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Effect of Cholesterol on the Sensitivity of *Mycoplasma laidlawii* to the Polyene Antibiotic Filipin

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ABSTRACT

WEBER, MORTON M. (St. Louis University School of Medicine, St. Louis, Mo.), AND STEPHEN C. KINSKY. Effect of cholesterol on the sensitivity of *Mycoplasma laidlawii* to the polyene antibiotic filipin. J. Bacteriol. **89**:306-312. 1965.—The polyene antibiotic, filipin, inhibited growth and caused lysis of *Mycoplasma laidlawii* cells which had been cultured in the presence of cholesterol. The antibiotic did not inhibit growth and did not promote lysis of the organism when grown in the absence of cholesterol. These results constitute strong support for the contention that the presence of sterol in the cell membrane is a necessary prerequisite for polyene sensitivity. Higher concentrations of filipin were required to inhibit growth when serum was added to the assay medium than when it was absent. These results suggest binding of the antibiotic to some component in the serum and may partially account for the previous inability to demonstrate growth inhibition by low concentrations of the polyene antibiotics. The extent of growth inhibition due to filipin decreased upon prolonged incubation. Subculture in the presence of high concentrations of antibiotic indicated that the apparent reversal of inhibition was caused by emergence of a filipin-resistant cell population. It was also observed that cells, which originally were rapidly lysed by filipin and digitonin, were no longer responsive to the action of these agents upon incubation in sterol-free medium at 25 or 37 C for several hours. This effect could be prevented by keeping the cells at 2 C. These results may indicate that filipin-resistant cells carry out a metabolic conversion of membrane-localized sterol to a form which can no longer react with the antibiotic. Other possible causes of resistance, which cannot be excluded on the basis of the present data, are discussed.

The role of sterols in membranes is not yet understood. Since sterols have not been found in bacteria and blue-green algae (Levin and Bloch, 1964), they are obviously not essential for all membrane systems. Except for these microorganisms, however, sterols are ubiquitously distributed in nature. In the cells which do contain sterols, they apparently serve an indispensable function in the cytoplasmic membrane, because low concentrations of sterol-complexing agents produce marked permeability alterations. The hemolytic activity of saponins, e.g., digitonin, may be cited as a classic example.

Several studies indicate that the polyene antibiotics probably act in a manner similar to the saponins. These antibiotics induce permeability changes, characterized by the loss of essential cell constituents, in molds (Kinsky, 1961), yeasts (Gottlieb et al., 1961; Lampen, 1962), and protozoa (Ghosh, 1963; Kusel and Weber, Biochim. Biophys. Acta, *in press*). Polyene-sensitive organisms bind nystatin, whereas cells, protoplasts, and particulate fractions of polyene-insensitive bacteria do not. The evidence which implicates the involvement of ergosterol at the polyene-binding sites in *Neurospora crassa* and *Saccharomyces cerevisiae* has been recently reviewed (Kinsky, 1964). On this basis, it was originally suggested that the selective toxicity of these antibiotics was due to interaction with a component (sterol) present only in the membrane of sensitive organisms (Kinsky, 1962*a*; Lampen et al., 1962).

The demonstration that mammalian erythrocytes are lysed in isotonic saline by several of the polyene antibiotics is in accord with this hypothesis and led to the prediction that other cells with sterols in the cytoplasmic membrane, particularly some strains of *Mycoplasma*, also should be polyene-sensitive (Kinsky et al., 1962). These *Mycoplasma* are especially interesting in this regard, because, while they possess many morphological

and biochemical properties in common with bacterial spheroplasts and L-forms, they do contain sterols (Kleineberger-Nobel, 1962). Lampen and co-workers (1963) recently showed that growth of *M. gallisepticum* is inhibited by filipin and amphotericin B. *M. gallisepticum*, however, will not grow in the absence of sterol, and therefore is not a suitable strain for critical examination of the "sterol hypothesis." *M. laidlawii*, on the contrary, does not synthesize sterol, but will grow almost equally well in the presence and absence of a sterol such as cholesterol. When grown in the presence of cholesterol, the cells rapidly accumulate the sterol. Because the *Mycoplasma* cultured in this manner are lysed by digitonin, Smith and Rothblat (1960) have concluded that cholesterol is functionally incorporated into the cell membrane. Accordingly, if the basis for the selective toxicity of the polyene antibiotics described above is correct, these agents should inhibit growth and induce lysis of *M. laidlawii* grown in the presence of sterol, but have no effect on cells grown in its absence.

