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ANTIBIOTIC RESISTANCE OF MYCOPLASMA

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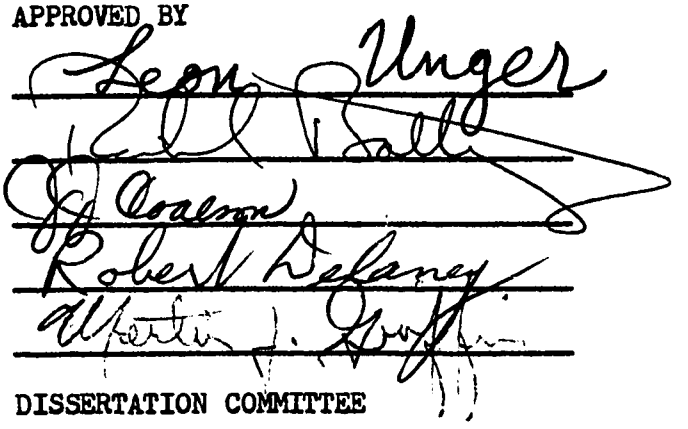

DISSERTATION COMMITTEE

This dissertation is dedicated to my parents, Audrey and J. E. Schneider, who have supported me in every way possible and who inspired me to love Truth.

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CHAPTER I

INTRODUCTION

The research reported in the following pages deals with the unique ability of mycoplasmas to resist the cidal activity of antibiotics and to adapt and grow in their presence. The question asked is: what is the basis for mycoplasmas' resistance to antibiotics?

Characteristics of Mycoplasma

Description of Mycoplasmas

Classification. The term mycoplasma refers to a group of prokaryotic organisms without cell walls comprising the order Mycoplasmatales in the recently created class Mollicutes (34). Thus, these organisms, formerly known as PPLO (pleuropneumonia-like organisms), now occupy a rank of classification equivalent to bacteria, blue-green algae, and the rickettsia and viruses (48). The fact that the majority of mycoplasmas contain sterol in their membrane, as do eukaryotes, and their lack of cell walls stimulated the argument that mycoplasmas should be reclassified from the plant kingdom to the animal kingdom (50). The family mycoplasmataceae contains two genera that are defined by their sterol growth requirements: the genus Mycoplasma, which requires sterol, and the genus Acholeplasma, which does not require sterol for

growth. Other distinguishing characteristics among mycoplasma groups are the requirement for urea, possessed by T-strain mycoplasmas, and the thermophilic, acidophilic nature of a recently isolated strain of mycoplasma (26).

Identification. The strict requirement an organism must meet in order to be defined as a mycoplasma is the ability to form, on solid growth medium, colonies with a "fried egg" morphology (49). This fried egg appearance of the mycoplasma colony is due to the penetration into the agar of a central portion of the colony that is dense in mycoplasma cells, with less dense growth at the agar's surface surrounding the central growth. Whittlestone (125) distinguishes umbonate morphology from the fried egg morphology of mycoplasma: umbonate refers to colonies with raised centers, whereas the center of fried egg colonies grow down into the medium.

Other identifying characteristics (48) of mycoplasma are their extremely small size (discussed below) and the lack of a cell wall, which accounts for the polymorphism of individual cells, their susceptibility to osmotic lysis, their resistance to penicillin, and their ability to squeeze through filters of pore sizes smaller than the cell size of the mycoplasma (85). Mycoplasma are also inhibited by their specific antibodies (33).

The smallest free-living organisms. A fascinating feature of mycoplasmas is that they are the smallest free-living organisms. The irreducible amount of DNA required by a self-replicating organism would appear to be around 5×10^8 molecular weight (3). According to Maniloff (69), if this amount of DNA were enclosed in a unit membrane

with minimal hydration (one gram H_2O per gram DNA) the resulting form would be 0.15 nm in diameter, theoretically the smallest cell possible. The other cell components that should be necessary for cellular functions would raise the size limit to 0.2 nm in diameter. A mycoplasma is known that is within a factor of 2 (a factor of approximately 10 in total mass) of the theoretically smallest size possible (68). The organism is Mycoplasma Sp. kid.

Limited complexity. Simplicity of mycoplasmal structure is reflected by their small genome sizes with low G + C content in mycoplasmal DNA ranging from 23 to 41 percent (76). DNA of Acholeplasma species have 30 - 36 percent G + C, mycoplasma species (except for M. pneumoniae) have a 23 - 36 percent G + C, and M. pneumoniae, a 39 to 41 percent G + C. The T-strain mycoplasmas studied by Bak and Black (2) all had G + C content equal to about 28 percent. As determined by renaturation studies, sizes of the DNA genome are in the range of 4.2 to 6.9 x 10^8 daltons for mycoplasma, and around twice that size for Acholeplasmas with chromosome weights of 7.6 to 11.0 x 10^8 daltons (3). Morowitz, by setting an average cistron equal to 800,000 daltons (estimated by assuming that an average cistron encodes a protein of 40,000 molecular weight, and using a coding ratio of DNA to protein of 20:1), calculated the number of cistrons in mycoplasma to range from 600 to 1,000 per cell. This compares with about 5,000 cistrons for the genome of E. coli and points to the limited complexity of mycoplasma (73). A demonstration of this is that Mycoplasma sp (Kid) was shown to have only one cistron each for the 16S and 23S rRNA species, and only about 44 cistrons for tRNA molecules (99). E. coli has 5 cistrons, each for

its 16S and 23S rRNA.

Simple membrane. One other unique feature of mycoplasma is their simple outer membrane. According to Razin (84) the majority of evidence favors a membrane structure of the subunit type (43), as opposed to the model proposed by Danielli and Davson (25), which suggests a bimolecular leaflet of lipid coated on both sides with protein. The subunit model for membrane structure consists of repeating lipoprotein subunits that interact among themselves, possibly through hydrophobic bonding, to result in an organized arrangement of units in a structure which comprises the membrane.

Significance of Mycoplasma

Although 14 of the 36 named mycoplasma species in the class Mollicutes are known plant or animal pathogens (35), only one of them, Mycoplasma pneumoniae, is known to be the cause of a human disease, primary atypical pneumonia. Due to their ubiquitous, albeit inconspicuous, distribution in nature as normal flora, and the lack of optimum conditions for their detection (49), mycoplasmas are suspected of being involved in more pathogenesis than is recognized.

Human clinical entities in which mycoplasmas have been suggested to play a role are include leukemia, periodontal disease, rheumatoid arthritis, autoimmune diseases, and Reiter's syndrome (49). In addition, their ever-present threat to cell cultures in the form of contamination has brought attention to their ability to alter the results of experiments and to resist elimination by antibiotic treatment. The following brief discussion of attempts to understand the pathogenicity of M. pneumoniae helps underscore the possible manifestations of

mycoplasma-host relationships.

Primary atypical pneumonia. The agent shown to be the etiological agent of primary atypical pneumonia (13) was determined to be a mycoplasma by Hayflick (47). The syndrome of atypical pneumonia is also associated with viral etiological agents such as influenza A, adenoviruses, B virus, parainfluenza type 3, and respiratory syncytial virus. The mycoplasma pneumonia disease caused by Mycoplasma pneumoniae can be distinguished by the production of cold agglutinin antibodies produced in the host (81).

The prevalence of respiratory disease caused by Mycoplasma pneumoniae has brought about a concerted effort to discover the mechanism of pathogenesis of this mycoplasma.

Autopsies of fatal cases have demonstrated that the ciliated respiratory epithelium is the target of the disease (79). However, since the disease is usually self-limiting (30), observation of human lung material is rarely available. Therefore, several host-parasite systems have been designed. One of them involves the interaction between M. pneumonia and the erythrocyte, producing hemolysis (15) shown by Somerson to be caused by production of a peroxide (115). The existence of a cell attachment phenomenon was shown by the adsorption of M. pneumonia colonies to erythrocytes (29) and the development of the direct hemagglutination reaction by Feldman and Suhs (37). It was subsequently shown by Sobeslavsky et al. (114) that M. pneumoniae attaches to neuraminic acid receptors of various cells. The combination of peroxide secretion and host cell-attachment may be important determinants of the virulence of M. pneumoniae (17). That such a mechanism

is not the only one to be considered in pathogenesis is indicated from results demonstrating no quantitative differences in peroxide production in a virulent and an avirulent strain of M. pneumoniae (18).

Another system that has been very useful in studying the pathogenesis of atypical pneumonia is the trachea of either the human or hamster in organ culture (18, 20). Tracheae from both sources show the interference of M. pneumoniae with normal ciliary activity and epithelial damage which includes disruption of the terminal bars, exposing the tracheal lumen to the intercellular space. Woodruff et al. (126) correlated the cessation of hamster tracheal ciliary beating with the number of M. pneumoniae organisms present, and also, in electron microscope studies, noticed a marked increase in the number of lysosomes in macrophages. The altered forms of many of the lysosomes suggested ingestion of M. pneumoniae. In human trachea, Collier and Clyde (19) noticed that specialized structures of M. pneumoniae are involved in the attachment site to the target cell membrane. The specialized structure contains an electron dense core surrounded by a clear zone and bound by the organism's membrane (16). A similar electron dense "terminal structure" has been described in M. gallisepticum, which is thought by Maniloff (68) to be the region containing the growing point for DNA replication in that organism.

In attempting to recognize pathogenicity markers, investigators have also exploited the fact that M. pneumoniae tends to lose its virulence when subcultured using solid or liquid, cell-free medium (23,109). In addition, Lipman and Clyde (63) were able to reverse the phenomenon when they restored an attenuated strain to full

virulence by 8 successive intranasal passages in hamsters. It was also shown (64) that of the characteristics monitored (growth, glycolysis, protein electrophoretic patterns, peroxide formation, morphology and cytadsorption) changes in only two characteristics, morphology and cytadsorption (adhesion of the mycoplasma cell to a target cell), could be correlated with total loss of virulence. Both virulent and attenuated strains, when examined by light microscopy, showed smooth, refractile, spherical clusters up to 400 nm in diameter, while the two completely avirulent strains showed clusters of organisms that were smaller, amorphous, granular and non-refractile. These strain differences could not be seen by electron microscopy. Also, they were stable and may represent mutations. Other possible explanations for the pathogenicity of M. pneumoniae infection include mechanical interference with normal beating of the cilia, disturbance of the surface charges necessary for the synchronization of ciliary motion, nutritional deprivation of host cells due to competition for metabolites or membrane injury, and cell damage, caused by antigen-antibody-complement reactions at the surface of epithelial cells that are associated with infecting mycoplasma (19,21,125).

Animal and plant diseases. In addition to the threat of mycoplasma in the area of human disease, mycoplasmas cause a great deal of economic loss as etiological agents of animal and plant diseases. In animals, the major diseases are contagious bovine pleuropneumonia (CBPP), contagious caprin pleuropneumonia (CCPP), and contagious agalactia of sheep and goats. These diseases are caused by (respectively) M. mycoides var mycoides, M. mycoides var capri and M. agalactia

(22). Mycoplasma infections most frequently involve the respiratory and, to a lesser extent, the urogenital system of animals.

In plants, over 50 disorders classed as "yellows" diseases are suspected of having a mycoplasmal etiology. These diseases are transmitted by insect vectors and in plants are characterized by yellowing of the leaves, phyllody and virescence of flowers, general stunting and proliferation of axillary shoots (28). In two yellows diseases, corn stunt disease and mulberry dwarf disease, evidence based on electron microscope studies and suppression of the disease by tetracycline has led to the hypothesis that these diseases are caused by mycoplasmas (28, 31). Based on the observation that yellows diseases resemble gibberelic acid (a plant growth-promoting substance) deficiency and the report that symptoms of corn stunt disease can be partly reversed by the application of gibberelic acid (70), Lemcke has suggested a pathogenic mechanism (61). Since plant gibberellins and sterols share a common biosynthetic pathway from acetate to farnesyl pyrophosphate (6), sterol-requiring mycoplasmas could possibly deplete a host plant of necessary intermediates for gibberellic acid synthesis. Hendrikson and Smith (51) have shown that among mycoplasmas there are at least two types of pathways, as represented by A. laidlawii, B and avian strain J, that possess the biosynthetic step in gibberellic acid synthesis that converts isopentylpyrophosphate to γ,γ-di-methylallyl pyrophosphate.

Tissue culture contamination. The frequency of tissue culture contamination over a ten year period ending in 1971 ranged from 12 to 87 percent of the cultures tested (116). Mycoplasmas in cell cultures have

caused biochemical changes in host-cell metabolism, altered cell growth and morphology, karyologic changes, and effects on viral synthesis (116). Mycoplasmas' requirements for nucleic acid precursors and amino acids, particularly arginine, are a probable cause for most of the effects seen in tissue culture. Requirements for nucleic acid precursors can be met by supplying the free bases, nucleosides, oligonucleotides, DNA or RNA, depending on the mycoplasma involved (83, 88, 89 and 90). Mycoplasmas are known to possess nucleases which provide their capability to utilize nucleic acid polymers (75, 82, 91). The most important amino acid depletion leading to cell changes or death in tissue cultures is that of arginine. Nonfermenting strains of mycoplasma utilize large amounts of arginine as a major energy source (100). The pathway responsible for arginine degradation in mycoplasma is the arginine dihydrolase pathway (5) and the end products of the pathway are ornithine and one mole of ATP per mole of arginine (100). In M. hominis, 14 percent of the soluble protein of cell extracts consisted of enzymes of the arginine dihydrolase pathway. A specific effect of arginine depletion by mycoplasmas has been observed in the replication of adenoviruses (97). An arginine dependent step appears to occur in the assembly of the virus and involves a component of a virus capsid antigen, called P antigen (98). A close mycoplasma-cell association is required, in many instances, for viability of contaminating mycoplasmas which indicates mycoplasmas have mechanisms for deriving nutrients from their hosts that are not supplied by the cell-free tissue culture medium (117). Another result of the association of mycoplasmas with tissue culture cells is the probable role it plays in protecting mycoplasma from

the action of antibiotics and specific antisera (5, 52).

Antibiotic Resistance of Mycoplasmas

Mycoplasmas *in vivo*

In man. The unique ability of mycoplasmas to resist eradication by chemotherapy is most dramatically demonstrated by *M. pneumoniae* infection and its treatment. Although the *in vitro* sensitivity of this mycoplasma to tetracycline and erythromycin is great (30), these antibiotics fail to eradicate *M. pneumoniae* from patients suffering from atypical pneumonia. Tetracycline may shorten the clinical course of the disease, but it neither cures the symptoms completely, nor abolishes the carrier state (104). Balassarian and Robbins (4) found that despite therapeutic doses of tetracycline, many patients harbored the mycoplasmas four to six weeks. *M. pneumoniae* cultured before and after antibiotic therapy had the same sensitivity to the tetracycline. Tetracycline did not eradicate the organism from the throat, decrease the frequency of its isolation, or shorten the length of the carrier state. Thus, although the morbidity of *M. pneumoniae* infections is effectively reduced by treatment with tetracycline and erythromycin, since the mycoplasmas are not eradicated by these antibiotics, antibiotics currently available do not seem to be the answer to controlling these infections (30).

Other results concerning the response of mycoplasma infections in man to antibiotic therapy involves the treatment of nongonococcal urethritis. The etiological role of T-strain mycoplasmas is suggested

by the fact that erythromycin treatment is successful in patients with nongonococcal urethritis and this can be correlated with the sensitivity of T-strains to erythromycin. In such patients, T-strains cannot be isolated from patients successfully treated, but when the antibiotic treatment fails, T-strain mycoplasmas can be isolated (39). The latter instance suggests the existance of T-strain variants resistant to the antibiotic used in the treatment. In contrast to results of antibiotic therapy for M. pneumoniae infection, eradication of T-strains has been demonstrated in cases where men and women were treated with tetracycline (105). M. hominis also has been isolated from patients with nongonococcal urethritis, but this mycoplasma is resistant to erythromycin, showing that mycoplasmas vary in their sensitivities to antibiotics (106).

In animals. Numerous examples exist of mycoplasmal infections in animals that resist eradication by antibiotic treatment. Infectious synovitis in chickens and turkeys is caused by M. synoviae and results in swollen joints, bursae and synovial sheaths, splenomegaly and an enlarged, discolored liver (12). Feed containing chlortetracycline will prevent the development of new cases of infectious synovitis as long as it is administered, but this treatment can neither cure diseased birds nor eradicate the organism from affected birds (36). Mycoplasma gallisepticum which causes chronic respiratory disease in chickens and turkeys, is sensitive to many antibiotics. Although antibiotic treatment alleviates the symptoms of the disease, the mycoplasmas are not eliminated and the birds are susceptible to recurrence and the carrier state (36). However, treatment of eggs carrying M. gallisepticum with Tylosin

results in elimination of the ability of the eggs to transmit the infection (62). Finally, treatment of very early clinical cases of acute synovitis-arthritis in swine -- caused by M. granularum -- with either Tylosin or lincomycin usually alleviates disease symptoms. Treatment is usually not satisfactory, however, if begun later than 72 hours after the first symptoms (121).

Mycoplasmas in vitro

Mycoplasmas in cell cultures. Mycoplasmas have been eliminated from cell cultures by treatment with kanamycin, tetracyclines, tylosin, erythromycin and lincomycin (116). However, in addition to the ability of mycoplasmas to develop resistance (80), the observation of mycoplasma-like forms by electron microscopy in tissue cultures supposedly cured of mycoplasma with tetracycline (52) indicates the failure of antibiotic treatment of mycoplasmal contamination. Cross et al. (24) were unable to culture mycoplasma from their tissue culture system following treatment with aureomycin, but succeeded in isolating them from disrupted cell pellets. Such results indicate an extremely close association of mycoplasma with cells -- perhaps an intracellular association -- which contributes greatly to mycoplasmas' resistance to complete elimination by antibiotic treatment. Except for instances where mycoplasmas have been observed in cytoplasmic vacuoles, or free in the cytoplasm of necrotic cells (127), most electron microscopy studies give evidence for an extracellular attachment of the mycoplasma to the cell membrane (116). It is also possible that cytoplasmic extensions of cells surround mycoplasmas and antibiotics are unable to establish contact with the mycoplasma in its sequestered environment (38). Another suggestion is that

mycoplasmas that are able to form microcolonies on a particular cell surface might allow protection for cells in the center of the microcolony. Also, a mycoplasma's sensitivity to an antibiotic could be related to its rate of metabolism; the slower the metabolism, the less sensitive to the antibiotic (86).

