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EFFECT OF POLYENE ANTIBIOTICS ON PHOSPHOLIPID SPHERULES CONTAINING VARYING AMOUNTS OF CHARGED COMPONENTS

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SUMMARY

Polyene antibiotics, such as filipin, nystatin, and amphotericin B, alter the gross permeability to previously captured anions or glucose of artificial phospholipid spherules prepared with long-chain polar compounds. To determine whether this effect was due to the interaction of polyenes with ovolecithin alone or with highly charged components, the composition of such artificial spherules was varied. Neither variations in the sign or amount of the charged component (anionic dicetylphosphate or cationic stearylamine in 5–20% molar ratios) altered the response of spherules to polyenes in the direction expected if the antibiotics interacted preferentially with long-chain polar lipids. When increasing amounts of cholesterol were incorporated into the spherules, leakage of anions or glucose induced by nystatin or amphotericin B was proportionately increased, whereas the action of filipin was independent of the presence of cholesterol. The amount of anions captured by the spherules varied directly with the molar ratios of the charged components, being greater for negative than for positive spherules. The experiments suggest that polyene antibiotics alter the gross permeability of lipid spherules by virtue of their direct interaction with phospholipids, and not with long-chain polar compounds, and that their effects are independent of the overall charge of the target structures. Nystatin and amphotericin B appear to differ qualitatively from filipin in their mode of action by preferentially disrupting membrane systems which contain cholesterol.

INTRODUCTION

Polyene antibiotics alter the permeability of sensitive cells, leading to the loss of essential cytoplasmic components and subsequent cell death[1]. Indirect evidence points to the cell membrane as a primary target of the polyenes, and furthermore suggests that sterols are the unique component of the cell membrane with which the antibiotics react[2–4]. Indeed, studies of the interaction between polyene antibiotics and purified lipids in artificial membrane systems support this view. Thus DEMEL,

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VAN DEENEN AND KINSKY[5] found that polyenes such as filipin and nystatin readily penetrated monolayers of ergosterol or cholesterol but failed to penetrate monolayers of natural and synthetic phospholipids. Recently, VAN ZUTPHEN, VAN DEENEN AND KINSKY[6] demonstrated that lipid bilayers prepared from solutions of pure lecithin were unaffected by filipin, whereas bilayers prepared from equimolar solutions of cholesterol and lecithin were rendered unstable by the antibiotic. Each of these studies has therefore indicated that the polyenes share a common mode of action, and that susceptibility to polyenes is determined by an interaction between the antibiotic and sterols present in cell membranes.

On the other hand, previous studies from this laboratory[7] have demonstrated that polyenes differ at least qualitatively in their mode of action on artificial lipid spherules and may be divided into two groups on this basis. Using phospholipid spherules prepared with and without cholesterol, it was found that whereas polyenes of relatively low molecular weight (filipin, etruscomycin) altered the gross permeability of phospholipid spherules prepared either with and without cholesterol, polyenes of relatively higher molecular weight (amphotericin B, nystatin) induced only modest increases in the permeability of spherules prepared in the complete absence of cholesterol. The activity of amphotericin and nystatin increased 2–3-fold as the cholesterol content was raised to 20% of the total lipid. This division of polyene antibiotics into two groups is in agreement with the earlier suggestions of LAMPEN AND ARNOW[8] who found differences in the action of these two groups on susceptible fungi. However, since in our earlier studies the lipid spherules were always prepared with 20% dicetylphosphate, it remained entirely unclear whether the effect of filipin and the other polyenes was due to their reaction with ovolecithin or with long-chain anion. Therefore the present studies were initiated, which are based upon the assumption that increases in permeability of the spherules to their previously captured markers should accompany increases in the molar percentage of the lipid component of the spherules with which the polyenes preferentially react. Since increased leakage of anions and glucose from spherules exposed to amphotericin B or nystatin did indeed accompany increases in the molar lipid ratio of cholesterol, the assumption seemed valid. Finally it should be noted that artificial spherules do not trap marker ions or molecules unless at least 5% (ref. 9) of the total lipid is constituted by long-chain anions or cations, presumably because these charged components produce repulsive forces between the lipid layers sufficient to permit aqueous channels to form in which the markers exist in solution.