MATERIALS AND METHODS

Growth of the organism. *M. laidlawii*, strain A, was obtained from the American Type Culture Collection. The growth medium, described in Table 1, was devised by combination of some of the constituents recommended by Razin and Knight (1960) and Smith (1963). When indicated, Difco pleuropneumonia-like organism (PPLO) serum fraction was added as the source of cholesterol. In medium devoid of serum fraction, sterol could not be detected by Liebermann-Burchard, $FeCl_3$, or digitonin assay. The rate of growth was essentially the same in media with or without serum fraction. Growth was quantitated by determination of the absorbancy at 550 mμ in a Zeiss PMQII spectrophotometer by use of 1-ml cuvettes with a 1-cm light path. Uninoculated medium was used in the reference cuvette.

Determination of antibiotic sensitivity. A 24- to 48-hr culture with an absorbancy of approximately 0.25 was diluted 1,000-fold with fresh medium. Screw-capped tubes (16 × 125 mm) containing 5 ml of medium and filipin, as indicated, were inoculated with 0.1 ml of the diluted cellular suspension and incubated at 37 C without shaking for various time periods.

Kinetics of cell lysis. Cells were grown for approximately 36 hr in 125-ml screw-capped flasks containing 50 ml of medium. The organisms were harvested by centrifugation at 4 C (approximately 7,500 × *g* for 15 min) and washed twice with cold fresh medium. All cells, whether cultivated in the presence or absence of the serum fraction, were washed with serum-free medium. They were then resuspended in this medium to give an absorbancy of approximately 0.5 and kept at the indicated temperature (see Results). Cell lysis was measured, in 3-ml cuvettes having a 1-cm light path, by the decrease in absorbancy at 550 mμ, by use of a Bausch and Lomb 505 spectrophotometer equipped with a constant speed Varicord No. 43 recorder. Reference cuvettes contained medium.

TABLE 1. *Medium for Mycoplasma laidlawii strain A**

Constituent	Final concn
Tryptose	2.0 g
NaCl	0.5 g
Glucose	0.5 g
Fraction V albumin	1.0 g
Sodium acetate	75 mg
Deoxyribonucleic acid (DNA)	5 mg
Ribonucleic acid (RNA)	5 mg
Deionized water	100 ml
Penicillin	200 units/ml
Difco PPLO serum fraction†	1.0% (v/v)

* Tryptose, NaCl, and sodium acetate were dissolved in deionized water, the *p*H adjusted to 8.3, and sterilized at 120 C for 15 min. Glucose was autoclaved separately and added aseptically. DNA and RNA, sterilized as indicated in the text, and penicillin were added aseptically to the autoclaved medium.

† Added when indicated in Results.

Materials. Sperm deoxyribonucleic acid and yeast ribonucleic acid, obtained from Calbiochem, Los Angeles, Calif., were dissolved in deionized water to a concentration of 5 mg/ml, and the solutions were sterilized by steaming for 20 min. Bovine Fraction V albumin (Armour and Co.) was dissolved in deionized water (100 mg/ml), the *p*H adjusted to 8 with KOH, and sterilized by Seitz filtration. Filipin was generously supplied by G. M. Savage of The Upjohn Co., Kalamazoo, Mich. Stock solutions were prepared with dimethylformamide (DMF) and stored at −20 C. Stock solutions of digitonin (Sigma Chemical Co., St. Louis, Mo.) were prepared with 50% ethanol.