Cell-free cultures of mycoplasmas. In broth cultures containing 20 percent horse serum, Jao and Finland (54) determined the susceptibility of 5 strains of M. pneumoniae to 21 commonly used antibiotics. The 5 strains were all similar in their response to the antibiotics. Erythromycin was distinguished as being the most potent antibiotic in terms of both growth inhibition and mycoplasmacidal activity. From an initial titer of around 1.0×10^6 colony forming units per ml, 10 µg/ml of erythromycin in the culture resulted in complete mycoplasmacidal activity within 6 hours. As expected, penicillin was ineffective. Cephaloridine, cephalothin and polymixin B were inhibitory only at high concentrations. Oleandomycin, 7 analogues of tetracycline, chloramphenicol, 3 glycoside antibiotics (streptomycin, gentamicin, and kanamycin) and lincomycin were all mycoplasmacidal in concentrations of 12.5 µg/ml or less. Dimethylchlortetracycline and tetracycline were the most effective and chlortetracycline the least active of the tetracyclines tested. The minimal inhibitory (M.I.C.) and minimal cidal (M.M.C.) concentrations of chlortetracycline were greatly increased as the inoculum size increased. The M.I.C. and M.M.C. of tetracycline were moderately increased and, of erythromycin, unaffected over the same 10,000-fold range of inoculum tested. From these data the correlation can be made that increased activity of antibiotic

action is associated with lack of influence by the number of organisms in the culture. At low concentrations (2.5 μg/ml or less) the activity of the tetracyclines is primarily mycoplasma static. In other studies with M. pneumoniae, Larin et al. (60) observed that the amount of serum in the culture can determine whether the nature of the activity of oxytetracycline, tetracycline or methacycline will be static or cidal. The normal 20 percent (v/v) horse serum allowed only mycoplasmacidal activity by 2.5 μg/ml of tetracycline. When horse serum was reduced to 1 percent in the broth culture, mycoplasmacidal activity was observed with methacycline as the most cidal of the analogues tested.

In the search for chemotherapeutic agents active against mycoplasma a broad spectrum of inhibitory substances have been tested. Aurothiomalate inhibits the growth of some mycoplasmas at concentrations of 16 to 128 μg/ml (93). Antiprotozoal agents found to be inhibitory to some mycoplasmas include the following: quinine, chloroquin diphosphate, atabrine, ethidium bromide, prothidium bromide, and antrycide. Acholeplasma laidlawii growth can be inhibited by steroid inhibitors that most likely interfere with carotenoid biosynthesis which shares in part the sterol biosynthetic pathway. Such inhibitors include benzmalecene, triparanol, farnesenic acid, vanadium trichloride, tolbutamide, geraniol, and farnesol (113). Experiments by Braun et al. (7) demonstrated the differential susceptibilities among mycoplasma strains to antibiotics. Erythromycin was more active against T-strains than against M. hominis or M. fermentans. Lincomycin, olindamycin, and nitrofurantoin had greater activity against M. hominis and M. fermentans than against T-strains.

Resistance mechanisms. Investigation of mechanisms responsible for mycoplasmas' resistance to antibiotics is sorely lacking. The evidence that has been gathered indicated a dominant role for the mycoplasmal membrane. Schwartz and Perlman (102) showed that polyuridylic acid directed cell-free protein synthesis by ribosomes from tetracycline resistant A. laidlawii, B cells was sensitive to tetracycline. Subsequent studies on the resistance of several strains of mycoplasma to chloramphenicol, dihydrostreptomycin and tetracycline indicated that reduced uptake of the antibiotic was involved rather than specific degradation of the antibiotic (41).

Potential mechanisms include mutation to resistance of the primary target of the antibiotic. The ability of mycoplasmas to mutate has been established. Steinberg has obtained mutants of M. pneumoniae which are able to grow at 32-34 C, but not at 40-42 C (119). Nitrosoguanidine (NTG) was used by Steinberg to induce the mutations, and NTG has also been used to obtain mutants constitutive for the inducible enzyme glucosidase (108). Mutants resistant to antibiotics have also been reported (58, 67). So far, however, most mutations in mycoplasma are either unstable, or have not been well characterized with regard to the mutational alteration (69).

The possibility exists that mycoplasma plasmids carry resistance factors similar to the RTF plasmids of bacteria. Extra Chromosomal DNA molecules have been observed in M. arthritidis (44, 73) and A. laidlawii, B (27, 32) and could represent plasmids. In addition, viruses have been isolated from A. laidlawii, B (65) which gives further weight to the evidence that functioning plasmids

exist in mycoplasma.

Plan of the Dissertation

The objectives of the work presented in this dissertation are to characterize the response of mycoplasma to selected antibiotics and to attempt to understand the basis for the responses observed. The experimental approach involves measurements of labeled uridine incorporation into acid insoluble material and of growth as determined by viable counts in order to determine responses to RNA synthesis inhibitors of cultures of mycoplasma. Two species of mycoplasma were used, Acholeplasma laidlawii, A and Mycoplasma orale. The RNA synthesis inhibitors used were rifampin, rifamycin SV, streptolydigan, streptovaricin, steffimycin, and lomofungin. A major emphasis of this research was to examine the response of A. laidlawii, A to rifampin. Some of the results to be presented here have also been presented at the Meeting of the American Society for Microbiology, April 1972, in Minneapolis, Minn.

CHAPTER II

MATERIALS AND METHODS

Mycoplasma Strains

Acholeplasma laidlawii, A (A. laidlawii, A) PG-8, ATCC #23206 was obtained from Leonard Hayflick, and Mycoplasma orale, I ATCC #15539 was obtained from the American Type Culture Collection, Rockville, Maryland.

Mycoplasma were maintained in stock as frozen (-96 C) 2.0 ml samples of log phase organisms in PPLO broth culture. The PPLO broth constituents differed for A. laidlawii, A and M. orale and are described below. Upon thawing, the titer of A. laidlawii, A was approximately 2.0×10^8 colony forming units per ml (CFU/ml).

Samples of a growing culture of M. orale I, taken at two different times during the interval when turbidity was increasing were frozen. This procedure insured that stock cultures contained healthy cells.

Media and Reagents

Media

PPLO medium. PPLO broth, containing serum and 10 percent (v/v) fresh yeast extract as supplements, was prepared essentially as described by Hayflick (47), using a commercial dehydrated medium (Bacto-PPLO

broth), with the following modifications. When used for culture of A. laidlawii, A, the serum supplement was 10 percent (v/v) rather than 20 percent. The usual 20 percent serum supplement was used for cultivation of M. orale. In addition, the medium was supplemented with 0.5 percent arginine-HCl and 0.5 percent glucose, and phenol red was included as described below when M. orale was cultured.

PPLO broth medium, containing 10^3 units per ml potassium penicillin G and 0.001 percent thallium acetate (final concentrations in the medium) was prepared as follows. Dehydrated PPLO broth was rehydrated by dissolving 21.0 g in 1 l; to this was added 100 ml of 25 percent fresh yeast extract and 0.5 ml of a 2 percent (w/v) solution of thallium acetate, and the solution was autoclaved for 15 minutes at 121 C. After cooling, the medium was supplemented with 100 ml of gamma-globulin-free thawed horse serum and 1.0 ml of a sterile solution of potassium penicillin G, 10^6 units/ml to give a final concentration of 10^3 units/ml. When required phenol red was added aseptically to the sterile, supplemented medium to a final concentration of 0.002 percent from a stock solution of 0.5 percent phenol red. PPLO agar plates were prepared by adding 14 g/l of agar prior to autoclaving.

Casitone medium. Casitone broth consisted of 20 g dehydrated casitone, 10 g dehydrated commercial yeast extract, and either 2.5 g or 5.0 g NaCl all dissolved in 1 l of doubly distilled water. Thallium acetate was added as described above for PPLO broth. The pH was adjusted to 8.0 with NaOH prior to sterilization. The broth was autoclaved, cooled, and glucose was added from a sterile 25 percent stock solution to give a final concentration of 0.5 percent glucose. Penicillin G was

added as described for PPLO broth. Casitone agar was prepared by adding 14 g/l agar to the broth prior to autoclaving. Where indicated in Results, serum was added (10 percent, v/v) subsequent to autoclaving and cooling of the medium.

Soy peptone-yeast extract (SP-YE) medium. SP-YE broth (14) was prepared similarly to casitone medium except that glucose was not added, and the salt concentration was always 0.5 percent. The broth was prepared by adding 20 g dehydrated soy peptone, 10 g dehydrated yeast extract, and 5.0 g NaCl in 1.0 l water. Thallium acetate and penicillin G were added as previously described. SP-YE agar was prepared by adding 14 g/l agar to the broth prior to autoclaving. Where indicated serum was added (10 percent v/v) subsequent to cooling the autoclaved medium.

NIH agar medium. This medium, used for monitoring cultures for contamination, was prepared by adding the dehydrated medium to water as directed on the bottle.

Sabouraud agar medium. This medium was used to monitor for fungal contamination and was prepared by rehydrating the powdered medium as per directions on the bottle.

25 percent fresh yeast extract. Dried Fleischmann's yeast (Saccharomyces cerevisiae, 250 g) was added to 1.0 l doubly distilled water and brought to a boil. The boiled mixture was centrifuged at 10 thousand rpm (16,300 x g) for 45 minutes at 4 C in a Sorvall RC2-B centrifuge. The supernatant was adjusted to pH 8.0 with 2N NaOH and autoclaved for 15 minutes at 121 C. The yeast extract was again centrifuged as before, and the non-sterile supernatant was dispensed

into 50 ml plastic containers and stored frozen at -20 C. The 25 percent fresh yeast extract was thawed and added to medium, prior to autoclaving.

Reagents

Dilution buffer. Dilution buffer consisted of 0.9 percent NaCl plus 0.02 M Tris buffer, pH 8.0 in doubly distilled water. The dilution buffer was sterilized and supplemented with 10^3 units/ml of potassium penicillin G.

Tris-maleate (TM) buffer. TM buffer (1) consisted of the following in g/l; Tris HCl, 6.10; maleic acid, 5.80; $(NH_4)_2SO_4$, 1.00; $MgSO_4 \cdot 7H_2O$, 9.10; $Ca(NO_3)_2 \cdot 4H_2O$, 5.0×10^{-4}; $FeSO_4 \cdot 7H_2O$, 2.5×10^{-4}. The pH was adjusted to 6.0 with NaOH before autoclaving.

Rifampin (Rif) and rifamycin SV (Rif SV) solutions. In order to add rifampin to culture media, solutions of rifampin were prepared by one of three methods: 1) rif was prepared as a 1 mg/ml solution in complete medium plus 10 percent ethanol (v/w) by dissolving the rif first in 95 percent ethanol (10 mg/ml), then adding complete medium to make the ethanol 10 percent (v/v); 2) dissolving rif in DMSO in any concentration desired up to 100 mg/ml; 3) for agar media, the desired total amount of rif was dissolved in 95 percent ethanol, which was added directly to the medium. When rif-ethanol solutions were added, the ethanol comprised not more than a final concentration of 0.5 percent in the media. The final concentration of DMSO never exceeded 1.0 percent. The solutions were prepared at a concentration such that, when added to the culture media, the desired final concentration of the rifampin in the culture was attained.

Other antibiotic solutions. The following antibiotics and agents were purchased from Sigma Chemical Co., St. Louis, Mo., and were dissolved in dimethylsulfoxide (DMSO): gramicidins J and S, polymyxin B, bacitracin, mycostatin, and mellitin. Valinomycin, 2-4-dinitrophenol (DNP) and 2-heptyl-4-dydroxy-quinoline-N-oxide (HHQO), also purchased from Sigma, were added as slurries of acetone to liquid broth medium. The RNA synthesis inhibitors streptolydigin (stlgn), streptovaricin (strvn), steffimycin (steff) and lomofungin (lomo) were kindly donated by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich., and were dissolved in DMSO. Actinomycin D (Act.D), generously provided by Dr. Mary Carpenter, was from Merck, Sharpe and Dohme, Rahway, N. J. Amphotericin B, purchased as Fungizone from Gibco, East St. Louis, Ill., was in the form of a sterile aqueous solution of 250 μg/ml Amphotericin B.

Final concentration of solvents. The maximum final concentrations of solvents in the medium were: ethanol, 0.5 percent; DMSO, 1.0 percent; acetone, 0.1 percent; KCl, 0.015 M.

Growth of Mycoplasma Cultures

Culture Conditions

Culturing of mycoplasma was initiated by adding two ml of thawed stock culture to the growth medium. When log phase cells were required, for inoculum, growing cells were transfered to fresh medium. Broth cultures for growth experiments usually consisted of 5 or 10 ml-1M screw cap tubes of 15 ml capacity. Alternatively, Roux bottles were used which allowed for cultures of larger volumes. Incubation was at 37 C in a stationary water bath. When change in color of phenol red

or change in turbidity were criteria for growth (Tables 1 and 2), tubes of uninoculated broth were also incubated and served as the standard for comparison of growth. Phenol red bleached upon prolonged incubation. At the conclusion of experiments involving growth of mycoplasma, cultures were streaked on NIH agar and on Sabouraud agar to check for bacterial and fungal contamination.

Viable Counts

The number of colony forming units (CFU) was determined by counting the number of colonies following application to the agar of 0.02 ml of appropriate dilutions of the sample to be titered. The method is essentially that of Butler and Knight (8a). An aliquot (0.1 ml) was withdrawn with a 0.2 ml glass, long-tipped, sterile pipette after the pipette had been rinsed 5 times with the sample. The aliquot was then added to 0.9 ml of sterile dilution buffer contained in a Wasserman tube with closure, and the pipette rinsed 5 times again with the dilution buffer. The addition of the 0.1 ml of sample to 0.9 ml dilution is the first of a series 10-fold serial dilutions. Transfer of cell suspensions during dilution was always accompanied by 5 rinses of the pipette.

Four dilutions of a sample were chosen to be plated, depending upon the estimated titer range of the sample. Each dilution was added as three 0.02 ml circular spots to one quadrant of an agar medium contained in a 100 mm x 15 mm plastic petri plate. The four quadrants of a plate, therefore contained the four dilutions spotted in triplicate. Usually 2 plates were used, the second plate serving to increase the number of spots of each dilution from triplicate to sextuplicate.

Alternatively, 50 x 12 mm petri dishes were used which allowed application of only one 0.02 ml spot per quadrant. In cases where an estimate of the titer range was in doubt, the second plate could be used to plate dilutions not spotted on the first plate.

The application of the 0.02 ml drops to the plate requires attention to several procedural points. Firstly, the pipettes were prerinsed 5 times with the dilution of sample to be plated. Secondly, the initial 0.02 ml drop was discarded onto an agar surface to eliminate any slight difference that might exist between the first drop out of the pipette and those that follow. When applying the 0.02 ml drop, care was taken to touch the agar very lightly so as to avoid damage to the surface. The drop was spread with the pipette tip over a circular area of approximately 5 mm diameter. This distributes the cell suspension thinly over the agar surface, allowing the liquid to be absorbed quickly into the agar, thereby immobilizing the mycoplasmas before they can reproduce in suspension. Moreover, spreading thinly prevents the 0.02 ml drop from running over the surface of the agar. After the spot was absorbed, the plates were placed in an incubator set at 37 C. Colonies grew enough in 48 hours to be seen with the aid of a low power lens. However, usually 4 days incubation were allowed before the colonies were counted. The colony forming units were usually expressed per ml of the original sample by multiplying the number of CFU counted in a 0.02 ml drop by 50 and then by the dilution factor.

Total CFU count. The titer of the total population of a culture was determined by plating on medium containing no selective agents and counting the CFU after 4 days of incubation.

Titer of rif-resistant variants. The same procedure for determining viable counts was followed except that rif was included in the medium on which the cells were plated. Concentrations of rif in the medium varied and are indicated in each experiment.

Titer of rif-inhibited cells. The same procedure was followed as for the titer of rif-resistant variants except that the agar contained rif and the incubation time allowed was 2 weeks. Rif-sensitive cells grow out on PPLO agar in the presence of rif in a two-week period.

Contamination Controls

Samples (0.1 ml) were streaked on NIH and Sabaraud agar and incubated 1 week at 37 C in order to detect the presence of any bacterial or fungal contamination. In experiments presented in Tables 1 and 2, uninoculated broth was incubated as a check for contaminating organisms.

Isotope Incorporation Measurements

Standard Pulsing Procedure

Cultures were prepared for pulsing by inoculation of 2 ml thawed A. laidlawii, A, stock culture into 5 ml of fresh, prewarmed, PPLO broth. After incubating for 24 hours at 37 C, this 7 ml culture was inoculated into 150 ml of fresh prewarmed medium (an approximate 1:20 dilution) and incubated for 3 or 5 hours to yield a titer of approximately 2×10^8 or 4×10^8 CFU/ml, (late log phase) respectively. A small sample was withdrawn for determining the presence of contaminants and CFU/ml. Five ml samples were pipetted from the 5 hour culture into 13mm Wasserman tubes. All experiments were designed to provide results in triplicate or quadruplicate. Each pulse consisted of rapidly adding 0.1 ml uridine-5-^{3}H (final concentration of 10 μC/ml) to the 5 ml culture,

inverting the parafilm-sealed tubes twice for mixing, and incubating for 2-8 minutes to allow incorporation of uridine into RNA. Incorporation was stopped by the addition of 10 or 20 μg/ml (final concentration) of Actinomycin D. The samples were mixed immediately for 2 seconds on a Vortex mixer and placed in an ice bath for at least 30 minutes. Zero time incorporation was attained by simultaneous addition of uridine-5-^{3}H and Actinomycin D. Effects of various antibiotics on incorporation were measured by pre-incubating the 5 ml sample with the antibiotic (added as a 0.1 ml solution in DMSO or ethanol) prior to the uridine pulse. The concentration of antibiotic and the length of the pre-incubation period varied and are described for each experiment in the Results section.