From such experiments, evidence has been obtained that the effect of polyene antibiotics on artificial lipid spherules depends neither upon the sign or amount of charged component, and is therefore presumably due to the interaction of polyenes with phospholipid. Finally, it has been clearly shown that the disruption by filipin of lipid structures is independent of their content of cholesterol, whereas amphotericin B and nystatin preferentially disrupt lipid spherules which contain cholesterol, even when charged components are present in minimal amounts.

MATERIALS AND METHODS

Artificial phospholipid spherules of varying lipid composition were prepared by methods previously described[10]. In brief: ovolecithin, cholesterol, dicetylphosphate,

or stearylamine, in molar ratios described below, were dissolved in chloroform, dried *in vacuo* to remove solvent, and permitted to 'swell' in 0.145 M K_2CrO_4 or 0.290 M glucose for 6 h at 23°. Anions or glucose not trapped within the spherules (16 μmoles of lipid/ml) were removed by dialysis against equimolar NaCl/KCl by methods described before. The dialysed spherules, now containing only sequestered ions or glucose, were dispensed as 1-ml aliquots into smaller dialysis bags to which 0.05 ml filipin, amphotericin B, or nystatin, in dimethylsulfoxide were added. To control samples, 0.05 ml of dimethylsulfoxide alone were added. One sample of each set was incubated with the non-ionic detergent Triton X-100 (0.2%, v/v); this detergent physically disrupts the spherules and is used to measure the maximum rate of leakage possible under these conditions, independent of variations peculiar to the charged ionic species under study. This concentration, at 90–120 min, releases all of the trapped ions[7]. Leakage of anions or glucose from the spherules, through the sacs, into smaller test tubes, was measured at 30 and 60 min. Amphotericin B and nystatin were obtained from the Squibb Institute for Medical Research, New Brunswick, N.J.; filipin from the Upjohn Company. Dicetylphosphate, stearylamine, and cetyl alcohol were obtained from K and K Laboratories, Plainview, N.Y.. Dimethylsulfoxide was obtained from Sigma Biochemicals, St. Louis, Mo.

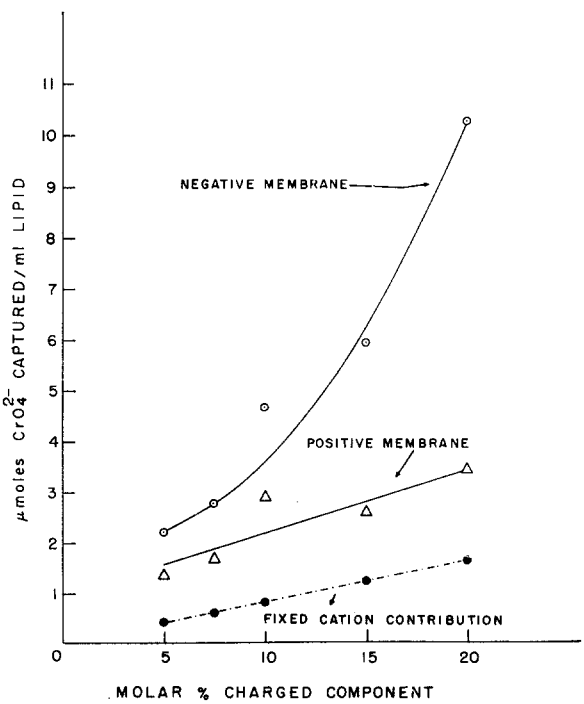

Fig. 1. Effect of varying molar percentage of charged components upon capture of CrO_4^{2-} by phospholipid spherules. Spherules were prepared with ovolecithin and increasing amounts of dicetyl phosphate (negatively charged spherules) or stearylamine (positively charged spherules). The amount of CrO_4^{2-} incorporated is expressed as μmoles captured/ml lipid (16 μmoles total lipid/ml) and represent total anions which remained in the spherules after 18 h dialysis at 23° against 0.145 M NaCl/KCl. Molar percentage of charged component was calculated assuming complete ionization, and therefore represents maximum possible values (see discussion in ref. 9).