RESULTS

Effect of growth in the absence and presence of serum fraction on filipin sensitivity. Filipin had no effect on the growth of *M. laidlawii* when cells, which had been previously cultivated in the absence of serum fraction, were used as inoculum (Table 2). However, when cells, which had been grown in the presence of serum, were employed, the antibiotic had a marked influence (Table 3). After 36 hr of incubation, growth was significantly depressed by concentrations of filipin as low as 5 μg/ml, and, at 40 μg/ml, the antibiotic produced almost complete growth inhibition.

In both experiments, the absorbancy of the cultures decreased from the maximum after 76 hr

TABLE 2. *Effect of filipin on cells previously grown in absence of sterol**

Compound	Concn	Absorbancy at 550 mμ		
		36 hr	48 hr	76 hr
	μg/ml			
No addition	—	.099	.254	.164
Dimethylformamide (DMF)	†	.100	.249	.169
Filipin	5	.105	.251	.164
Filipin	10	.118	.239	.169
Filipin	20	.095	.256	.160
Filipin	40	.096	.259	.169

* Organisms used were grown in absence of PPLO serum fraction and inoculated into similar medium for determination of antibiotic sensitivity.

† All concentrations of filipin (dissolved in DMF) used were added in 0.01-ml amounts, and, therefore, 0.01 ml of DMF was added as a control.

TABLE 3. *Effect of filipin on cells previously grown in presence of sterol**

Compound	Concn	Absorbancy at 550 mμ		
		36 hr	48 hr	76 hr
	μg/ml			
No addition	—	.114	.302	.203
Dimethylformamide	†	.122	.303	.201
Filipin	5	.097	.296	.194
Filipin	10	.043	.256	.198
Filipin	20	.024	.187	.198
Filipin	40	.018	.105	.203

* Organisms used were grown in presence of PPLO serum fraction and inoculated into medium lacking the serum fraction for determination of antibiotic sensitivity.

† See Table 2 for concentration.

of incubation. During this interval, the *p*H of the medium had dropped from an initial value of 8.3 to 6. It seems quite probable that the decline in optical density was due in part to cell lysis induced by the acidic conditions resulting from glucose fermentation by the organism.

The above experiments demonstrate that growth of *M. laidlawii* in the presence of serum fraction conferred filipin-sensitivity to the cells. Because the serum fraction contains a high content of cholesterol, it was assumed that these results were due to the presence of sterol in the medium used to cultivate the inoculum. This was confirmed by growing cells in medium containing 20 μg/ml of cholesterol as a substitute for the serum fraction and subsequently testing for filipin sensitivity. Results, similar to those described in Table 3, were obtained.

Selection of filipin-resistant cells. Table 3 indicates that the effect of filipin was less pronounced after 48 hr of incubation. Thus, the antibiotic, at a concentration of 10 μg/ml, produced 65% growth inhibition after 36 hr, but only 15% after 48 hr. This apparent reversal of filipin inhibition was observed over a wide range of antibiotic concentration, and, for reasons cited in the Discussion, was probably not due to inactivation of the antibiotic. A more plausible explanation is that in the beginning, filipin killed a significant portion of the cells and that the subsequent growth was due to the emergence of an antibiotic-resistant cell population. Preliminary experiments did, in fact, show that incubation with filipin for 24 to 36 hr resulted in a reduction of the number of viable cells when the organism was plated out on medium (Table 1) containing 1.5% agar. The existence of filipin-resistant *Mycoplasma* was established in the following manner. Cells were cultivated in the presence of serum in media containing no filipin or high concentrations of the antibiotic (40 μg/ml). Growth was delayed by the antibiotic, but cultures were incubated for sufficient time to achieve reversal of growth inhibition. After maximal growth had been obtained, each of these cultures was again tested for antibiotic sensitivity. Table 4 shows that cells grown in the presence of serum, but in the absence of filipin, were sensitive to the antibiotic. However, cells which had been grown with serum and exposed to filipin were now completely resistant to the antibiotic.

