene antibiotics and vitamin A do not share a common mechanism is supported by the present finding that, unlike the vitamin, the antibiotics give essentially no interaction with phospholipids.

The extensive studies of Schulman and Rideal (28), Goddard and Schulman (29), and Matalon (30) have shown that saponins give a much greater increase of surface pressure with monolayers of sterols than with lecithins. Although the structures of saponins and polyene antibiotics are quite different, the present data suggest that these compounds may have a similar mode of action. Both saponins and the polyenes interact more strongly with cholesterol than with ergosterol. The reasons for this difference and for the peculiar "bell-shaped" curve obtained with ergosterol are not yet known. The curve may be due to difference in the type of film produced by cholesterol and ergosterol at low surface pressures.

The present results give some clue to the molecular basis of polyene antibiotic action and to the differences in biological potency of filipin and nystatin. Although preliminary experiments have shown that filipin can solubilize cholesterol, treatment of Neurospora mycelial mats did not result in a reduction of the total sterol content or of the ratio of unesterified to esterified sterol (11). These results exclude a "detergent" action of the antibiotics that results in the selective extraction of the sterol from the membrane. The slight increase in area of sterol monolayers obtained after interaction with exceedingly low concentrations of the polyenes suggests that the presence of only a few antibiotic molecules is sufficient to produce a larger increase in surface pressure. Therefore it seems quite probable that the polyene antibiotics produce a reorientation of the sterol molecules in the membrane and a consequent alteration in cell permeability. The lytic effect of saponin has been shown by electron microscopy to involve a reorientation of the lipids in the erythrocyte membrane (32-35).

Nystatin did not interact as strongly as filipin with the sterol monolayer, particularly at low initial pressures. However, under these conditions, nystatin did produce a greater increase in film area (Fig. 5). Inspection of the force-area curves indicates that at pressures between 12 and 12 dynes per cm the nystatin molecule occupies more space than filipin (Fig. 1). Thus, the greater increase in area was probably not due to a larger number of molecules penetrating the sterol monolayer. It seems more likely that nystatin molecules produce a less extensive reorientation of the sterol than does an equivalent number of filipin molecules. Of the polyene antibiotics used in this study, the structure of filipin only has been established (24). Until the structures of the others, especially the clinically important nystatin and amphotericin B, have been determined, it seems premature to attempt a definition of sterol reorientation in terms of physical models.

**SUMMARY**

The ability of the polyene antibiotics to penetrate lipid monolayers of varying composition was examined, and the following

1 At high pressures, cholesterol has been shown to give a condensed film (15). It is worth emphasizing that the phospholipids, (tetrahexanoyl)-l-$\alpha$-phosphatidylethanolamine and (tetrodecanoyl)-l-$\alpha$-phosphatidylcholine, did not interact appreciably with filipin (Fig. 2, Curves 4 and 5) although these compounds, like cholesterol, give condensed monolayers (31).

2 As in all monolayer studies of this type, however, reactions occurring at the air-water interface may not be valid for biological membranes.

3 Thus, when 2 mg of filipin in 0.5 ml of dimethylformamide were added to 20 ml of an aqueous suspension of cholesterol (5 mg per ml), approximately 25 mg of cholesterol were solubilized, whereas the corresponding value for the solvent control was 5 mg.
observations were made. (a) Filipin and nystatin readily penetrate monolayers prepared from cholesterol and ergosterol at initial surface pressures greater than the collapse pressures of the antibiotics. Interaction was stronger with filipin than with nystatin. (b) Under the same conditions, there was essentially no interaction with a variety of pure synthetic phospholipids unless sterol was present. (c) Filipin did not penetrate monolayers prepared from polyene-insensitive bacteria. (d) Filipin did penetrate monolayers prepared from lipid extracts of beef erythrocytes, which are rapidly lysed by the polyenes. When the extract was separated into a neutral lipid and a phospholipid fraction, filipin did not penetrate a monolayer of the phospholipids but did interact with the neutral lipids consisting primarily of cholesterol. (e) At high initial surface pressures, penetration of either antibiotic was accompanied by a very small percentage increase in the area of the monolayer. From these results, it is concluded that (a) the polyene antibiotics interact specifically with sterols, and (b) penetration of only a few antibiotic molecules into the cell membranes of sensitive organisms causes "reorientation" of the sterol molecules.

REFERENCES

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