RESULTS

Variations in molar ratios of charged components

Spherules containing chromate were prepared with increasing amounts of dicetylphosphate or stearylamine preincorporated, and the total amount of divalent anion which remained trapped in the spherules after prolonged dialysis was determined. BANGHAM, STANDISH AND WATKINS[9] had previously shown that the amount of cation trapped by phospholipid spherules was finite, and increased with surface charge increment whether positive or negative. The data in Fig. 1 indicate that these relationships also obtained for divalent anions of larger hydrated radius. Therefore trapping of ions by the spherules did not appear to depend upon the charge of the captured ions, but upon the charge (and resultant geometry) of the trapping spherules. Both from Fig. 1, and from data of BANGHAM, STANDISH AND WATKINS[9] it is clear that negatively charged spherules trap more ions that positively charged spherules, the amount of ions trapped by positive spherules still being well above those required by the fixed cation contribution of stearylamine.

Release of anions from lipid spherules (Fig. 2) did not vary with the amount of dicetylphosphate incorporated. Filipin (0.1 μmole/16 μmoles of lipid, approximate final molar concn. 10^{-4}) released anions to the same extent as the detergent Triton X-100 over the entire range of dicetylphosphate incorporation: amphotericin B and nystatin were considerably less active. The amount of anions released by polyenes did not increase as the molar ratio of dicetylphosphate was increased; such a relationship would have been expected were the polyenes preferentially to react with the charged component. Evidence that the long-chain anion was in fact incorporated into the

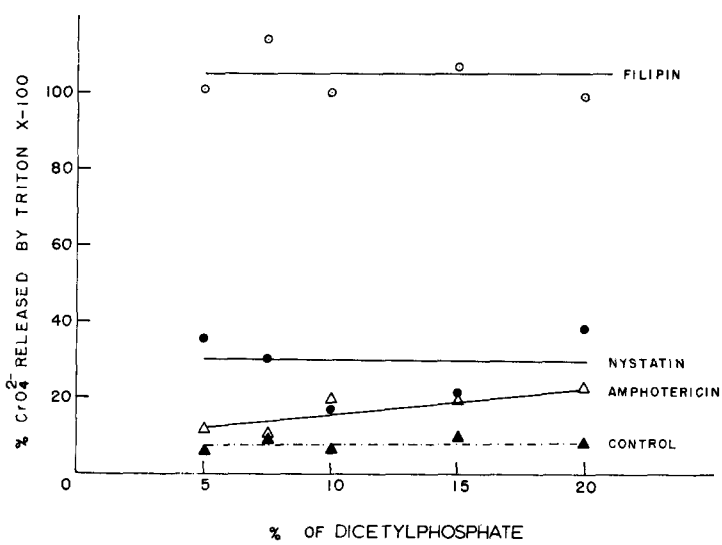

Fig. 2. Effect of varying molar percentage of dicetyl phosphate added on release of sequestered anions by polyenes antibiotics. The spherules were prepared with ovolecithin and increasing amounts of dicetyl phosphate. The amounts of anions (CrO_4^{2-}) released are expressed as percentage of the amount released by Triton X-100 in 30 min at 37°. The polyenes were added at a final approximate molarity of 10^{-4} (0.05 ml/16 μmoles lipid) in dimethylsulfoxide and an equal volume of dimethylsulfoxide was added to the control sample.

spherules is shown in Fig. 1: total ion-trapping did increase with increments of the charged component. The results of these experiments suggest that polyenes preferentially reacted with lecithin of the spherules, rather than with long-chain anion.