Tubes containing the samples were centrifuged in the 4 C room in a Sorvall type A angle tabletop centrifuge at a setting of 90 (approximately 6700 rpm) for 25 minutes. Supernatants were decanted and the tubes allowed to drain. The pellet was resuspended in 0.5 ml cold distilled water and 0.1 ml of a 1.0 mg/ml solution of yeast RNA added as carrier. The sample was treated with 4 ml ice cold 10 percent TCA, vortexed briefly, poured onto a Whatman filter paper disc (GF/C glass fiber paper) in a millipore filter box, and the filtrate washed three times with 5 ml 5 percent TCA. The first 5 ml were also used to rinse the sample tube. In experiments involving antibiotics, all filters received two additional washes of 5 ml of cold ethanol. Filters were then dried and placed into plastic counting vials to which were added 15 ml Aqua-sol (a xylene-based scintillation solution purchased from New England Nuclear, Boston, Mass.). Samples were cooled and the

tritium activity counted on a Nuclear Chicago Mark I liquid scintillation counter. The efficiency of counting was 29 percent.

A variation in the procedure was employed occasionally in measuring inhibition by rifampin of RNA synthesis. This variation consisted of washing the TCA precipitable material by 3 cycles of resuspension in 5 ml 5 percent TCA and centrifugation. The pellet from the final centrifugation was dissolved in 0.5 ml H_2O and transferred to a scintillation vial and scintillation solution was added as described above. This wash procedure in one experiment reduced counts from 247,605 cpm for the supernatant from the first wash, to 110 cpm for the supernatant for the third wash.

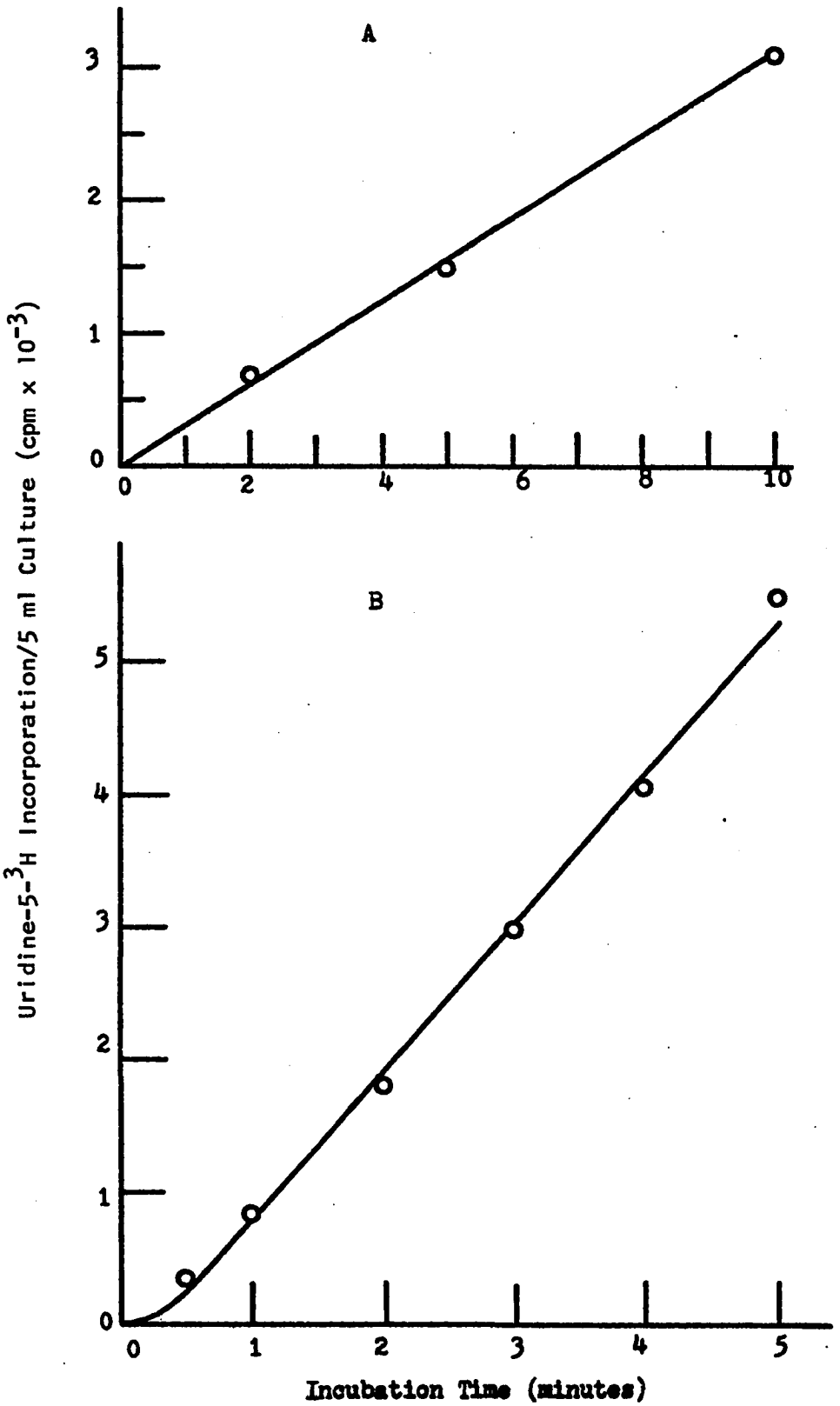
Preparation of Labeled Uridine Solution for Pulsing

Stock solution of uridine-5-^{3}H was diluted with sterile PPLO broth to give a concentration of 500 μCi/ml, and a specific radioactivity of approximately 25 Ci/mM.

Kinetics of Incorporation of Uridine-5-^{3}H by Log Phase Cells of A. laidlawii, A

The time allowed for incorporation of uridine varied within the linear ranges shown in Figures 1,A and 1,B. The experiments depicted in this Figure were performed as described above and the time allowed for incorporation was varied. Figure 1,A represents conditions for experiments dealing with mycoplasma sensitivity to rifampin in which cultures of approximately 2×10^8 CFU/ml were used. The results indicate that incorporation of uridine into cold TCA-precipitable material is linear for at least 10 minutes. Cultures of approximately 4×10^8 CFU/ml, used in the experiments involving antibiotics other than rifampin, show

Figure 1. Incorporation of Uridine-5-^{3}H by Log Phase Cells of A. laidlawii, A, as a Function of Time.


A
B
Uridine-5-^{3}H Incorporation/5 ml Culture (cpm x 10^{-3})
Incubation Time (minutes)
0
1
2
3
4
5
6
8
10

the incorporation kinetics presented in Figure 1,B. It was found that incorporation of uridine by a cell population of approximately 4×10^8 CFU/ml is linear for at least 5 minutes.

Mutagenesis Procedures

U.V. Irradiation

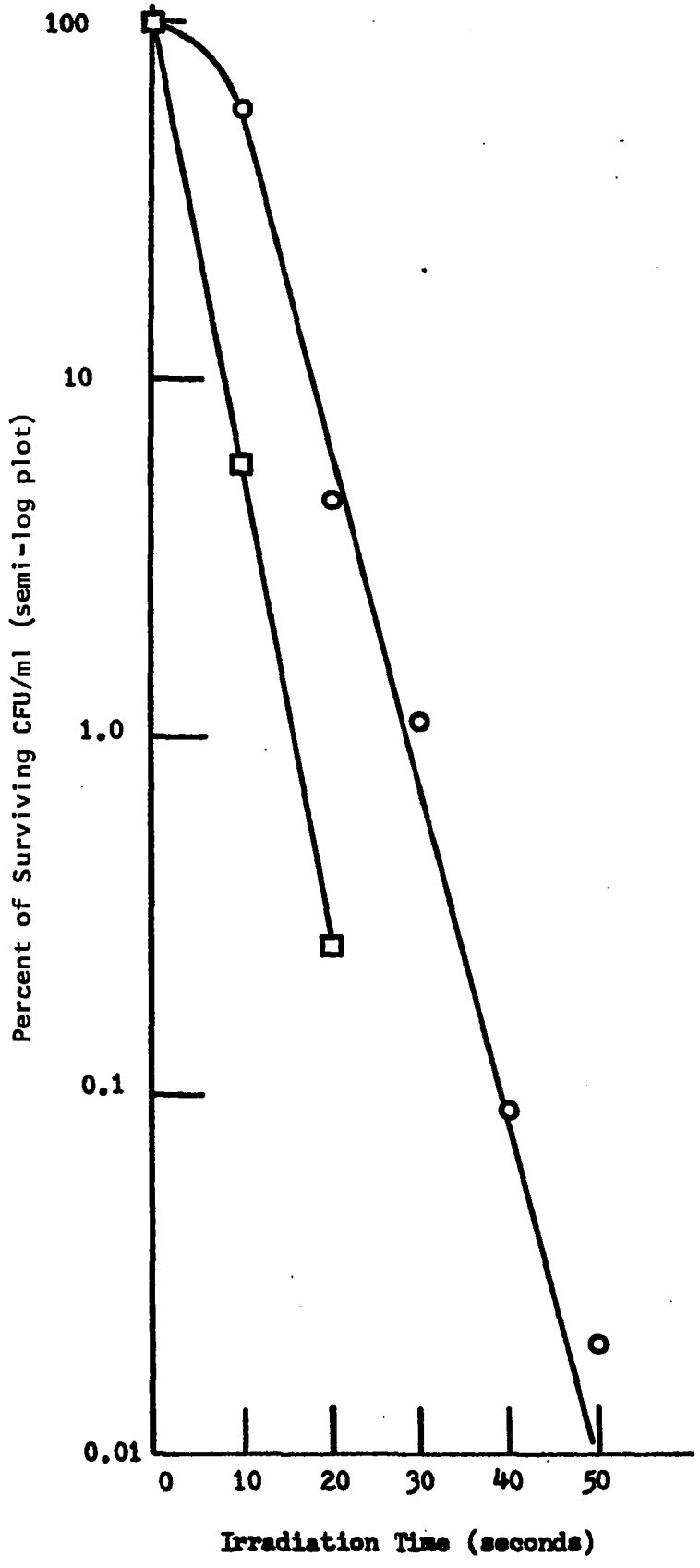
A. laidlawii. A, cells were thawed from frozen stock culture and 2 ml inoculated into 5 ml casitone broth. After incubation for 24 hours at 37 C, a turbid culture resulted and 1.0 ml was inoculated into 50 ml of fresh casitone broth - a 1:50 dilution of the inoculum. After 12 hours of incubation, this 50 ml culture served as an inoculum for 1.0 l of pre-warmed casitone broth - a 1:20 dilution of the inoculum. The culture was allowed to incubate for 3 hours, in order to ensure log phase cells. The cells were collected by centrifugation for 10 minutes at 5,000 rpm (3020 x g) in the small head of a Sorvall RC-B centrifuge at 25 C. The cell pellets were resuspended in 50 ml of dilution buffer (0.9 percent NaCl + 0.02M tris, pH 8.0), and allowed to remain for 5 hours. The purposes of this treatment were to: 1) allow cell clumps to disperse, 2) inhibit (by starvation) protein synthesis-dependent DNA repair processes that may exist in the cells (111), and 3) reduce the DNA content to its minimal amount per cell. A ten ml aliquot of a 1:10 dilution of this cell suspension was placed in a glass petri dish on a rotating platform 40 cm beneath an ultra-violet lamp for various time intervals. The exposure to irradiation was ended quickly by covering the petri dish after the desired time interval and removing it from the platform. Aliquots of 1 ml each were withdrawn from the irradiated cell suspension, inoculated into 9 ml of casitone broth, and

incubated until the culture was visibly turbid. Aliquots of appropriate dilutions of this culture (0.2 ml) were spread on plates containing selective media. Reduction of the shoulder on the inactivation curve and the dosage which gave approximately 95 percent kill served as the criteria in selecting the dilution and U.V. exposure time for mutagenesis. Figure 2 shows that there is no detectable shoulder for a 1:10 dilution of the cell suspension and that a 95 percent kill results from 10 seconds of U.V. irradiation under these conditions. It was also assumed, although not established, that the 5 hour period in dilution buffer would also decrease clumping and therefore the shoulder in the U.V. inactivation curve.

Nitrosoguanidine (NTG) Mutagenesis

The cells of a 5 ml sample of a late log phase A. laidlawii, A., culture in PPLO broth were pelleted by centrifugation (20 minutes centrifugation at a setting of 100 on a table top clinical centrifuge) and resuspended in 1.0 ml TM buffer. Mutagenesis was initiated by adding 1 ml of a fresh NTG solution to the 1 ml of cell TM buffer suspension and incubating at 37 C. The NTG solution consisted of NTG dissolved in TM buffer to a concentration twice that desired for mutagenic conditions. The NTG-cell suspension was incubated 45 minutes at 37 C. To terminate the mutagenesis 0.5 ml samples of the treated cells were pipetted into 4.5 ml of buffer composed of 0.01 M KH_2PO_4 + 1 percent (v/v) PPLO broth, centrifuged to pellet the cells, and resuspended in 1.0 ml of the same buffer. In order to segregate the presumed mutant cells, the 1 ml suspension of cells was inoculated into 9 ml PPLO broth and incubated for 15 hours. Samples from the

Figure 2. Survival of A. laidlawii, A, as a function of U.V. dosage. One hundred percent survival of the undiluted cell suspension represents 3.7×10^8 CFU/ml. Circles represent the undiluted cells, squares the 1:10 dilution of the cells. Viable counts were performed on PPLO agar.


100
10
1.0
0.1
0.01
Percent of Surviving CFU/ml (semi-log plot)
0
10
20
30
40
50
Irradiation Time (seconds)

resultant turbid culture were plated on selective media.

Spontaneous Mutation

Aliquots (0.2 ml) from a log phase culture in PPLO broth were spread onto 100 mm x 15 mm agar plates containing selective media. The plates were incubated for 4 days at 37 C.

The Fluctuation Test

Two ml thawed stock culture were inoculated into 5 ml casitone broth and incubated 24 hours. From this culture, 0.5 ml were inoculated into 50 ml of fresh casitone broth and incubated 24 hours. A sample of the resultant culture was diluted $1:10^3$ with dilution buffer, and 0.5 ml of this diluted cell suspension was added to 50 ml of fresh casitone broth to give a $1:10^5$ dilution of the 24 hour broth culture. To each of 20 15 ml screw cap tubes were pipetted 1.1 ml of the fresh casitone broth culture. The remaining 28 ml served as the batch culture control for the fluctuation test. The 20 test cultures and the control batch culture were incubated 24 hours at 37 C in a water bath. One ml from each of the 20 test cultures was plated on PPLO agar containing 100 μg/ml rifampin by adding 0.05 ml of the culture to agar in each quadrant of five 100 mm x 15 mm plastic petri dishes. From the batch culture 20 1 ml samples were plated in the same manner. All cultures were titered for total cell yield at the end of the 24 hour incubation and only cultures with a yield of between 5×10^7 and 2.5×10^8 CFU/ml were used for test cultures in the fluctuation test. The purpose of this procedure was to eliminate cell density as a variable factor in determining the fluctuations in mutant populations.

CHAPTER III

RESULTS

Investigation of the basis for the capability of mycoplasmas for developing resistance to various antibiotics was approached by allowing a confrontation of two alternate possibilities. Can the source of mycoplasmas' resistance to an antibiotic be attributed principally to rapid selection of resistant clones; or, on the other hand, does the entire mycoplasma population become resistant? Critical considerations implicit in this question are the nature of the antibiotic in question and the culture conditions. For the purposes of much of this research, a standard, rich serum-containing medium was chosen that would allow various mycoplasmas to be compared under very similar conditions. Moreover, such a medium more nearly simulates, in many cases, environments where mycoplasmas' resistance to antibiotics is most troublesome (e.g., tissue culture, animal hosts). The RNA synthesis inhibitors serving as antibiotics in this project were usually employed at concentrations of 100 μg/ml or less in expectation that the results observed would reflect the specificity of an agent's action.

Response of Mycoplasma to the Presence of Rifampin

A. laidlawii, A, and M. orale, I, were the strains used in the following experiments in order to compare organisms of basically different membrane structure. M. orale requires cholesterol for growth and incorporates

it directly into its membrane, whereas A. laidlawii does not require it for growth but will incorporate the sterol, if present, in the medium. Also A. laidlawii apparently incorporates carotenoids into its membrane instead of cholesterol.

Response of A. laidlawii, A, to Rifampin: Growth and RNA Synthesis

Qualitative Observations. One of the critical considerations bearing on the general problem of mycoplasmas' resistance to antibiotics is the antibiotic involved. Two preliminary qualitative experiments were performed to determine whether rifampin affects the growth of A. laidlawii, A. In the first, cells were inoculated into three tubes containing PPLO broth with phenol red. Two of the tubes also contained rifampin at concentrations of 25 and 100 μg/ml. The results are shown in Table 1. The culture without rifampin showed significant turbidity accompanied by a change in the color of the dye from red to yellow after 16 hours of incubation. Cultures containing rifampin are less amenable to observation of small color and turbidity changes due to the dark red color imparted to the medium by the rifampin. However, significant growth was detectable with certainty. The presence of 25 or 100 μg/ml of rifampin delayed considerably the growth of cultures to the approximate degree attained in the absence of rifampin. It was concluded from this experiment that onset or rate of growth of A. laidlawii, A, is inhibited significantly by rifampin at concentrations of 25 and 100 μg/ml.

Since the growth of cultures in the presence of rifampin required extensive incubation periods, it was possible that instability of the rifampin or changes in the constituents of the medium allowed eventual growth of the culture. Therefore, the cultures represented in Table 1 were used as sources of inoculum, after 96 hours of incubation, to determine whether rif-

TABLE 1

DELAYED GROWTH OF *A. laidlawii*, A, IN PPLO BROTH CONTAINING RIF[a]

Rif Concentration (μg/ml)	Time Required to Observe Growth (Hours)
None	16
25	60
100	96

[a]Rif was added from an ethanol-PPLO broth stock solution as described in Materials and Methods. Cultures were 10 ml each, contained 0.002% phenol red, and were inoculated with 0.05 ml of a turbid, untitered suspension of cells in PPLO broth. Growth was determined qualitatively as turbidity and color changes visible to the unaided eye.

cultivated cells would again show delayed growth in fresh rifampin-containing medium. Each of the two rif cultures served as the inoculum source for two parallel cultures without and with corresponding concentrations of rifampin. The cells grown in the absence of rifampin were subcultured into three parallel cultures containing 0, 25, and 100 μg/ml rif. The results of this experiment are shown in Table 2. The cultures previously grown without rifampin showed growth after 43 hours incubation only in fresh medium with no rifampin but not in the presence of 25 and 100 μg/ml rifampin even after 72 hours incubation. The two pairs of cultures previously cultivated with 25 and 100 μg/ml rifampin, respectively, showed growth after 43 hours whether or not rifampin was in the medium.