Effect of filipin on growth in media containing serum. In the above experiments, filipin sensitiv-

TABLE 4. *Selection of filipin-resistant Mycoplasma*

History of inoculum	Conditions of assay*	Absorbancy at 550 m		
		25 hr	41 hr	48 hr
Cells grown with serum fraction, never exposed to filipin	No addition	.072	.243	.208
	Filipin, 40 μg/ml	.000	.036	.104
Cells grown with serum fraction and 40 μg/ml filipin	No addition	.099	.224	.198
	Filipin, 40 μg/ml	.078	.247	.207

* In both cases cells were transferred to media devoid of serum fraction after maximal growth had been attained.

ity was determined by growing *M. laidlawii* in the presence of serum, and then transferring appropriate dilutions of these cells to serum-free medium. This procedure was employed because previous investigation had demonstrated that the hemolytic activity of the polyene antibiotic, amphotericin B, was markedly inhibited by serum (Kinsky, 1963). It was suggested that this latter effect may be a consequence of antibiotic binding by a component in the serum (probably sterol) which would result in a reduction of the effective free polyene concentration. This could account for the previous inability to demonstrate growth inhibition of *Mycoplasma* by low concentrations of these antibiotics (Kinsky, 1963, 1964). If this explanation is applicable, then the concentration of filipin required to inhibit growth of *M. laidlawii* in medium containing serum should be significantly greater than the amount necessary in medium devoid of serum. Table 5 shows that after 28 hr of incubation, 5 μg/ml of filipin inhibited growth by 66% in serum-free medium, whereas in medium supplemented with serum, a filipin concentration of 20 μg/ml was necessary to achieve a similar growth inhibition. The results after 40 hr clearly indicate that in medium containing serum, complete growth occurred in all of the tubes with filipin, although greater than 50% inhibition by 10 μg/ml was still exhibited in medium lacking serum.

Lysis of M. laidlawii induced by filipin and digitonin. The preceding experiment suggests that the sterol present in the serum binds filipin, thus making it unavailable for reaction with cellular sterol. It seems quite probable, as results obtained with other organisms have indicated (see introduction), that the primary site of filipin, action in *M. laidlawii* is the cell membrane. As a consequence of antibiotic interaction with sterol localized in the cell membrane, sensitive organisms manifest an altered permeability, ultimately culminating in cell death. If this hypothesis is valid, then filipin would be expected to induce a rapid lysis of *M. laidlawii*, but only with cells which have been previously grown in the presence of serum as a source of sterol. Fig. 1A shows that cells grown without serum fraction did not lyse upon exposure to filipin. Under these conditions, sterol was obviously not present in the cell membrane, because digitonin also had no effect. However, filipin caused a rapid lysis of cells which had been previously grown in the presence of sterol (Fig. 1B). Lysis by 17 μg/ml of filipin was not complete, because a further absorbancy decrease resulted upon subsequent addition of digitonin. Filipin, at a concentration of 67 μg/ml, caused all of the susceptible cells to lyse,

TABLE 5. *Effect of serum fraction on filipin sensitivity of Mycoplasma laidlawii grown previously in presence of sterol**

Conditions of assay medium	Additions	Concn	Absorbancy at 550 mμ		
			28 hr	40 hr	64 hr
		μg/ml			
Serum fraction absent	None	—	.082	.275	.195
	Dimethylformamide (DMF)	†	.084	.284	.198
	Filipin	5	.028	.206	.190
	Filipin	10	.012	.104	.189
	Filipin	20	.006	.048	.206
	Filipin	40	.001	.029	.241
Serum fraction present	None	—	.103	.221	.156
	DMF	†	.096	.219	.150
	Filipin	5	.100	.230	.164
	Filipin	10	.092	.237	.154
	Filipin	20	.044	.261	.159
	Filipin	40	.025	.232	.147

* Cells which were cultivated in the presence of PPLO serum fraction were used as inocula.

† See Table 2 for concentration.