To exclude the possibility that the action of polyene antibiotics depended upon the sign of the charged component, cationic stearylamine was incorporated into the spherules. The presence of 5% stearylamine (Fig. 3) did not effectively diminish the release of anion from the spherules induced by filipin. Indeed, the effects of filipin on spherules containing 5% stearylamine were comparable to the effects of this polyene on spherules containing equal amounts of dicetylphosphate. Nor did filipin induce greater leakage of anions as the molar ratio of stearylamine was increased. The effect of filipin on the spherules was actually diminished as the molar percentage of the long-chain cation increased. However, release of anions by amphotericin and nystatin did not vary at all with the amounts of cation incorporated.

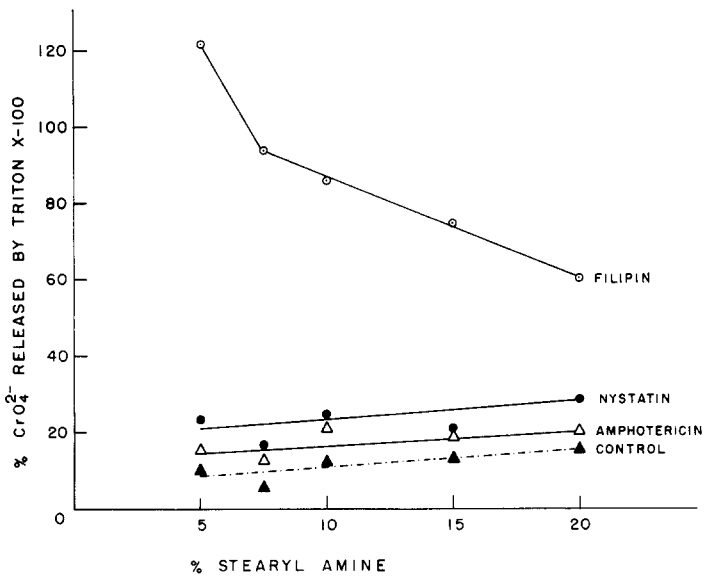

Fig. 3. Effect of varying molar percentage of stearylamine added on release of sequestered anions by polyene antibiotics. The spherules were prepared with ovolecithin and increasing amounts of stearylamine. The amounts of anions (CrO_4^{2-}) released are expressed as percent of the amount released by Triton X-100 in 30 min at 37°. The polyenes were added at a final approximate molarity of 10^{-4} in dimethylsulfoxide (0.05 ml/16 μmoles lipid) and an equal volume of dimethylsulfoxide was added to the control sample.

These experiments suggested that filipin reacted with stearylamine itself, but not with stearylamine that had been incorporated into forms which would influence anion trapping: in other words not all the stearylamine had necessarily been incorporated into the spherules. Therefore experiments were done in which stearylamine was added to the outside of the spherules after dialysis, shortly before filipin was added at approximate final molarities of 10^{-4} M, $5 \cdot 10^{-5}$ M and 10^{-5} M. At these concentrations, the addition of 5% stearylamine to the sacs diminished subsequent anion leakage induced by filipin to 88, 80, and 65% of values obtained without addition of the long-