We conclude that eventual growth of A. laidlawii, A, in medium containing rifampin is not due to decay of rifampin activity or to changes in the culture medium which occur during the long incubation period. The results also illustrate that antibiotic resistance need not depend upon the presence of a host organism in which the mycoplasma sequesters itself for protection from the deleterious agent.

Growth curve in viable units. In order to delve deeper into the nature of the growth response of mycoplasma to antibiotics, the accuracy and validity of growth measurements were increased by determining colony forming units per ml (CFU/ml). The normal growth of A. laidlawii, A, as a function of incubation time in PPLO broth is presented in Figures 3, 4 and 5. In all three experiments the doubling time was approximately 90 minutes during the exponential phase of growth. This rate appears to be maintained until the culture attains a titer of approximately 2.0×10^8 CFU/ml (Figure 3) at which point the growth rate decreases until a yield of essentially 10^9 CFU/ml is attained. Growth rates and yields tend to vary with different batches of

TABLE 2

UNINHIBITED GROWTH OF RIF-CULTURED CELLS
FOLLOWING TRANSFER TO FRESH MEDIUM CONTAINING RIF

Rif Concentration in Previous Medium [a] (μg/ml)	Rif Concentration in Fresh Medium (μg/ml)	Growth [b] (Presence of Visible Turbidity)
None	None	+
None	25	-
None	100	-
25	None	+
25	25	+
100	None	+
100	100	+

[a] Inoculum (0.1 ml) was taken from the corresponding cultures described in Table 1. These cultures were 4 days old when used as inocula.

[b] Growth was measured qualitatively, by the presence (+) or absence (-) of marked turbidity after 43 and 72 hours incubation. Other conditions are as described for Table 1.

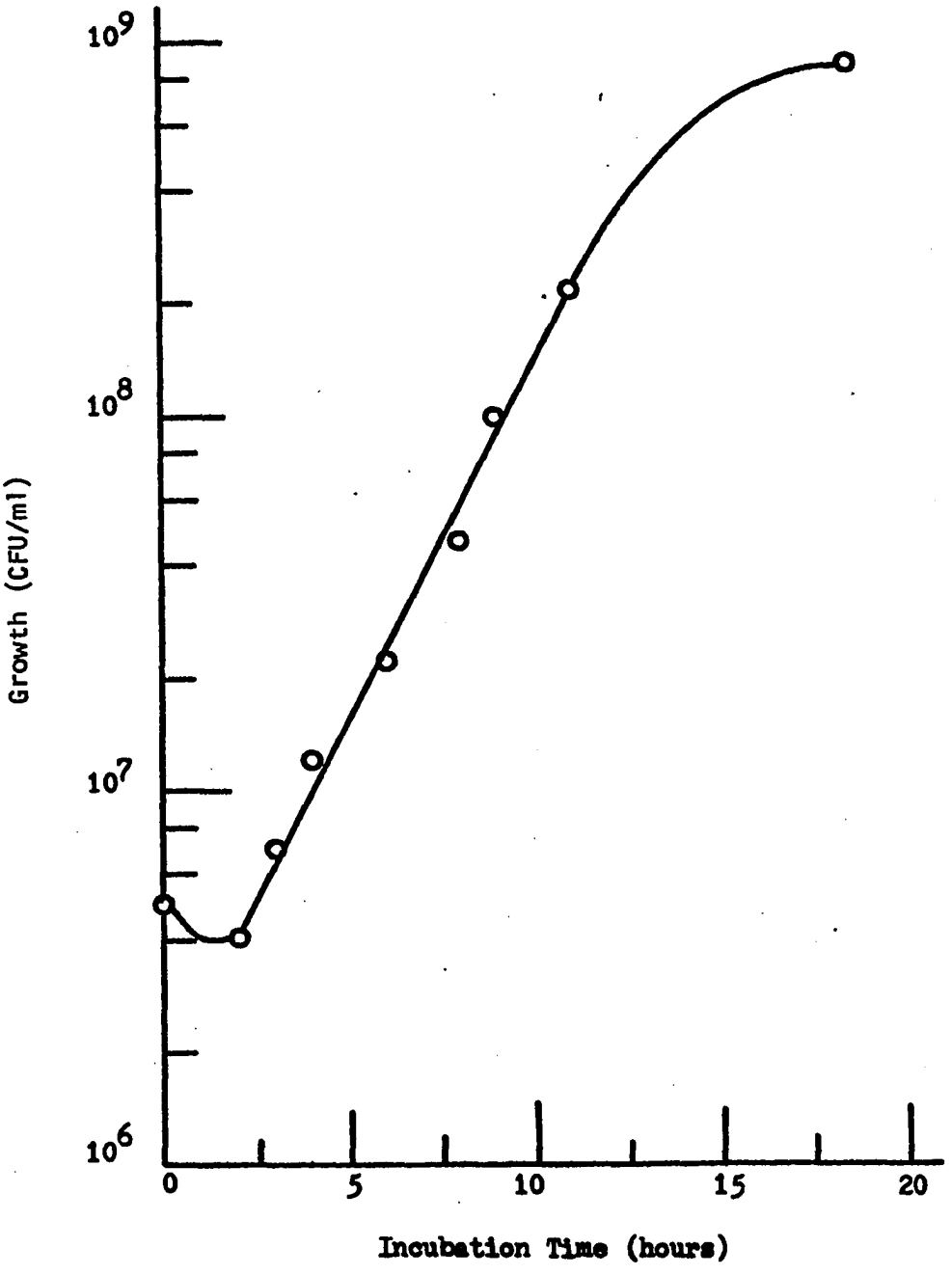


Figure 3. Growth curve of A. laidlawii, A, in PPLO broth. Inoculum was freshly thawed cells. Viable counts were performed using PPLO agar.

Figure 4. Growth of A. laidlawii, A in PPLO broth in the presence (+ rif) and absence (- rif) of 5 μg/ml rifampin. The inoculum was in log phase in PPLO broth not containing rif. Rifampin was added from a DMSO stock solution.

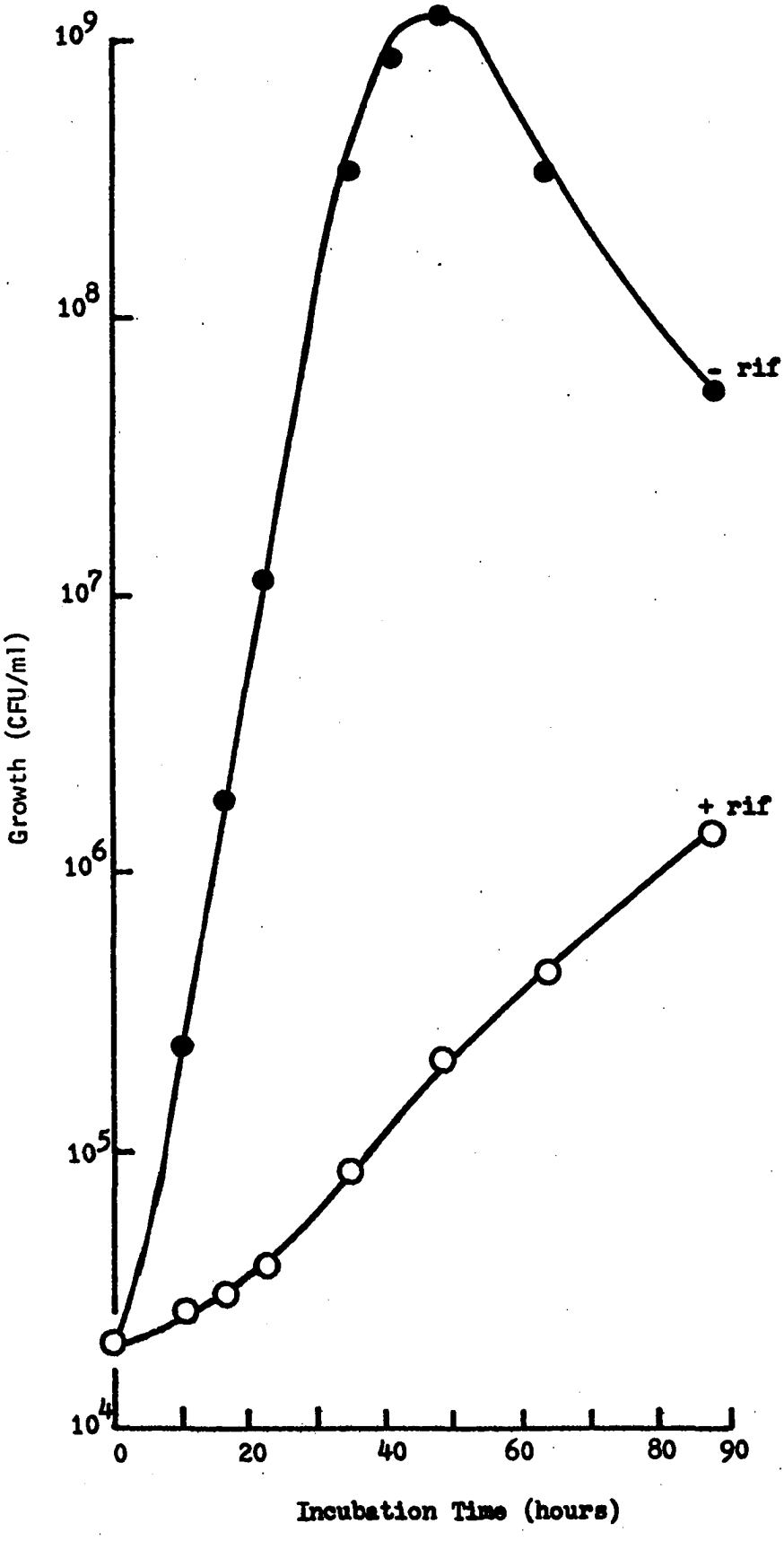
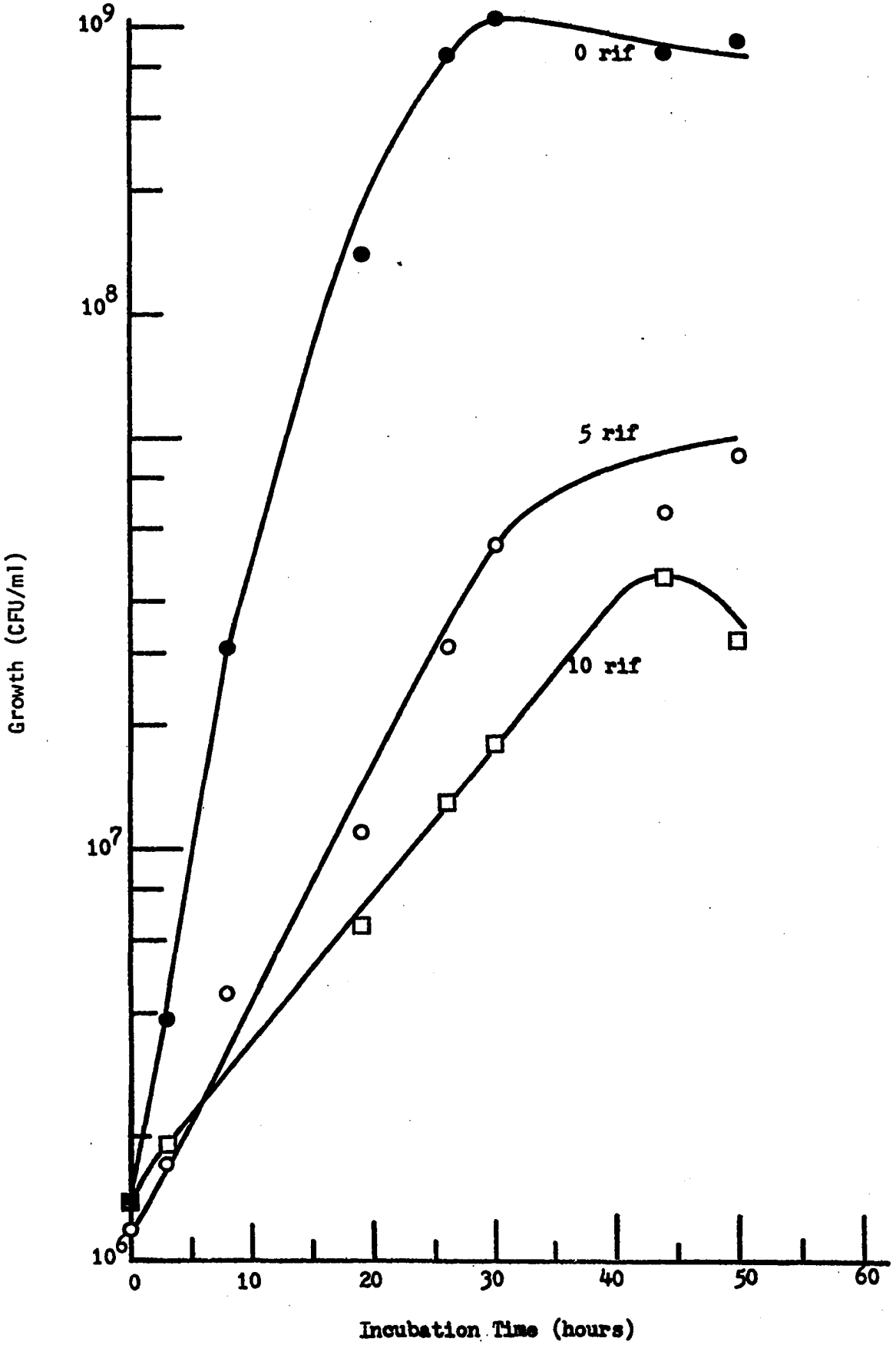

10^9
10^8
10^7
10^6
10^5
10^4
Growth (CFU/ml)
- rif
+ rif
0
20
40
60
80
90
Incubation Time (hours)

Figure 5. Growth response of A. laidlawii, A, to varying concentrations of rifampin in PPLO broth. The inoculum was taken from cultures of log phase cells in the corresponding medium. The designations 0 rif, 5 rif and 10 rif represent the concentrations (μg/ml) of rifampin present in each culture. The rifampin was added as a DMSO solution.

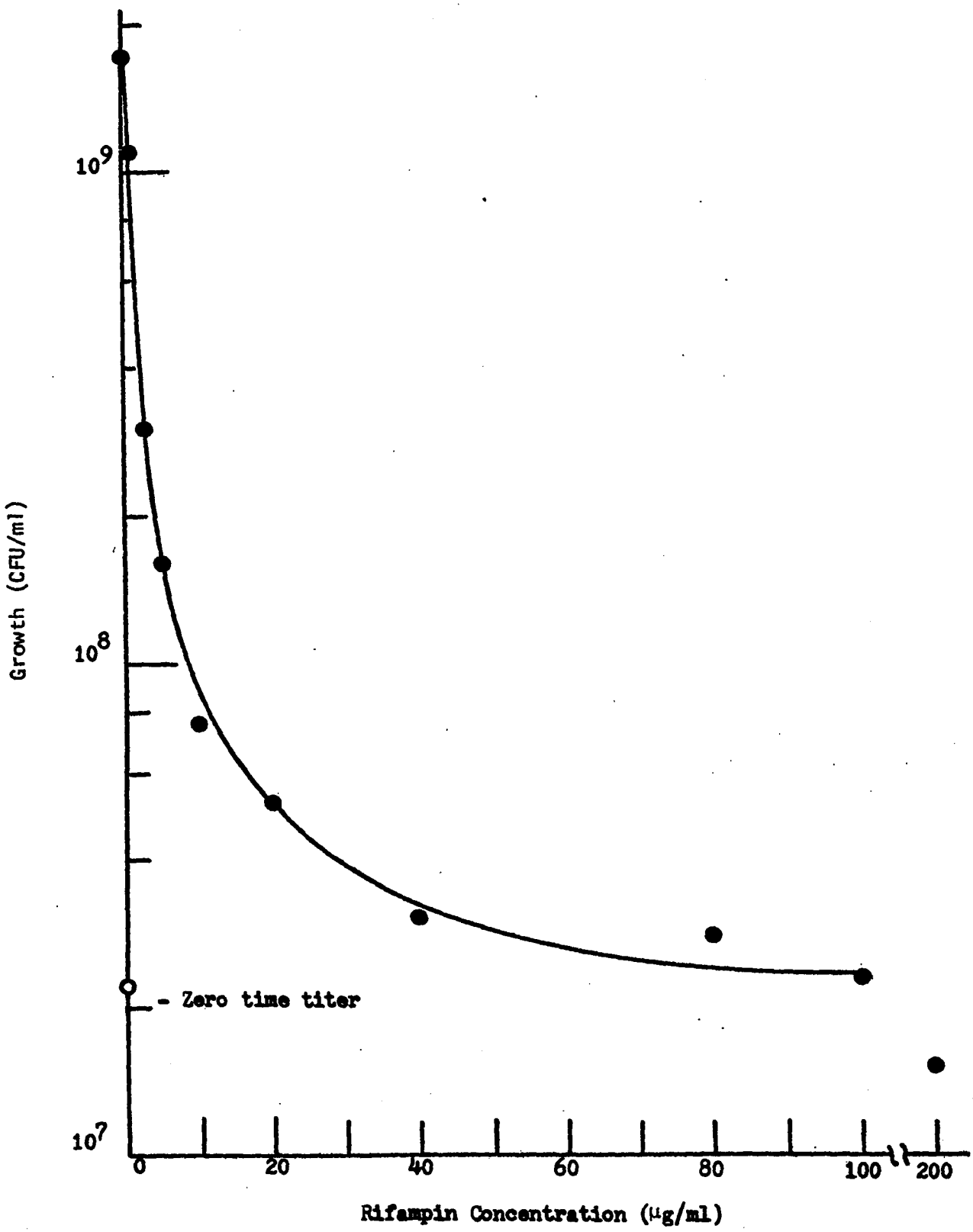

10^9
10^8
10^7
10^6
0 rif
5 rif
10 rif
Growth (CFU/ml)
0
10
20
30
40
50
60
Incubation Time (hours)

serum and fresh yeast extract.