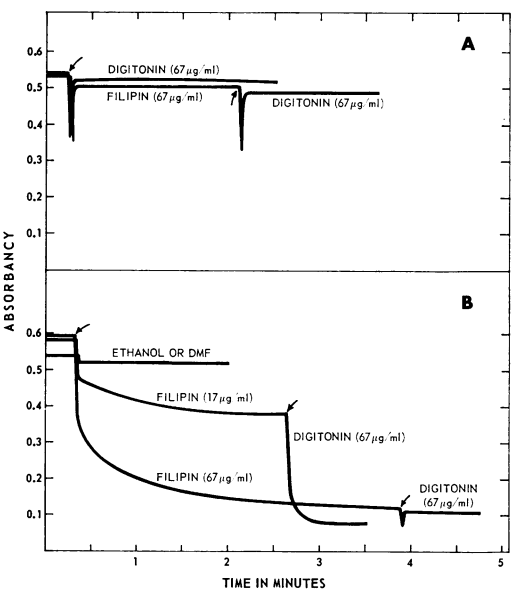

FIG. 1. *Effect of filipin and digitonin on lysis of Mycoplasma laidlawii. Cells used were grown in absence of serum (A) and presence of serum (B). At the times indicated by the arrows, either filipin, digitonin, or the appropriate solvent controls were added. The solvent controls contained 0.08 ml of 50% ethanol or absolute dimethylformamide. Concentrations greater than 67 μg/ml of filipin or digitonin did not result in more extensive lysis.*

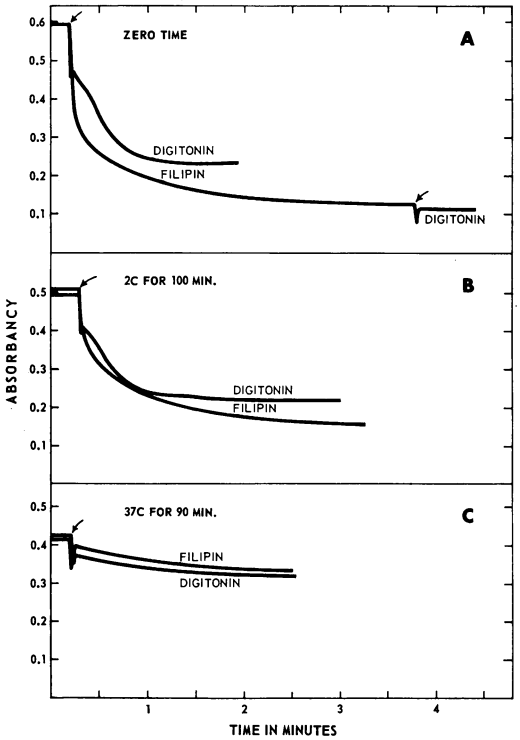

FIG. 2. *Effect of temperature on sensitivity of Mycoplasma laidlawii to lysis by filipin and digitonin. Cells used were grown in the presence of serum, harvested, and washed as indicated in the text, and incubated at 2 and 37 C for the times shown. At the times indicated by the arrows, filipin and digitonin (final concentration: 67 μg/ml) were added.*

and addition of digitonin had no further effect. Lysis was not observed with either of the solvent controls.

Effect of temperature on sensitivity of cells to lysis by filipin and digitonin. In the experiment just described, the cells were kept at approximately 25 C, and it was observed that after 2 hr the organism was no longer sensitive to the lytic action of either filipin or digitonin. This phenomenon could be almost completely circumvented by maintaining the cells at 2 C. At "zero time," *M. laidlawii* was rapidly lysed by filipin and digitonin (Fig. 2A). After 100 min of incubation at 2 C, the cells were still sensitive to the action of these agents, but cells which had been incubated for the same time period at 37 C had become almost fully insensitive to the lytic activity of filipin and digitonin (Fig. 2B and 2C). Antibiotic resistance was not due to renewed growth during incubation in the sterol-free medium. In fact, the absorbancy of the cultures declined slightly at both temperatures.