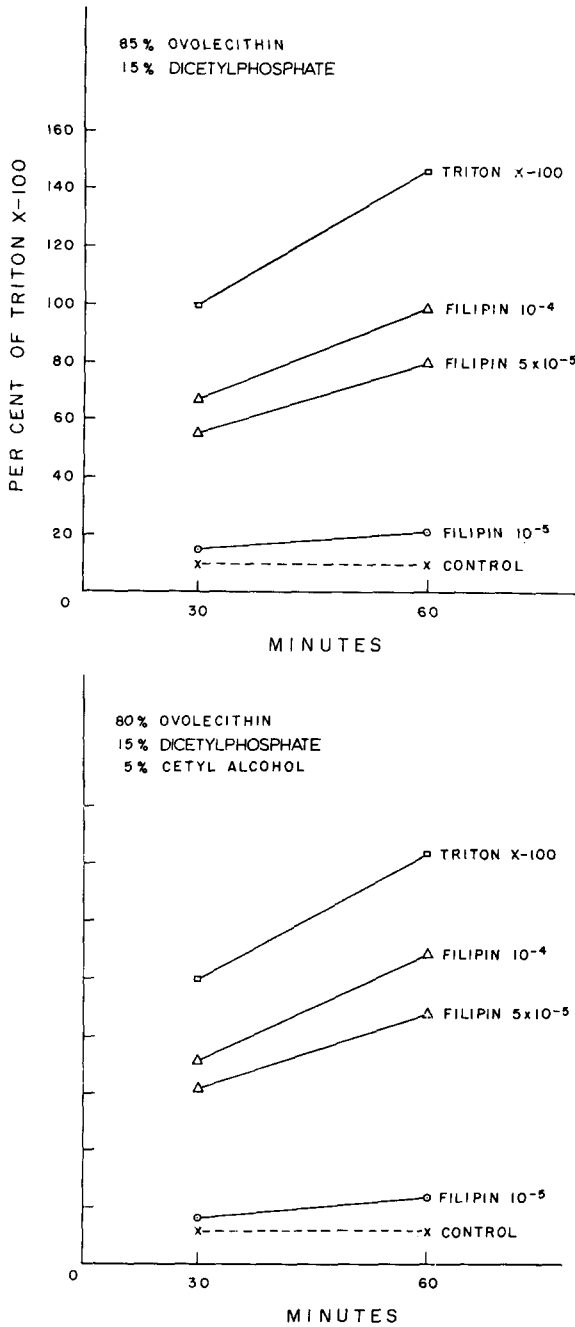

Fig. 4. Effect of cetyl alcohol upon release of chromate ions by polyene antibiotics. The spherules were prepared either with 85% ovolecithin and 15% of dicetylphosphate, or with 80% ovolecithin, 15% dicetylphosphate and 5% cetyl alcohol. The amounts of anions (CrO_4^{2-}) released are expressed as percent of the amount released by Triton X-100 in 30 min at 37°. The polyenes were added at a final approximate molarity indicated above in dimethylsulfoxide (0.05 ml/16 μmoles lipid) and an equal volume of dimethylsulfoxide was added to the control sample.

chain cation. In contrast, when filipin had been allowed to react with spherules at room temperature for 10 min before the addition of stearylamine, only minimal inhibition of anion leakage was found. These experiments suggested that any stearylamine which remained unincorporated into the spherules had either reacted with, or prevented access to, the spherules of filipin which had been subsequently added. On the other hand, when filipin had been allowed to interact with the spherules, its effect was not influenced by subsequent addition of stearylamine.

The effect of cetyl alcohol on polyene-induced anion leak

It has been suggested that polyenes can interact with monolayers of cetyl alcohol[11] and that filipin more readily entered such monolayers than did amphotericin B or nystatin. Were the commercial preparations employed to be contaminated by cetyl alcohol, the results previously presented could well be explained by the propensity of polyenes to interact with cetyl alcohol, rather than with ovolecithin or dicetylphosphate. Consequently spherules were prepared to contain up to 5% of cetyl alcohol, an amount which would represent 25% contamination of commercial batches of dicetylphosphate. Data shown in Fig. 4 show that the presence of 5% cetyl alcohol had no effect upon increases in gross permeability of the spherules induced by any of the polyenes. KINSKY, LUSE AND VAN DEENEN[11] have indeed themselves presented a brief description of experiments in which it was found that filipin did not appreciably interact either with dicetylphosphate or with cetyl alcohol.