After reaching a peak of approximately 10^9 CFU/ml, the culture retained this titer for variable periods of time. Figure 5 shows that the peak titer was retained for 20 hours, from 30 hours of incubation to 50 hours, at which time the experiment was concluded. The growth depicted in Figure 4, however, began declining after reaching a peak of 1.3×10^9 CFU/ml. This difference in post-stationary phase behavior could be due to the difference in size of the initial inocula in the two cultures. The beginning titer in Figure 4 is 1.4×10^4 CFU/ml, whereas that in Figure 5 is 2.0×10^6 CFU, a difference of over 100-fold. Other than a correlation of this phenomenon with inoculum size, and the ever present threat of differences in the medium, no explanation is apparent for why the one culture stabilizes its titer at the peak of growth for 20 hours whereas the other culture proceeds into a death phase shortly after reaching its peak. Further consideration of this problem will be dealt with in Chapter IV. The lag phase seen in Figure 3 is presumably due to the use of freshly thawed cells for inoculum. The significant, if any, lag phases of Figures 4 and 5 presumably reflect the use of fresh, exponentially-growing cells for inoculum.

Growth response to rifampin. The results presented in Tables 1 and 2 established that A. laidlawii, A, is indeed sensitive to 25 and 100 μg/ml of rifampin. For a quantitative determination of the sensitivity of A. laidlawii, A, to rifampin, an experiment was designed to measure the titer attained after 24 hours incubation by cultures containing varying concentrations of rifampin. The results are shown in Figure 6. Each point represents the growth attained by a culture containing the indicated concentration of rifampin 24 hours after receiving an inoculum that gave a zero time titer of 2.2×10^7 CFU/ml. As in the qualitative experiments of Table 1 and 2, the

Figure 6. Growth inhibition of A. laidlawii, A, in PPLO broth after 24 hours as a function of rifampin concentration. The inoculum source was a stationary phase culture, and the initial titer after inoculation is denoted by an open circle on the ordinate. Rif was added as an ethanol medium solution.


Growth (CFU/ml)
10^9
10^8
10^7
- Zero time titer
0
20
40
60
80
100
200
Rifampin Concentration (µg/ml)

inoculum source was a culture that had reached stationary phase. Rifampin at concentrations up to 100 μg/ml, is extremely effective in reducing the number of CFU observed after 24 hours. In the absence of rifampin, the culture reached a titer of 1.7×10^9 CFU/ml, whereas in the presence of 5 μg/ml rifampin it attained only 1.6×10^8 CFU/ml. This value represents 90 percent inhibition. The experiment also shows that after approximately 95 percent inhibition of growth was reached (by 10 μg/ml of rifampin), increasing concentrations of rifampin failed to sustain the steep inhibition slope. The slope of inhibition appeared to decrease in an asymptotic fashion with regard to the initial titer of 2.2×10^7 CFU/ml. Rifampin at a concentration of 100 μg/ml prevented any significant growth above the initial number of cells provided by the inoculum. When 200 μg/ml of rifampin was present in the culture, a decrease in the titer below that provided by the inoculum occurred, which indicated that killing had taken place.

The chief conclusion from the data represented in Figure 6 is that growth of A. laidlawii, A, is inhibited effectively by low concentrations of rifampin in serum-containing medium during the growth period of 24 hours. This sensitivity is well represented by the 90 percent inhibition of growth by 5 μg/ml rifampin. Another conclusion is that the response of mycoplasma to rifampin tends to be of a mycoplasma-static nature, rather than mycoplasmacidal. This conclusion is based on the inability of rifampin at concentrations up to 100 μg/ml to reduce the number of CFU below that of the inoculum. However, a rifampin concentration of 200 μg/ml did reduce the titer of the culture below the inoculum level. This indication of killing by high concentrations of rifampin is likely to reflect a non-specific activity of rifampin, and was not investigated further.

The significant sensitivity of A. laidlawii, A, to levels of

rifampin below 5 μg/ml in 24 hours (Figure 6) and, on the other hand, the eventual outgrowth of the culture even in the presence of as much as 100 μg/ml of rifampin (Tables 1 and 2) are two phenomena which were paradoxical and upon which the next experiment was based. In order to analyze the kinetics of inhibition and outgrowth, samples from cultures containing low concentrations of rifampin were titered at suitable intervals for a 36 hour period.

Figure 7 shows the dose response curves. Rifampin elicits a biphasic growth response. There is a transient phase of slow growth followed by a phase of faster growth. This can be seen most clearly perhaps for the culture containing 5 μg/ml rifampin. Growth after 7.5, 12 and 18 hours of incubation is negligible. However, after 24 and 36 hours, recovery and outgrowth ensues. Cultures containing 1.5 and 2.5 μg/ml of rifampin also show the biphasic growth pattern, whereas the culture containing 20 μg/ml rifampin shows a static growth response throughout the 36 hour incubation period. The observation that cell titers generally appear to increase above the inoculum levels before the onset of inhibition is not easily explained and will be discussed in Chapter IV.

In order to identify the early phase of growth inhibition by rifampin observed in Figure 7, the term, "transient inhibition" was instituted. Transient inhibition is defined as the growth inhibition delineated by points representing titers taken at 7.5, 12 and 18 hours incubation (Figure 7). The transient inhibition, therefore, was calculated from doubling times derived from these three points. The doubling times during transient inhibition are shown for all the cultures in Table 3. The reciprocals of these growth rates were converted to percent inhibition by setting the value obtained in the absence of rifampin equal to zero. For the cultures

Figure 7. Biphasic growth response of A. laidlawii, A to concentrations of rifampin in PPLO broth. The inocula were taken from a freshly thawed stock culture. Rifampin was added from an ethanol-medium solution. The various curves are designated according to the number of μg/ml of rifampin in the culture (e.g., 5 rif represents 5 μg/ml rifampin).

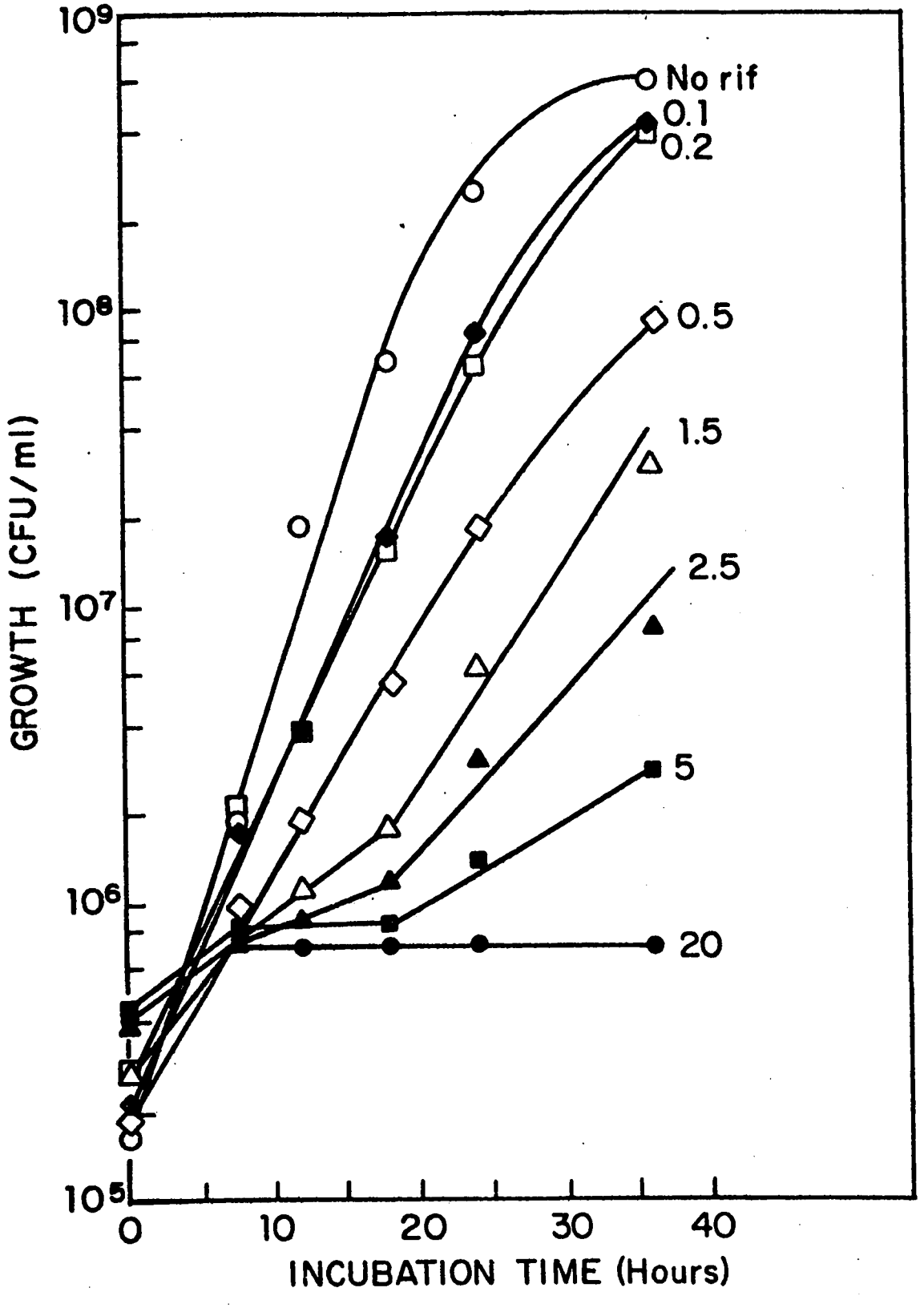

10^9
10^8
10^7
10^6
10^5
GROWTH (CFU/ml)
No rif
0.1
0.2
0.5
1.5
2.5
5
20
0
10
20
30
40
INCUBATION TIME (Hours)

TABLE 3

TRANSIENT INHIBITION BY RIFAMPIN OF GROWTH OF *A. laidlawii*, A, IN PPLO BROTH

Rifampin[a] (μg/ml)	Doubling Times During Transient Inhibition (minutes)	Transient Inhibition[b] (percent)
0	120	0
0.1	165 [c]	28
0.2	180 [c]	34
0.5	240	50
1.5	510	76
2.5	1,800	93
5.0	10,800	100
20.0	no growth	100

[a]Rif was added from a stock solution of ethanol-medium as described in Materials and Methods.

[b]The reciprocal of the doubling time during the transient period of growth inhibition was converted to percent inhibition by setting the value obtained in the absence of rifampin equal to zero.

[c]Calculated from overall growth rate.

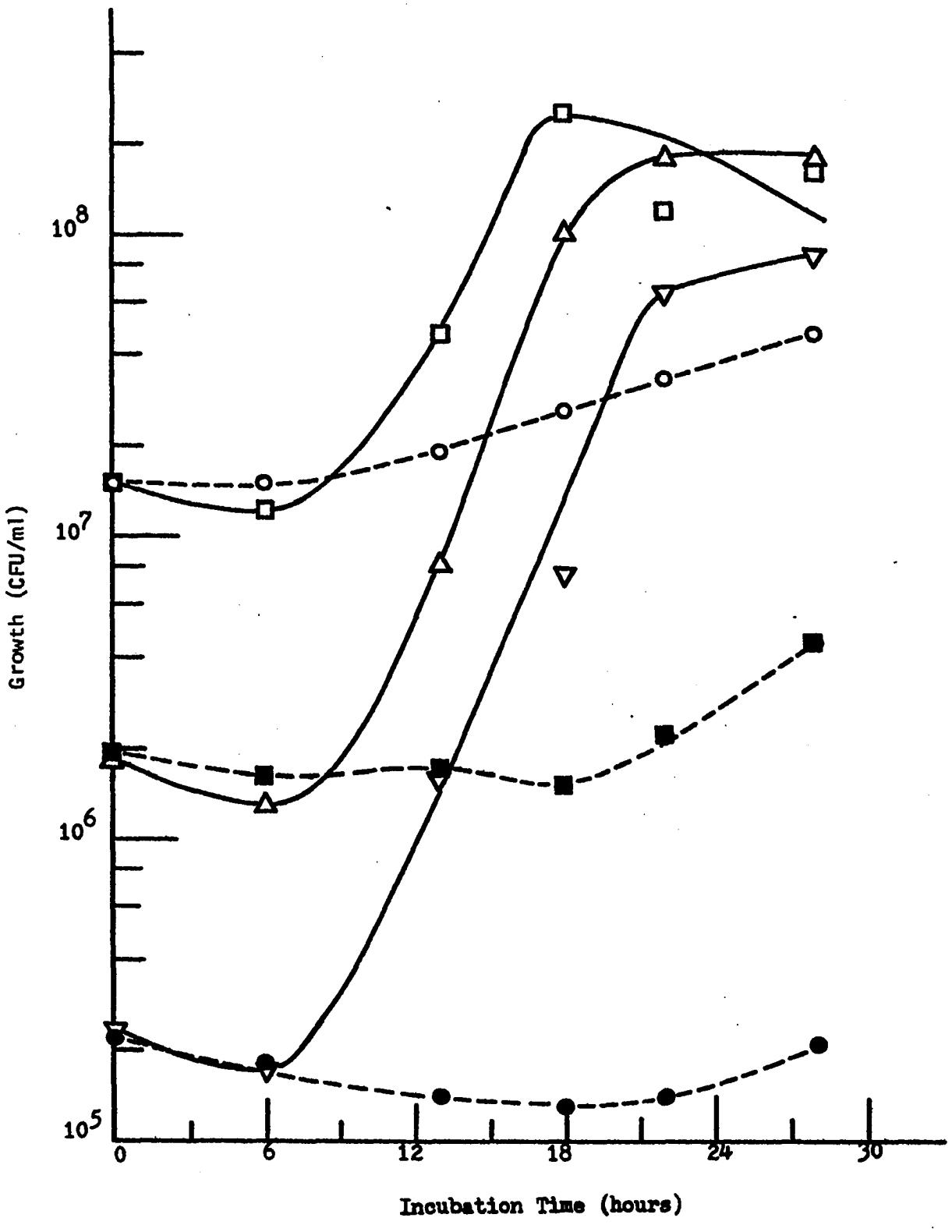
containing 0.1, 0.2 and 0.5 μg/ml of rifampin, a transient phase of inhibition was not well distinguished, so that a doubling time that corresponded to the overall growth rate was used. Inhibition is a function of rifampin concentration. Growth is transiently inhibited by relatively low concentrations of rifampin. As little as 0.1 μg/ml inhibits growth (28 percent inhibition). A concentration of 0.5 μg/ml causes 50 percent inhibition, whereas 1.5 μg/ml causes 76 percent inhibition. One hundred percent transient inhibition is attained by 5 μg/ml of rifampin. Doubling times for the second growth phases of cultures containing 1.5, 2.5 and 5 μg/ml rifampin were 254 minutes, 342 minutes and 600 minutes, respectively. Since the corresponding doubling times for these cultures during transient inhibition was 510 minutes, 1,800 minutes and 10,800 minutes (Table 3), the biphasic nature of the growth is well demonstrated.

It was concluded from the results represented in Figure 7 and Table 3 that the presence of rifampin in cultures inoculated with freshly thawed cells elicits a biphasic growth response consisting of a period of transient inhibition followed by a period of recovery and outgrowth. Rifampin does not appear to be mycoplasmacidal since there is no reduction in titer below that of the initial inoculum (Figs. 5, 6, 7) in the presence of up to 100 μg/ml rifampin.

The importance of the inoculum size in the response to rifampin of A. laidlawii, A, is suggested by comparing the percent inhibition after 24 hours incubation in the presence of 5 μg/ml rifampin observed in Figure 4 and Figure 5. In Figure 5, the initial number of CFU was approximately 100-fold higher (2.0×10^6 CFU/ml) than that in Fig. 4 (2×10^4 CFU/ml). Approximately 90 percent inhibition was produced by 5 μg/ml rifampin, whereas for the lower inoculum, the inhibition was over 99 percent. Since

this difference was observed by comparing results of two different experiments, a single experiment was designed to measure the effect of inoculum size on the growth response to rifampin. Three 20 ml portions of PPLO broth were inoculated with freshly thawed stock culture cells, the size of the inoculum varying ten-fold among each culture. After inoculation, each 20 ml portion was mixed and divided into a pair of 10 ml portions, and rifampin was added to one of each pair to give a final concentration of 5 μg/ml. Growth of the six cultures was followed by determining CFU/ml at intervals throughout a 28-hour incubation period in order to measure the variation in length of the period of transient inhibition as a function of inoculum size. The resulting growth curves are depicted in Figure 8. Growth in the absence of rif was approximately the same for all inoculum sizes, as indicated by the lag times (6-8 hours) and the doubling times (DT) which were 1.5 - 2.0 hours. Thus, neither lag times nor growth rates appear to be a function of inoculum size. Rifampin (5 μg/ml) in the medium caused delays in growth of 13, 22 and 28 hours for inoculum sizes of 1.6×10^7, 1.9×10^6 and 2.1×10^5 CFU/ml, respectively. Rif did not delay growth for the highest inoculum size culture even though it significantly decreased the growth rate. However, for inoculum sizes of 1.9×10^6 and 2.1×10^5, growth in the presence of rif was delayed by 12 and 16 hours, respectively, beyond the normal lag. Reduction by rifampin of titer below the lowest inoculum size of 2.2×10^5 CFU/ml cannot be attributed to killing by the antibiotic, since all the control cultures without rifampin showed a similar reduction in titer before exponential growth began. Inoculation into medium that was not pre-warmed may have contributed to the lag phase in this experiment. The main conclusion gained from this experiment is that decreasing inoculum sizes lead to increased duration of the transient

Figure 8. Transient inhibition by rifampin of growth of A. laidlawii, A, in PPLO broth as a function of inoculum size. The broken lines designate the growth of cultures containing 5 μg/ml of rifampin; the corresponding solid lines represent the growth of the control culture without rifampin. Symbols are identified by the CFU/ml designated at zero time, which represent the inoculum size for their respective cultures. The inoculum source was freshly thawed stock culture. Rifampin (final concentration, 5 μg/ml) was added from an ethanol-medium solution.


Growth (CFU/ml)
10^8
10^7
10^6
10^5
0
6
12
18
24
30
Incubation Time (hours)

inhibition by rifampin. It should also be pointed out that 5 μg/ml of rifampin are unable to kill A. laidlawii even when the cells are in the poor physiologic condition assumed to be associated with lag phase.