DISCUSSION

Lampen and co-workers (1963) have shown that growth of *M. gallisepticum* was inhibited by several polyene antibiotics. This particular strain of *Mycoplasma* has an absolute growth requirement for sterol. Polyene antibiotics are known to complex with sterols, and it has been suggested (Smith, 1964*a*) that inhibition occurred because the antibiotic level was sufficiently high to cause a deficiency of exogenous sterol. To test this possibility, the effect of the polyene antibiotic, filipin, on *M. laidlawii* was examined. This species was employed, because, though incapable of synthesizing sterol, it grows equally well in the presence or absence of cholesterol. Smith and Rothblat (1960) have shown that, when grown with cholesterol, this organism will incorporate the sterol into the cell membrane. The experiments demonstrate that filipin inhibits growth and causes lysis of *M. laidlawii* only when the cells have been cultivated in the presence of sterol. These results constitute the strongest evidence yet reported for the contention that the presence of sterol in the cell membrane is a necessary prerequisite for polyene sensitivity (Kinsky, 1962*a;* Lampen et al., 1962).

Evidence has also been obtained for the existence of a filipin-resistant population of *Mycoplasma* grown in the presence of sterol. The basis for this resistance must still be established, and could be due to several factors. (i) The resistant cells may have elaborated a mechanism, e.g., an enzyme, which leads to inactivation of the antibiotic. This alternative appears unlikely, because filipin, at a concentration of 40 μg/ml, had no apparent effect on growth of the resistant population (Table 4), whereas approximately the same amount of antibiotic induced lysis of sensitive cells within 2 min (Fig. 1B). Thus, destruction of the antibiotic must occur at an extremely rapid rate if this is the cause of resistance. (ii) The resistant cells either may be unable to concentrate the sterol and incorporate it into the cell membrane, or, if they do incorporate it, the sterol is no longer accessible to filipin. It is also possible that resistant cells may convert cholesterol to a form which is incapable of reacting with filipin and can no longer occupy a functional position in the membrane. Rothblat and Smith (1961) and Smith (1964*b*) have shown that certain strains of *Mycoplasma* can convert cholesterol to glucoside derivatives and that specific structural features are required in the sterol molecule to "fit" in the appropriate space of the cell membrane. Some support for this alternative comes from the observation that filipin-sensitive cells gradually become resistant to the

lytic action of the antibiotic if they are kept at 37 C in media devoid of sterol. Resistance to the lytic action of filipin was largely prevented by maintaining the cells at 2 C. Loss of sensitivity to filipin was accompanied by a decreased lytic activity of digitonin, as would be expected if these two agents had a common site of action.

The action of the polyene antibiotic, nystatin, on PPLO has been studied by Razin (1963), who compared the antibiotic-binding capacity of various strains of *Mycoplasma*, yeast, and bacteria which were cultured in the presence of Difco PPLO serum fraction as a source of cholesterol. Although significant and nearly comparable amounts of nystatin were absorbed by all these microorganisms, only growth of the yeast (*Candida albicans*) was inhibited by low concentrations of the antibiotic. It was thus concluded that differences in the ability of microorganisms to bind polyene antibiotics may not suffice to explain the selective toxicity of these agents. The evidence on which this conclusion is based may be questioned for several reasons. First, as Razin suggests, the appreciable nystatin-binding by *Escherichia coli* and *Staphylococcus aureus* undoubtedly was due to growth in the cholesterol-rich medium. However, there is no reason to believe that the sterol had been incorporated as part of the cell membrane structure, and, accordingly, the inability of nystatin to inhibit growth is not unexpected. Several attempts in our laboratories to sensitize *Bacillus megaterium* and *Mycobacterium phlei* to the action of digitonin and filipin by growing them in the presence of cholesterol have consistently met with failure, although sterols were "absorbed" by the cells. Secondly, nystatin is a relatively weak polyene antibiotic, compared with filipin and amphotericin B. This quantitative difference among the various antibiotics has been observed with yeast (Lampen and Arnow, 1963), *Neurospora* protoplasts (Kinsky, 1962*b*), and mammalian erythrocytes (Kinsky et al., 1962; Kinsky 1963), and it was the main reason that the present experiments were carried out with filipin. In this regard, it seems quite significant that Lampen and coworkers (1963) have shown that the growth of *M. gallisepticum* was not inhibited by nystatin concentrations as high as 100 μg/ml, but was completely blocked by low concentrations of filipin and amphotericin B.

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