Effect of variations in cholesterol content of lipid spherules

From the foregoing, it was clear that increases in the molar ratios of charged

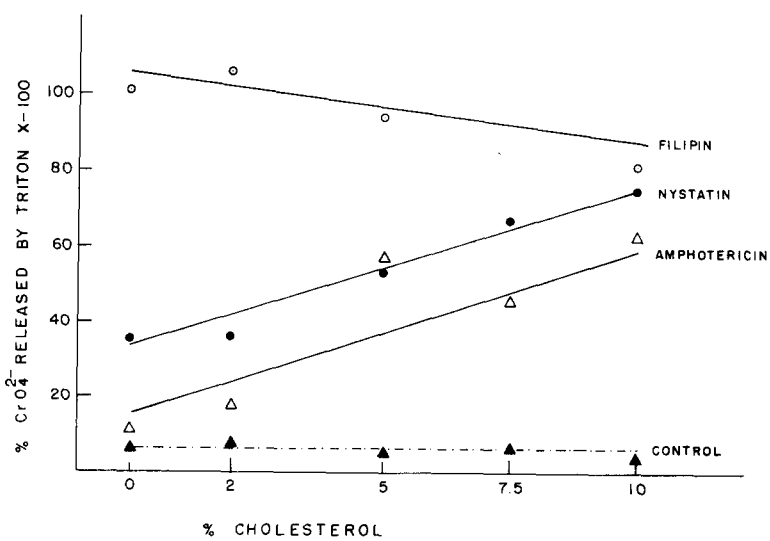

Fig. 5. Effect of varying molar percentage of cholesterol added upon release of anions by polyene antibiotics. The spherules were prepared with ovolecithin, 5% of dicetyl phosphate and increasing amounts of cholesterol. The amounts of anions (CrO_4^{2-}) released are expressed as percent of the amount released by Triton X-100 in 30 min at 37°. The polyenes were added at a final approximate molarity of about 10^{-4} in dicetylphosphate (0.05 ml/16 μmoles lipid) and an equal volume of dicetylphosphate was added to the control sample.