Resistance of individual CFU. The fact that there is no detectable killing by rifampin in broth (Figs. 6 & 7) suggests that essentially all the cells of the culture are resistant to a certain degree or that cell growth and death proceeded at the same rate. Moreover, it seems that the denser the cell population is before exposure to rifampin, the more resistant is the culture to the action of rifampin. The issue thus presents itself as to whether each cell is able to adapt and grow in the presence of rifampin. The experiment designed to resolve this question involved measuring the proportion of rif-resistant cells in a population which had not been previously exposed to rifampin. Samples of cells growing in the absence of rifampin were withdrawn at suitable intervals and plated on both rifampin-and non-rifampin-containing agar. Comparison of the titers from both types of plates should reveal the extent to which individual colony forming units are rifampin-resistant. The results are shown in Table 4. As indicated, samples taken from three different growth stages of the culture were compared. Early and late log phase cells appear to be best able to demonstrate rifampin-resistance, with 92 percent and 88 percent, respectively, able to grow on plates containing 10 μg/ml rifampin. However, only 73 percent of the late stationary phase cells survived the rifampin plating conditions. It is concluded that the absence of measurable killing in broth (Figs. 6 & 7) presumably reflects the ability of approximately 90 percent of the individual CFU to resist the action of rifampin. In other words, approximately 90 percent of log phase cells survive rifampin treatment and eventually grow. However, this percentage apparently decreases as the culture ages.

TABLE 4

PROPORTION OF POPULATION UNEXPOSED TO RIFAMPIN THAT ARE ABLE TO GROW ON RIFAMPIN-CONTAINING AGAR

Growth Stage[a]	CFU/ml		Percent of Population Able to Grow on Rif Agar[c]
	Rif Agar[b]	Non-Rif Agar	
Early log (3)	3.6×10^6	3.9×10^6	92
Late log (26)	7.6×10^8	8.6×10^8	88
Late stationary (44)	6.4×10^8	8.7×10^8	74

[a]Samples were all taken from the same PPLO broth culture without rif. The numbers in parentheses are the hours of incubation prior to plating. The zero time titer was 1.4×10^6 CFU/ml.

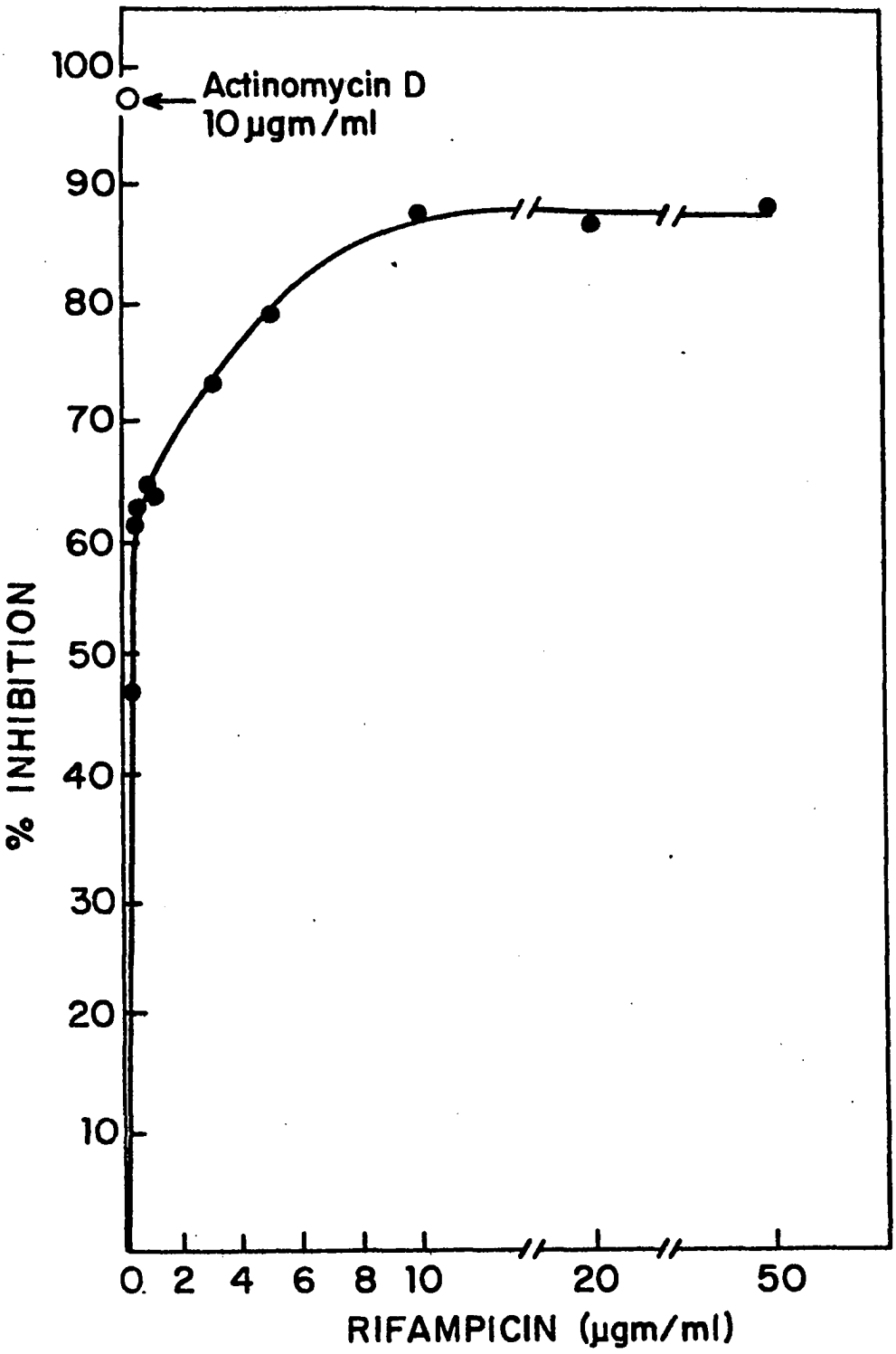
[b]The concentration of rifampin in the agar was 10 µg/ml. These plates were incubated for 2 weeks (see Materials and Methods).

[c]The percent of the mycoplasmal population able to grow on rif-containing agar was calculated by dividing the number of CFU/ml counted on unsupplemented plates and multiplying by 100.

Inhibition of in vivo RNA synthesis. The ability of rifampin to inhibit RNA polymerase activity in prokaryotes is well documented (refs. 124, 124a). To determine the effectiveness of rifampin in inhibiting in vivo RNA synthesis of A. laidlawii, A, we measured the amount of uridine-5-^{3}H incorporated into the cold TCA-precipitable material of growing log phase culture in the presence of increasing concentrations of rifampin.

Log phase cells were prepared for the pulse experiment by inoculating 500 ml PPLO medium with 2 ml freshly thawed stock and incubating for 17 hours at 37C. Samples (10 ml) were pipetted into screw-capped tubes and incubated for an additional hour before rifampin or actinomycin were added. After a 15 minute preincubation with the antibiotic, labeled uridine was introduced and the pulsing procedure followed as described in Chapter II. The percentage of uridine incorporated into antibiotic-treated cells was calculated by setting the corresponding value for untreated cells equal to 100. The results, expressed as percent inhibition as a function of rifampin concentration, are shown in Figure 9. The results indicate that in vivo incorporation of tritiated uridine into cold TCA-precipitable material (RNA) is inhibited 50 percent by 0.25 μg/ml rifampin, and 87 percent by concentrations of 10 to 50 μg/ml rifampin. It is concluded that RNA synthesis is sensitive to low concentrations of rifampin until 60-70 percent inhibition is reached (approximately 2 μg/ml); increasing amounts of rifampin slowly raise the percent inhibition to approximately 87 percent (10 μg/ml). Concentrations between 10-20 μg/ml rifampin do not elevate inhibition above 87 percent. Hence, inhibition by rifampin of RNA synthesis appears to reach a plateau at approximately 87 percent of the total RNA synthesis in the cell. Moreover, the residual 13 percent incorporation allowed by 10-50 μg/ml rifampin is essentially not observed when the cells

Figure 9. Inhibition of uridine-5-^{3}H incorporation into TCA-insoluble material in PPLO broth cultures of A. laidlawii, A, by varying concentrations of rifampin. Cells were preincubated with rifampin or actinomycin D for 15 minutes at 37°C prior to addition of labeled uridine. The pulse lasted 8 minutes. 100% incorporation equaled 4.6×10^{3} dpm/10^{9} CFU. Rifampin was added as an ethanol-medium solution. Actinomycin D was added from a 1 mg/ml solution of 1.5 M KCl.



INHIBITION OF URIDINE-5-H^3 INCORPORATION INTO TCA-INSOLUBLE MATERIAL BY RIFAMPICIN

are treated with 10 μg/ml actinomycin D (Figure 9), suggesting that there is a fraction of RNA synthesis which is resistant to rifampin, but not Actinomycin D.

The data appear to be consistent with the view that the mycoplasmastatic effect of rifampin is associated with its known activity as an inhibitor of RNA synthesis.

Growth response to rifampin of exponentially multiplying cells. Inhibition of RNA synthesis was determined by addition of rifampin to exponentially growing cultures (Fig. 9). Therefore experiments were designed to measure inhibition of growth by addition of the antibiotic to log phase (Figs. 4 and 5), rather than to freshly thawed (Figs. 7 and 8) or stationary phase (Fig. 6) cells. For these experiments pre-warmed PPLO broth without and with rifampin was inoculated with cells from a growing culture. The growth response to 5 μg/ml of rifampin of a very low beginning titer of 2.0×10^4 log phase CFU per ml is shown in Figure 4. There is some suggestion of the biphasic growth pattern observed previously (Fig. 7). However, even with such a low inoculum size, 5 μg/ml cannot produce 100 percent transient inhibition as was observed with a higher inoculum of thawed cells in Figure 7. The portion of the growth curve corresponding to transient inhibition reflects a doubling time of 29 hours (Fig. 4). This value represents 93 percent inhibition of the uninhibited generation time of 2 hours. The second phase doubling time which ensues following the onset of recovery from transient inhibition was also markedly longer (14 hours) than the uninhibited time. Two conclusions were drawn from the results shown in Figure 4: 1) exponentially-growing cells of A. laidlawii, A, appear to be more resistant to rifampin than lag phase cells and, 2) the growth rate following recovery from transient inhibition by rifampin remains substantially slower than that of the control

culture containing no rifampin.

The results of the experiment depicted in Figure 4 suggested another, similar experiment to investigate two hypotheses: 1) the possibility that a higher inoculum size would eliminate the transient phase of inhibition by rifampin and, 2) the slower overall growth rate of A. laidlawii, A, cultures induced by rifampin would produce lower cell yields at their peak of growth. Pre-warmed PPLO broth in three tubes containing 0, 5 and 10 μg/ml of rifampin, respectively, were each inoculated with exponentially growing cells to give initial titers of approximately 2.0×10^6 CFU/ml. The results presented in Figure 5 show that the growth rate is decreased in the presence of rifampin, but there is no evidence of a biphasic response to the antibiotic. Doubling times of the cultures in the presence of rifampin are approximately 5.5 hours with 5 μg/ml and 8.5 hours for 10 μg/ml rifampin. In addition, the growth yield obtained with 5 μg/ml rifampin was one log less than that of the control culture, whereas that obtained with 10 μg/ml rifampin is approximately 1.5 logs lower. It is concluded that rifampin, in concentrations of 5 and 10 μg/ml, reduces significantly the growth yield below that of the uninhibited culture under the conditions of this experiment. Whereas the lower inoculum size of 2×10^4 CFU/ml shows transient inhibition (Fig. 4), the 100-fold higher inoculum size of 2×10^6 CFU/ml shows no such biphasic growth (Fig. 5). Therefore, it appears that transient inhibition is overcome as the inoculum size of cells in log phase increases. A similar conclusion was reached with regard to the effect of inoculum size on transient inhibition when freshly thawed cells were used (Fig. 7). On the other hand, uridine incorporation was inhibited significantly by 5 μg/ml rifampin in a rapidly growing culture of high titer (1.1×10^8 CFU/ml) as seen in Figure 9.

Based on the hypothesis that high concentrations of rifampin

could evoke a biphasic growth response even from high titers of exponentially growing cells, samples were titered at short time intervals to increase the resolution of measurements during transient inhibition. The results, shown in Figure 10, allow a close look at the initial events in the growth response to rifampin of a log phase culture. The high inoculum of 8.5×10^7 CFU/ml responded to the addition of 100 μg/ml rifampin by giving evidence for transient inhibition. However, the evidence is based upon one point, taken at three hours, that gave a titer of 1.5×10^8 CFU/ml.

We conclude that transient growth inhibition of A. laidlawii, A, appears to be a general characteristic of the organism's growth response to rifampin.

Release of inhibition by rifampin of labeled uridine incorporation in log phase cultures of A. laidlawii, A. Therefore, it seemed reasonable to ask whether the inhibition by 5 μg/ml rifampin of RNA synthesis in a log phase culture of high titer (Fig. 9) is transient or is persistent for a significant period of time. To help solve the question, uridine incorporation into acid-insoluble material of a high titer, log phase culture of A. laidlawii, A, was measured. Prior to addition of the labeled uridine, the cells were allowed to incubate for various lengths of time in the presence of 3 μg/ml rifampin. The results are given in Table 5.

Inhibition by 3 μg/ml of rifampin remains quite constant throughout 20 minutes of incubation with the antibiotic giving 72-77 percent inhibition. After 30 and 45 minutes, however, the inhibition is less, at 53 and 55 percent, respectively.

From Table 5 we concluded that 3 μg/ml rifampin significantly reduces RNA synthesis in a log phase culture at high titer and that at 30 and 45 minutes of incubation recovery appears to begin.

Figure 10. Initial growth response to rifampin (100 μg/ml) of A. laidlawii, A, in PPLO broth inoculated with a high titer of log phase cells. Rifampin was added from a DMSO solution. The broken line represents growth of the culture containing no rifampin, and the solid line represents growth of the culture in the presence of 100 μg/ml of rifampin.

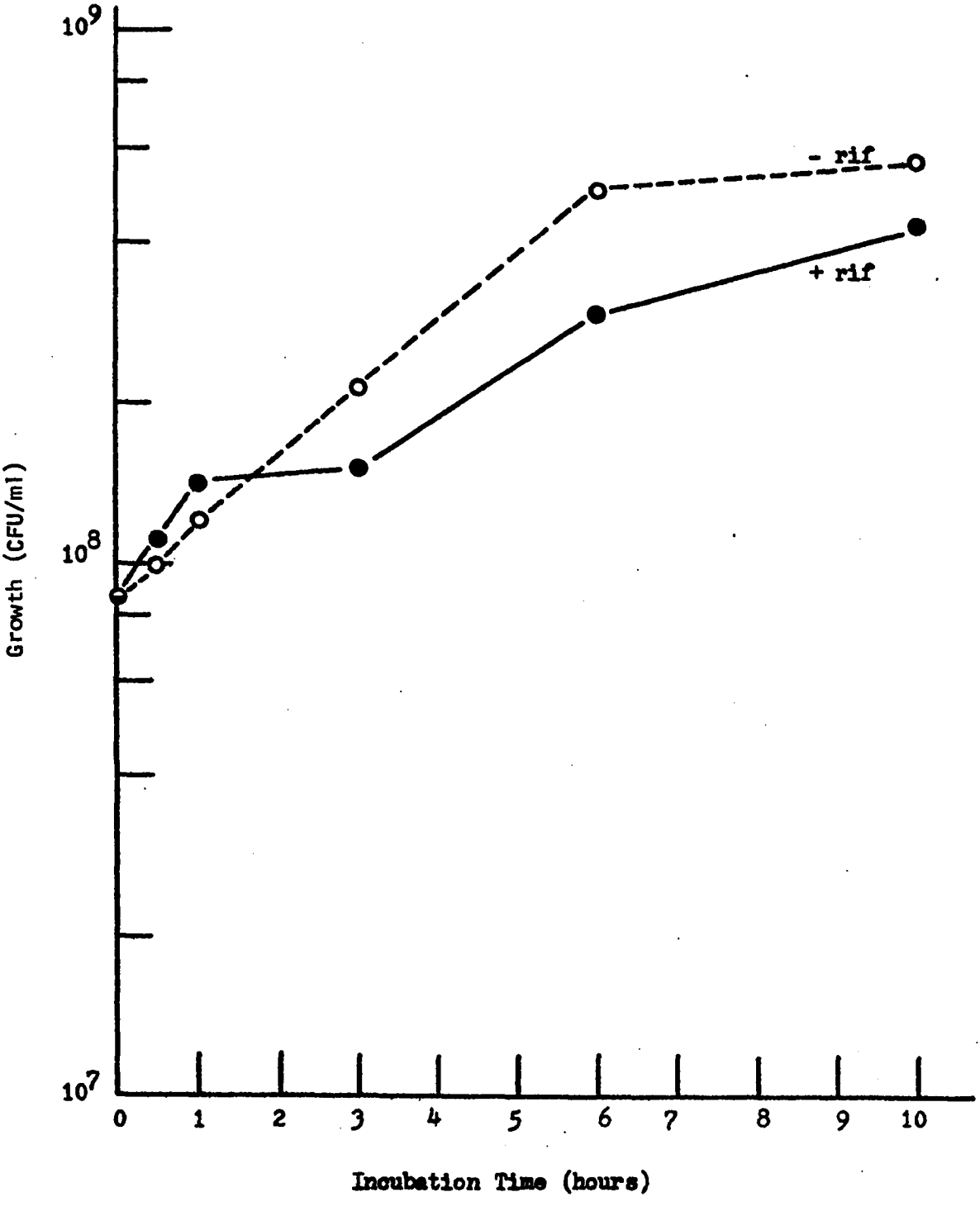

10^9
10^8
10^7
Growth (CFU/ml)
- rif
+ rif
0
1
2
3
4
5
6
7
8
9
10
Incubation Time (hours)

TABLE 5

RELEASE OF INHIBITION BY RIFAMPIN OF URIDINE-5-H^3 INCORPORATION IN LOG PHASE[a] CULTURES OF *A. laidlawii*, A

Rifampin (μg/ml)[b]	Pre-incubation Time (minutes)	Mean cpm[c]	Percent Inhibition
3	5	707	77
None	5	2954	
3	10	791	72
None	10	2760	
3	15	673	72
None	15	2388	
3	20	666	72
None	20	2304	
3	30	506	53
None	30	1080	
3	45	566	55
None	45	1241	

[a]Titers for the experiment were taken from the culture without rifampin from which the samples for pulsing were taken. One titer was taken at the beginning and another at the end of the experiment that lasted 1.5 hours. The titer at the beginning was 3.1×10^8 CFU/ml, the titer at the end was 6.0×10^8 CFU/ml. Titers in the Table represent the average of two different samples taken immediately prior to pulsing.