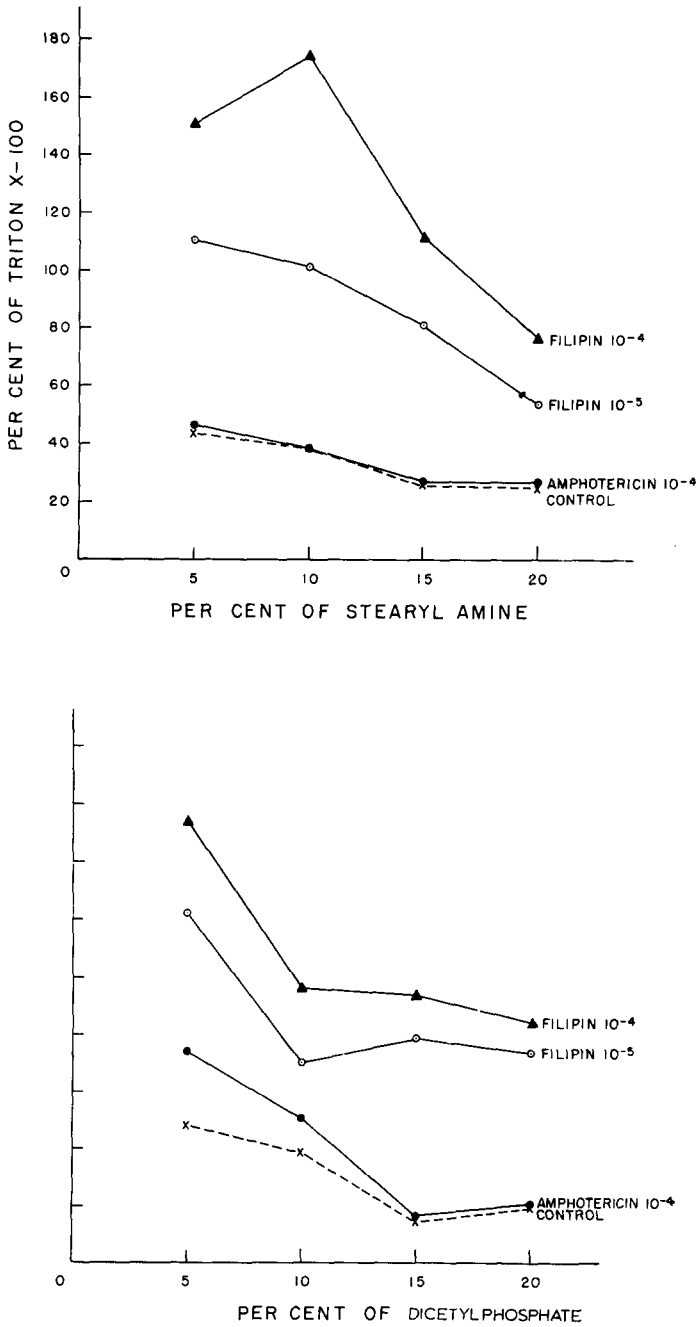

Fig. 6. Effect of variations in the molar percentage of charged components upon release of glucose by polyene antibiotics. Spherules were prepared either with lecithin and increasing amounts of stearylamine or with ovolecithin and increasing amounts of dicetylphosphate. The polyenes were added at final approximate molarity indicated above, and the release of glucose is expressed as the percent of the amount released by Triton X-100 in 30 min at 37°.

components were not associated with increases in the permeability of spherules induced by polyenes. It had previously been shown that amphotericin B and nystatin react preferentially with cholesterol-containing spherules prepared in the presence of maximum amounts of long-chain anions or cations. Therefore increasing amounts of cholesterol were incorporated into spherules which had been formed with minimum amounts of dicetylphosphate (5%). When the spherules' content of cholesterol was raised from 5–20% (Fig. 5), dicetylphosphate remaining constant, leakage of anion induced by amphotericin B and nystatin was augmented significantly. These experiments support the premise that agents which preferentially react with discrete lipid components of the spherules induce changes in the permeability of the structures which vary directly with the molar percentage of the critical component.

Release of glucose from lipid spherules

To exclude the possibility that polyenes released only charged ions or molecules from the spherules, the structures were prepared with sequestered glucose. Increases in the molar ratios of charged components (Fig. 6) were not accompanied by increases in polyene-induced changes in gross permeability. Indeed, decrements in filipin-induced leakage of glucose from the spherules were observed as the molar ratios of dicetylphosphate and stearylamine were increased.

Such decrements were also observed in control samples (Fig. 6). Since the control: filipin ratio of glucose leakage remained relatively constant these results indicate that increases in the molar percentage of charged components rendered the spherules less permeable to the uncharged marker.

Finally it appeared possible that the action of filipin was only apparently independent of the nature or amount of charged component because the concentrations of antibiotic were far in excess of those at which variations in the charged component were critical. Therefore the concentration of filipin was lowered to 0.01 μmole/16 μmoles of lipid. At this level (Fig. 6), filipin-induced leakage of anions or glucose approached that induced by the less active polyenes such as amphotericin, the behavior of which was critically determined by the molar percentage of cholesterol. Nevertheless, increases in the molar percentage of charged components did not augment filipin-induced release of glucose.

DISCUSSION

The results presented above indicate that the action of polyene antibiotics on artificial lipid spherules is independent of the sign or amount of long-chain anion or cation within the structures. Presumably therefore, the antibiotics react with the other component of the spherules: phospholipid. Such reasoning is based upon the premise that increases in permeability of the spherules should vary directly with the molar percentage of the lipid component with which the polyenes preferentially react. Evidence that this premise is warranted is afforded by experiments in which it was shown that amphotericin B and nystatin did indeed release more anion from spherules as the molar percentage of cholesterol was increased. Nor was it possible to show that the polyenes reacted with cetyl alcohol, a possible contaminant of dicetylphosphate. Finally the experiments have shown that the action of filipin was independent of the molar ratios of charged components at all concentrations of the polyene, and under

conditions at which critical increases in the molar percentage of a minor component (cholesterol) were reflected by increments in gross permeability of the spherules to other polyenes (*e.g.* amphotericin).

It is quite unlikely that polyenes affected the spherules because of specific effects upon one or another of the markers used. Not only have previous studies shown that anions, cations, glucose, and glycine, are each released from such structures by several membrane-active agents[7,9,10], but it is clear from the present studies that both chromate and glucose were released in similar fashion by the several polyenes studied.