[b]Rifampin was added as an ethanol-medium solution.

[c]Average cpm values were calculated by averaging two determinations, then subtracting the average zero time incorporation as described in Materials and Methods. The time allowed for incorporation of label was 5 minutes.

Growth Response of M. orale to Rifampin

Since the requirements for cholesterol that distinguish classes of mycoplasmas may be reflected in membrane structure, the effect of rifampin on the growth of M. orale, a cholesterol-requiring mycoplasma, was measured. PPLO broth cultures were inoculated with cells growing logarithmically. Growth was followed throughout an extended incubation time by measuring CFU after 12, 26, 38, 60, and 89 hours of incubation. The results are shown in Figure 11. From an inoculum level of 2.0×10^7 CFU/ml, the uninhibited culture increased by 1.5 logarithms to approximately 3.5×10^8 CFU/ml before decreasing in titer with prolonged incubation. An important observation is that M. orale grew in the presence of up to 100 µg/ml rifampin, just as did A. laidlawii, A, (Fig. 10). Additionally, the results in Figure 11 indicate that the peak growth yields attained by the cultures in the presence of rifampin are generally decreased as the concentration of rifampin is increased. It should be kept in mind, for purposes of comparison, that the PPLO medium for M. orale contains 20 percent serum compared with 10 percent used in PPLO medium for cultures of A. laidlawii, A. Although the membrane of A. laidlawii, A, is structurally different from that of M. orale, both strains show reduced cell yields with up to 100 µg/ml rifampin. Therefore, the effect of rifampin on cell yields appears to be unrelated to the differences in membrane structure.

Environmental factors influencing the response of A. laidlawii, A, to rifampin

Growth Response in Enriched Medium and at Lower Temperatures

Iancini et al (59) observed that upon methionine starvation E. coli underwent a reversal of inhibition of RNA synthesis due to rifampin. This reversal was associated with a reversal of the cidal effect of rifampin. A

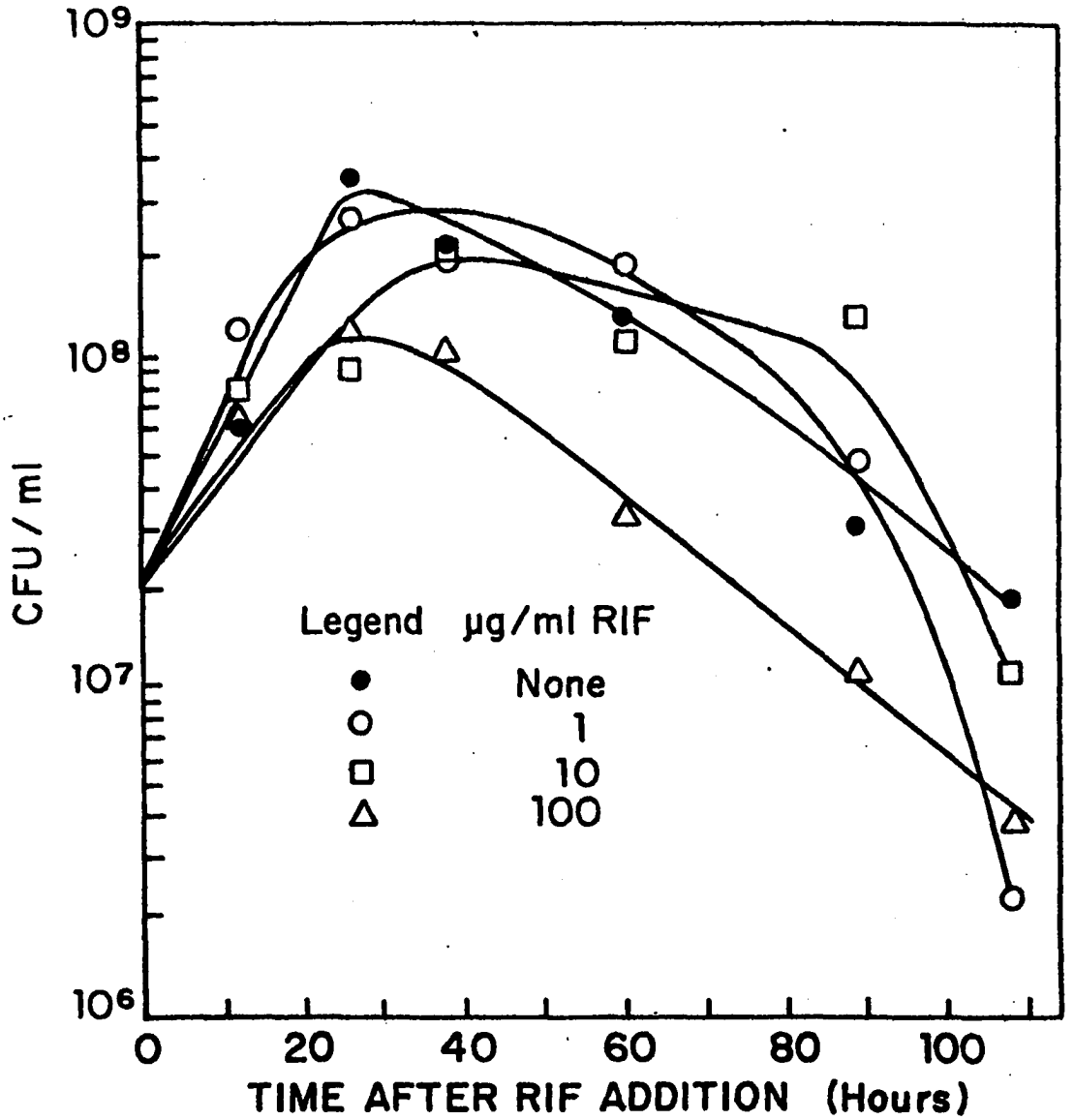
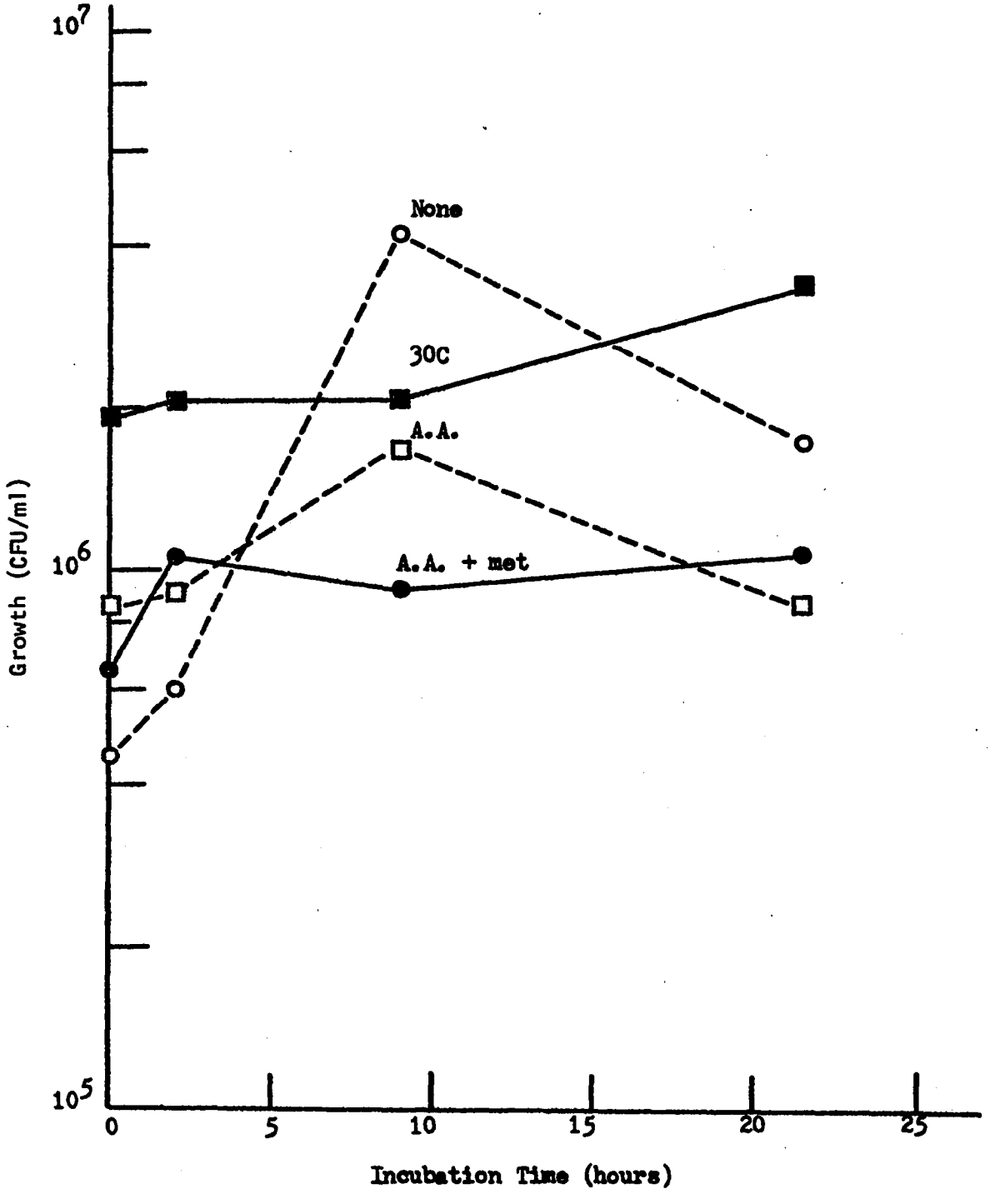


Figure 11. Growth response to various concentrations of rifampin of M. orale in PPLO broth (20% serum). The curves are identified by the number of µg/ml of rifampin. Rifampin was added from a DMSO solution. Inoculum source was a log phase culture.

possible explanation for the static, rather than cidal, effect of rifampin on growth of A. laidlawii, A, is that if the amino acid requirements of the organism were not fully met by the growth medium, a slowdown in RNA synthesis might result in a fashion similar to the stringent control of RNA synthesis in E. coli and reduce the organism's sensitivity to inhibition of RNA synthesis. The experiment designed to investigate this possibility consisted of reducing the drain on the amino acid pool in two ways: 1) addition of more amino acids, especially methionine, into the growth medium; and 2) reduction of the metabolic rate of the culture by incubation at a lower temperature (30°C rather than 37°C). The purpose of the experiment was to determine if rifampin could kill the cells under the following conditions: 1) PPLO broth medium supplemented with 1 percent (w/v) NZ-case amino acids; 2) PPLO broth medium supplemented with 1 percent NZ-case amino acids plus 200 μg/ml methionine; and 3) the usual PPLO broth medium but incubation of the culture at 30°C. A fourth culture in PPLO broth incubated at 37°C served as the control. All four cultures contained 5 μg/ml rifampin. Cultures were inoculated with log phase organisms and titers taken after 2, 9 and 21.5 hours. The results (Fig. 12) show that under none of the conditions in the experiment were titers reduced below the inoculum size which would indicate killing by rifampin. However, an effect on rifampin-inhibition of growth was observed. The three cultures containing supplements to the PPLO broth medium allowed increased inhibition by rifampin with increasing additions to the medium. After 8 hours of incubation, inhibited growth of the culture containing no additions to the medium reached a titer approximately 1 logarithm above the inoculum level, the culture containing the addition of 1 percent NZ-case amino acid yielded an increase of 0.5 logarithm above inoculum level, and the richest medium, containing PPLO broth supplemented with

Figure 12. Inability of rifampin to become mycoplasmacidal for A. laidlawii, A, under altered culture conditions in PPLO broth. The four conditions as designated in the figure are: none, PPLO broth incubated at 37C; 30C, PPLO broth incubated at 30C; A.A., PPLO broth supplemented with 1 percent N-Z case amino acids, incubated at 37C; and A.A. + met, PPLO broth supplemented with 1 percent N-Z case amino acids plus 200 μg/ml methionine, incubated at 37C. All four cultures contain 5 μg/ml rifampin added from an ethanol-medium solution.


10^7
10^6
10^5
Growth (CFU/ml)
None
30C
A.A.
A.A. + met
0
5
10
15
20
25
Incubation Time (hours)

NZ-case amino acids plus 200 μg/ml methionine, allowed virtually no growth compared to the titer taken after 2 hours of incubation. The latter culture did show an initial increase in titer within the first 2 hours of incubation, however. This finding is another instance of an initial increase in titer in a culture in the presence of rifampin, the meaning of which is unclear. It is concluded that addition of amino acids to PPLO broth medium increased the sensitivity of A. laidlawii, A, to growth inhibition by rifampin. Moreover, lowering the metabolic rate of the organism by reducing the incubation temperature to 30C, or supplementing with 1.0 percent (w/v) of NZ-case amino acids with or without additional methionine did not provide conditions allowing rifampin to exert a cidal effect on A. laidlawii, A. Methionine added in overabundance also rules out methionine starvation as the cause of a mycoplasmastatic effect due to possible requirements of methionine as a methyl group donor for modification of DNA or RNA.

Response of Growth to Rifampin in Serum-less Media

In order to investigate the nature of mycoplasma's ability to resist killing by rifampin, A. laidlawii, A, was grown in medium that was more defined than the PPLO broth required for most mycoplasma strains.

Casitone medium. Casitone medium (described fully in Chapter II) containing 2 percent casitone and 1 percent dehydrated commercial yeast extract requires no serum supplement to support growth of A. laidlawii, A. Glucose was added (0.5 percent, final concentration) to casitone medium as a carbon source. Growth of A. laidlawii, A, in casitone broth is similar to that in PPLO broth in that the doubling time is 90-120 minutes, the maximum yield of cells is approximately 10^9 CFU/ml, and the titer remains stable for several hours before declining.

Information regarding the response of A. laidlawii, A, growth

to the presence of rifampin in casitone medium was needed, since the experiment depicted in Figure 12 indicated nutrients influenced the cells' response to the antibiotic. Casitone broth differs significantly in nutritive content (Chapter II) from PPLO broth, one major difference being the lack of serum in casitone broth. An experiment was performed to measure the titers of A. laidlawii, A, cultures throughout a 24 hour incubation period in rifampin-containing casitone broth. The effect of ionic strength on inhibition by rifampin was tested by varying the concentrations of NaCl. Rifampin was used at a concentration of 100 μg/ml in order to elicit as definitive a result as possible. The results of this experiment are recorded in Table 6. The result of major significance is that, after incubation for 24 hours, cultures containing rifampin were found to be reduced in titer by several orders of magnitude below the inoculum level of 3.1×10^7 CFU/ml. These drastically reduced titers are evidence for a mycoplasmacidal effect by rifampin and are underlined in Table 6. Whereas growth ensued more rapidly in the presence of 0.30 and 0.55% added NaCl, killing was slightly less efficient than at 0.075% added NaCl. The cidal effect was evident for all the variations in ionic strength tested. Control cultures without rifampin showed the maximum growth expected, attaining titers of around 10^9 CFU/ml after 24 hours incubation. The titers of the cultures after 8 hours incubation offer additional information about the growth response of A. laidlawii, A, to rifampin. There is no reduction by rifampin in CFU below the inoculum level of 3.1×10^7 CFU/ml after 8 hours of incubation. The value that does decrease below inoculum level in a rifampin-containing culture, 2.2×10^7 CFU/ml for the culture containing 0.075 percent NaCl, cannot be attributed to rifampin activity since the control culture without rifampin also showed a similar decrease, to 2.8×10^7

TABLE 6

MYCOPLASMACIDAL ACTIVITY OF RIFAMPIN ON A. laidlawii, A, IN CASITONE BROTH CONTAINING VARYING NaCl CONCENTRATIONS

Supplements			CFU/ml		
Rifampin[a] (100 μg/ml)	Added NaCl[b]		Hours of Incubation		
	Percent	(Molarity)	0	8	24
-	0.075	(6.1 mM)	3.1×10^7	2.8×10^7	9.1×10^8
+	0.075	(6.1 mM)	3.1×10^7	2.2×10^7	7.4×10^3
-	0.15	(1.7 mM)	3.1×10^7	4.9×10^7	1.0×10^9
+	0.15	(1.7 mM)	3.1×10^7	4.7×10^7	3.7×10^4
-	0.30	(3.3 mM)	3.1×10^7	1.9×10^8	1.3×10^9
+	0.30	(3.3 mM)	3.1×10^7	5.0×10^7	3.1×10^4
-	0.55	(6.1 mM)	3.1×10^7	2.8×10^8	1.6×10^9
+	0.55	(6.1 mM)	3.1×10^7	4.4×10^7	2.4×10^4

[a]Rif was added from a stock solution dissolved in DMSO.

[b]Each of the eight cultures (10 ml) was inoculated by adding 1.0 ml of log phase cells from casitone broth culture containing 0.5 percent NaCl to 9 ml of fresh casitone broth to give the final added concentrations of NaCl indicated.

CFU/ml, after 8 hours incubation. Titers of the cultures without rifampin after 8 hours of incubation are increasingly higher as the percent of NaCl is increased. Since the culture used for the inoculum source for this experiment contained 0.5 percent NaCl, the lack of growth indicated by the titers after 8 hours in cultures of lower concentration of NaCl, (0.075 and 0.15 percent) probably represents a lag phase induced by change in ionic strength. The occurrence of such a lag phase allows another observation to be made: even cells put into lag phase by the shock of a sudden decrease in ionic environment from a high (0.5 percent NaCl) to a 6.7-fold lower (0.075 percent NaCl) ionic strength are apparently not susceptible to significant cidal activity by rifampin during the initial 8 hours. Conclusions made from the data in Table 6 are: 1) rifampin has a very definite mycoplasmacidal action against A. laidlawii, A, in casitone broth after 24 hours of incubation; 2) rifampin does not, however, kill the organisms after exposure to the antibiotic for 8 hours in casitone medium; 3) even organisms in lag phase growth due to their introduction into medium of lower ionic strength are not susceptible to killing by rifampin.