Although these experiments have shown that filipin acts upon spherules composed of ovolecithin and dicetylphosphate by interaction with phospholipid alone, the same cannot be said for spherules prepared with higher amounts of stearylamine. At molar ratios of 5:95 (stearylamine:ovolecithin), the effects of filipin were entirely comparable to the effects of filipin upon spherules prepared at similar ratios of dicetylphosphate:ovolecithin. Higher molar ratios of stearylamine, however, resulted in diminished changes in gross permeability after addition of filipin, but not after amphotericin B or nystatin.

The latter observation would exclude any effect of the excess stearylamine upon the spherules themselves. Indeed, a portion of the stearylamine which was added at the time of preparation of the spherules may not have entered the structure of the spherules. Since addition of stearylamine to the dialysed spherules diminished the subsequent changes in permeability induced by addition of filipin, it is probable that some stearylamine remained unassociated with the spherules and prevented access of filipin in an as yet unidentified manner. Studies of anion trapping showed that positively-charged structures trapped less anions than did negatively-charged structures[9], a finding also compatible with the premise that some long-chain cations did not become incorporated into the spherules. BANGHAM, STANDISH AND WATKINS[9] were forced to conclude that the trapping of lesser amounts of cations by positive spherules was due to differences in the effect of screening ions as required by electrical double-membrane theory. Such an interpretation would not hold true for the equally diminished trapping of anions, and therefore the simpler explanation is offered that not all the stearylamine became incorporated.

It can reasonably be concluded from the above experiments that filipin, amphotericin B, and nystatin induce leakage of marker molecules from lipid structures by virtue of their interaction with phospholipids. Such a conclusion is at variance with the studies of DEMEL, VAN DEENEN AND KINSKY[5] and of VAN ZUTPHEN, VAN DEENEN AND KINSKY[6] quoted above. However each of the artificial systems used to study the interactions of polyenes with purified lipids differs considerably from the others and from any ideal model of naturally occurring membranes. Studies with monolayers yield imperfect information as to the disruptive effects of agents which might require bilayers or other geometric arrangements of lipid in water. The model bilayer system[6] suffers from having the majority of lipid molecules unincorporated in the black membrane, the optical properties of which serve as an endpoint in determining the reaction of its components with test agents.

Indeed, discrepancies in the several systems may also be due to differences in the concentration of the polyenes (10^{-4} M in these studies in contrast to 10^{-8} M in those of DEMEL, VAN DEENEN AND KINSKY[5]) and thus in one sense the spherules are less 'sensitive' to these agents. Each of these points has been discussed in detail

before[7], but it should be clear that studies in each of the artificial systems are needed to evaluate the action of membrane-active agents.

Filipin appeared to react with phospholipids and not with long-chain polar compounds since variations in the latter did not alter the effects of filipin. On the other hand, amphotericin B and nystatin appeared to react preferentially with cholesterol since the amount of markers released by these polyenes varied only with the level of sterol. These observations are consonant with the suggestion made previously that the two groups of polyenes(filipin *vs.* amphotericin B) differ at least qualitatively in their mode of action[7]. Thus the division of these fungicides into two groups[8], which has been made upon functional grounds, is borne out in a purely artificial system, as is the hypothesis that some of the polyenes require an optimum phospholipid:cholesterol ratio in order to disrupt lipid-bounded structures[6]. The division of polyenes into two groups has also been attributed[5,6,11] to differences in their relative affinities for sterols (*e.g.*: filipin>etruscomycin>nystatin). Furthermore there are modest quantitative differences in the response of natural membranes and the model spherules; for example, although amphotericin B is more potent a hemolysin than is nystatin, the former agent is somewhat less disruptive than the latter for the artificial spherules. Finally it must be stated that these studies in no way contradict the hypothesis that sterols are the unique component of lipid membranes with which polyenes react in order to disrupt such biological membranes as those which bound fungi, erythrocytes, lysosomes, *etc.* It is however evident that the polyenes have the capacity to disrupt lipid structures composed entirely of phospholipids, thereby producing functional changes in their overall permeability.

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