Response of the cells to the presence of rifampin in casitone medium supplemented with 10 percent serum is recorded in Table 7. The cultures without serum supplement, showing the mycoplasmacidal effect of rifampin, are the same ones as are represented in Table 6. As seen by the titers at the 24-hour time for the cultures containing serum, 100 μg/ml of rifampin did not reduce the titer below the inoculum level (Table 7). The titers of the culture containing both serum and rifampin were lower than those of the serum-containing culture without rifampin after 8 and 24 hours of incubation. These titers indicated that, although cells were not killed,

TABLE 7

PROTECTION BY SERUM AGAINST MYCOPLASMACIDAL ACTIVITY OF RIFAMPIN IN CASITONE BROTH

Supplements		CFU/ml		
Rifampin (100 µg/ml)	Serum (10%)	Incubation Time (hrs)		
		0	8	24
-	-	3.1×10^7	1.9×10^8	1.3×10^9
+	-	3.1×10^7	5.0×10^7	3.1×10^4
-	+	3.1×10^7	2.9×10^8	9.0×10^8
+	+	3.1×10^7	5.3×10^7	1.6×10^8

NOTE: The conditions for this experiment are exactly as described for Table 6 except for the addition of serum; the NaCl concentration used for the casitone broth in this experiment was 0.30 percent (final, added amount).

their growth rate was slower in the presence than in the absence of rifampin. Therefore, it is concluded that serum antagonizes the mycoplasmacidal activity of rifampin in casitone broth.

SP-YE medium. Another serum-less medium that supports growth of A. laidlawii, A, is SP-YE broth (Chapter II). Information concerning the response of A. laidlawii, A, to rifampin in a serum-less medium, other than casitone, was desired in determining the uniqueness of serum as an antagonist of the mycoplasmacidal action of rifampin. Data were obtained by measuring titers attained in 24 hours of incubation in SP-YE cultures containing 100 μg/ml rifampin. The inoculation of the cultures consisted of adding 1.0 ml of a late log phase culture in SP-YE broth to 9 ml of fresh SP-YE medium. The data, collected from four different experiments, are shown in Table 8. The experiments are shown in order of increasing inoculum size which resulted from use of cultures of different titers for inocula. Cultures show a static growth response to rifampin since their titers were not reduced to below the inoculum level after 24-hour incubation. The very slight decrease in the rifampin-containing culture of Experiment 2 was considered insignificant. The conclusion made from these data is that components of growth media other than serum antagonize the cidal activity of rifampin toward A. laidlawii, A.

Agents which Potentiate Rifampin Killing in SP-YE Medium

Membrane function should be included as a possible factor responsible for the difference between the cidal and static response to rifampin by mycoplasma. Experiments were therefore designed to detect a potentiating effect, if any, of the cidal effect of rifampin due to addition to SP-YE medium of agents likely to increase permeability of rifampin into the mycoplasmal cell. Agents were selected on the basis of their presumed

TABLE 8

GROWTH RESPONSE TO 100 μg/ml RIFAMPIN OF *A. laidlawii*, A, IN SP-YE MEDIUM

Experiment	Rifampin* (100 μg/ml)	Titer (CFU/ml)	
		Zero Time	24 Hr. Incubation
1	-	2.7×10^7	6.8×10^8
	+	2.7×10^7	8.0×10^7
2	-	3.3×10^7	1.3×10^9
	+	3.3×10^7	3.1×10^7
3	-	8.8×10^7	5.3×10^8
	+	8.8×10^7	9.4×10^7
4	-	1.0×10^8	2.3×10^8
	+	1.0×10^8	2.0×10^8

*Rifampin was added from a DMSO stock solution.

or known effects upon membrane or transport systems, or, alternatively, their capacity to increase the solubility of rifampin in the growth medium. The rationale of the experiment is based upon the results depicted in Table 8. Since rifampin has a static rather than cidal effect on A. laidlawii, A, in SP-YE broth, an agent that could potentiate the cidal activity of rifampin should be easily detected by a titer significantly reduced below inoculum levels in cultures containing both rifampin and the agent. Potentiation by agents that themselves cause killing would show their potentiating effect by increased killing when combined with rifampin. The results, expressed as CFU/ml before and after 24 hours incubation, can be seen in Table 9. The effects of added agents on the state of a culture, both in the presence and the absence of rifampin, can be generally catagorized as follows: 1) allowing growth or no growth in the absence of rifampin and producing significant killing in the presence of rifampin; 2) producing killing in the absence of rifampin, but increased killing in the presence of rifampin; 3) allowing growth in the absence of rifampin, and no growth in the presence of rifampin. The first two categories would suggest that the agent acted as a potentiating agent for the cidal activity of rifampin. The third category suggests the reverse, that the agent did not help rifampin kill the target organism. According to the results in Table 9, agents effecting the results considered to belong in the first two categories are Gramicidins J and S, DMSO at concentrations of 7.5 and 10 percent, Tris, pH 8.0, at 0.06 M, Tween 80, melletin, and, possibly, mycostatin. Since these potentiating agents are known to act on membranes and, since DMSO probably makes cells more permeable to rifampin, impermeability of cells to rifampin is suggested as the basis for absence of killing in SP-YE broth.

TABLE 9

POTENTIATION OF RIFAMPIN KILLING BY VARIOUS AGENTS IN SP-YE BROTH

Added Agent	Amount	Rifampin* (100 µg/ml)	Titer (CFU/ml) Zero Time	Titer (CFU/ml) 24 Hours
		Experiment 1		
None	—	—	2.7×10^7	6.8×10^8
None	—	+	2.7×10^7	8.0×10^7
Tris,pH 8.0	0.02 M	—	2.7×10^7	8.6×10^8
Tris,pH 8.0	0.02 M	+	"	1.0×10^8
Tris,pH 8.0	0.04 M	—	"	2.7×10^8
Tris,pH 8.0	0.04 M	+	"	9.5×10^7
Tris,pH 8.0	0.06 M	—	"	2.7×10^8
Tris,pH 8.0	0.06 M	+	"	5.9×10^5
DMSO	5% (v/v)	—	"	6.6×10^8
DMSO	5% (")	+	"	4.4×10^7
DMSO	7.5% (")	—	"	4.0×10^8
DMSO	7.5% (")	+	"	3.5×10^6
DMSO	10.0% (")	—	"	6.1×10^6
DMSO	10.0% (")	+	"	1.9×10^6
		Experiment 2		
None	—	—	3.3×10^7	1.3×10^9
None	—	+	3.3×10^7	3.1×10^7
Amphotericin B	50 µg/ml	—	3.3×10^7	4.3×10^8
Amphotericin B	50 "	+	"	2.7×10^7

*Rifampin was added from a DMSO solution of 10 mg/ml

TABLE 9 (Continued)

Added Agent	Amount	Rifampin* (100 µg/ml)	Titer (CFU/ml) Zero Time	Titer (CFU/ml) 24 Hours
		Experiment 3		
None	—	—	8.8×10^7	5.3×10^8
None	—	+	8.8×10^7	9.4×10^7
Mycostatin	1.0 µg/ml	—	8.8×10^7	3.4×10^8
Mycostatin	1.0 "	+	"	9.0×10^7
Mycostatin	10 "	—	"	4.4×10^8
Mycostatin	10 "	+	"	3.2×10^7
Melliten	1.0 "	—	"	2.7×10^7
Melliten	1.0 "	+	"	1.4×10^5
Melliten	10 "	—	"	1.7×10^8
Melliten	10 "	+	"	4.5×10^7
		Experiment 4		
None	—	—	1.0×10^8	2.3×10^8
None	—	+	1.0×10^8	2.0×10^8
Gramicidin J	0.1 µg/ml	—	1.0×10^8	1.6×10^8
Gramicidin J	0.1 "	+	"	1.4×10^5
Gramicidin S	0.1 "	—	"	7.7×10^3
Gramicidin S	0.1 "	+	"	5.0×10^2
Polymixin B	1.0 "	—	"	9.9×10^8
Polymixin B	1.0 "	+	"	1.4×10^8
Valinomycin	1.0 "	—	"	3.6×10^8
Valinomycin	1.0 "	+	"	1.8×10^8

*Rifampin was added from a DMSO solution of 10 mg/ml

TABLE 9 (Continued)

Added Agent	Amount	Rifampin * (100 µg/ml)	Titer (CFU/ml) Zero Time	Titer (CFU/ml) 24 Hours
	Experiment 4 (Continued)			
Bacitracin	1.0 µg/ml	—	1.0×10^8	9.2×10^8
Bacitracin	1.0 "	+	"	1.3×10^8
HHQO	1.0 "	—	"	9.5×10^8
HHQO	1.0 "	+	"	1.2×10^8
DNP	1.0 "	—	"	1.1×10^9
DNP	1.0 "	+	"	2.0×10^8
Tween 80	1:500 dilution	—	"	$<5.0 \times 10^2$
Tween 80	1:1000 "	—	"	2.8×10^3
Tween 80	1:5000 "	—	"	4.1×10^7
Tween 80	1:5000 "	+	"	$<5.0 \times 10^2$

*Rifampin was added from a DMSO solution of 10 mg/ml

Genetic Factors Involved in Resistance of *A. laidlawii*, A, to Rifampin

Pre-existence of Rifampin-Resistant (Rif-R) cells in Cultures Unexposed to Rifampin: The Fluctuation Test

The nature and source of occurrence of rifampin-resistant variants was further investigated by use of the classical Luria-Delbruck fluctuation test (66). This statistical test is designed to answer the following question: are the observed variants due to an adaptive response of a constant proportion of the population, or are they the result of random, spontaneous, genetic events? If the latter is true, then the number of variants measured in individual parallel cultures, each growing from an inoculum of a very few cells, should fluctuate widely. If, however, an adaptive response causes the rifampin-resistance, then the number of variants in a series of parallel cultures should fluctuate no more than that found in a series of samples taken from the same bulk culture. Table 10 shows that the variance (the fluctuation measurement) in the number of rifampin-resistant CFU is much greater (11,245) for the 10 samples from the parallel cultures than that (693) for the 17 samples from the same bulk culture. Hence, the rifampin-resistant variants in population of *A. laidlawii*, A, fluctuate widely from culture to culture. The results strongly suggest that the basis of these variants is a random, spontaneous mutagenic event and that the mycoplasmal population is not homogeneous. This conclusion cannot be held strictly, however, due to theoretical assumptions required in the application of the fluctuation test that cannot be assured in the case of *A. laidlawii*, A, whose reproductive cycle is not well characterized. It is also possible to calculate the mutation frequency for the single bulk culture (control) and the parallel test samples. The mutation frequency for the

TABLE 10

RANDOM NATURE OF EVENTS LEADING TO
RIF-RESISTANT CFU: THE FLUCTUATION TEST

Control Samples	Test Samples
210	53
201	363
197	154
234	143
227	164
211	119
195	84
213	14
231	13
156	55
209	
232	
199	
147	
173	
185	
168	
Sum 3,388	1,162
Mean ($\bar{x}$) 199	116
$\Sigma(x-\bar{x})^2$ 11,093	101,209
Variance 693	11,245

Note: The formula by which variance was calculated is:

$$\text{Variance} = \frac{\Sigma(x-\bar{x})^2}{n-1}$$

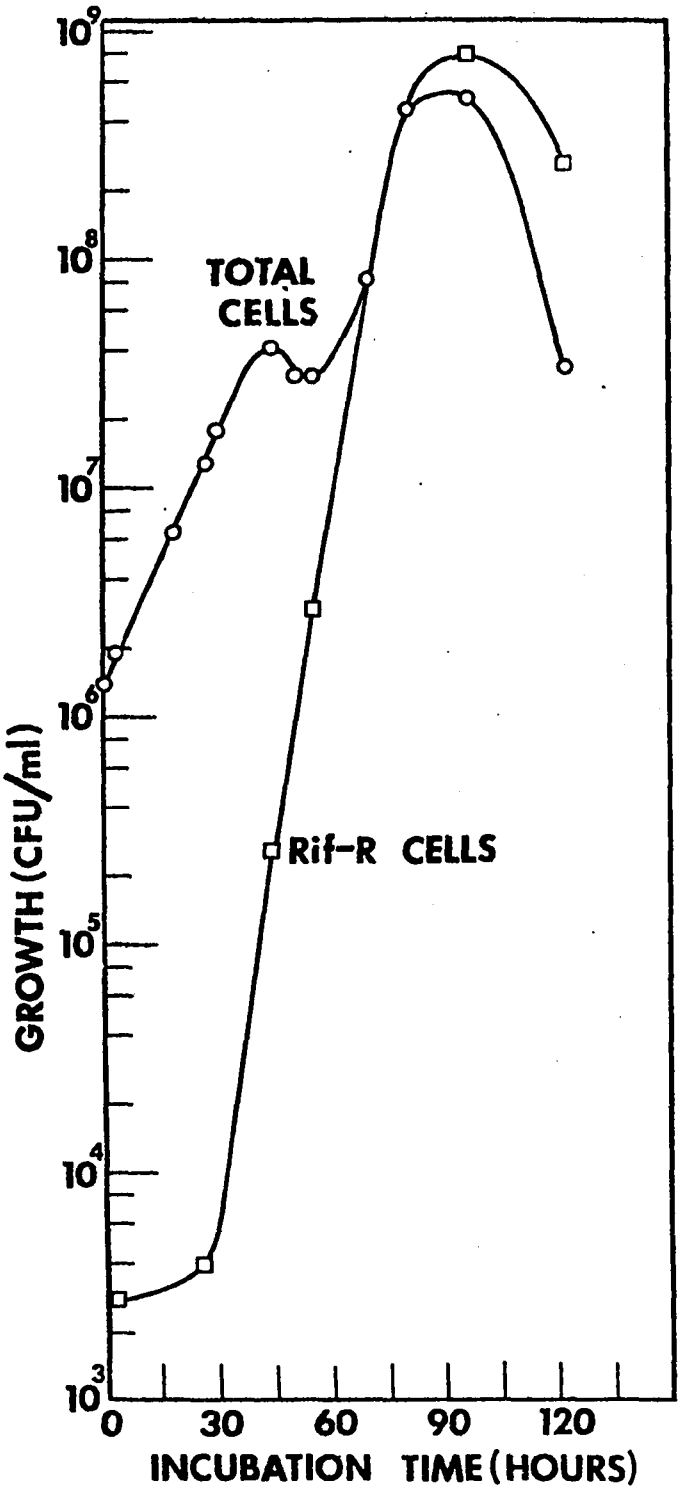
$\bar{x}$ = mean of the observed numbers of rif-resistant CFU
x = observed number number of rif-resistant CFU
n = number of observations (samples)

control is 3.3×10^{-7}, and the average mutation frequency for the ten parallel cultures is 8.1×10^{-7}. Also, the mutation rate, calculated from the formula $r = aNt \log(aNtC)$ is 2.5×10^{-7}. In this equation (66) r = the average number of mutants per sample, C is the number of cultures or samples (10 in this case), a is the mutation rate, and Nt equals the number of bacteria per sample.

Selection by Rifampin for Pre-Existing Rif-R Population

We asked whether outgrowth from rifampin inhibition is due to selection for a pre-existing population of Rif-R cells in the predominantly Rif-R population which had not been exposed previously to the antibiotic. Cells were cultivated in PPLO broth with 10 μg/ml rifampin. At suitable intervals, samples were plated on PPLO agar without and with 10 μg/ml antibiotic. The results are presented in Figure 13. The viable cell count found on agar without rifampin is a measure of the total number of cells. The count observed on agar containing rifampin reflects the number of Rif-R cells in the total population. The total number of cells in the initial inoculum was approximately 1.5×10^6. Of these, 3×10^3 CFU/ml, or 0.2% were Rif-R. In the presence of 10 μg/ml rifampin, the total population shows a biphasic growth pattern. The initial doubling time of the total population, reflecting the predominantly Rif-S component, is approximately 8-9 hours. The titer declines slightly before resuming rapid growth and eventually attains a titer of approximately 5×10^8 CFU/ml. The doubling time of the uninhibited Rif-R component is approximately 4 hours during the slow initial growth of the total population. Eventually, the faster growing Rif-R cells outgrow the Rif-S cells, take over the culture, and comprise the total population. The titers at 96 and 122 hours suggest that much of the Rif-R population may actually be rifampin-dependent since titers measured on agar containing rifampin were slightly higher than those measured on agar without rifampin.

Figure 13. Selection for a rif-resistant population in a PPLO broth culture of A. laidlawii, A, containing 10 μg/ml of rifampin. Inoculum consisted of log phase cells. Viable counts were performed on PPLO agar without and with 10 μg/ml rifampin. Plates were incubated for eight days. Rifampin was added as a DMSO solution. Circles represent the total cell population; squares represent the Rif-resistant population.



SELECTION BY RIFAMPIN FOR RIF-R POPULATION

Therefore, we conclude that recovery and outgrowth from rifampin inhibition is due to selection by the antibiotic for a pre-existing population of Rif-R mutants.

Stability of Outgrown Rif-R Population

We observed that rifampin (100 μg/ml) has a static effect on log phase A. laidlawii, A, in serum-containing SP-YE medium; the period of inhibition is followed by outgrowth which reaches a peak titer of approximately 1.9×10^9 CFU/ml after 60-70 hours of incubation (data not presented here). These findings are essentially similar to those for PPLO broth. Demonstration of stable resistance to rifampin would implicate a genetic origin for the resistant characteristic. Therefore, a culture of A. laidlawii, A, grown in SP-YE medium containing 100 μg/ml rifampin for 97 hours was subcultured three times for a total of 24 doublings (62 hours) in the same medium in the absence of rifampin. Hence, the resulting culture could contain a maximum of only 4.5×10^{-6} percent of the original inoculum from the first outgrown culture. Upon renewed exposure to rifampin, cells from this culture should show static growth if the rif-resistance of the original culture was due to a reversible "adaptation". However, if the resistance was due to a stable genetic change, the resistant phenotype should express itself by undelayed growth upon re-introduction into rifampin-containing medium. The results are shown in Figure 14.

The undelayed growth is evidenced by an exponential increase in CFU of over one logarithm in 12 hours of incubation in medium containing 100 μg/ml rifampin. The similar growth rate (doubling time, 2 hrs) of the cells in non-rifampin medium further illustrates the integrity of the rifampin resistant character of the original outgrown cells. After 24 hours of incubation, however, the culture containing rifampin was reduced in titer below that measured at 12 hours, whereas the culture without rifampin

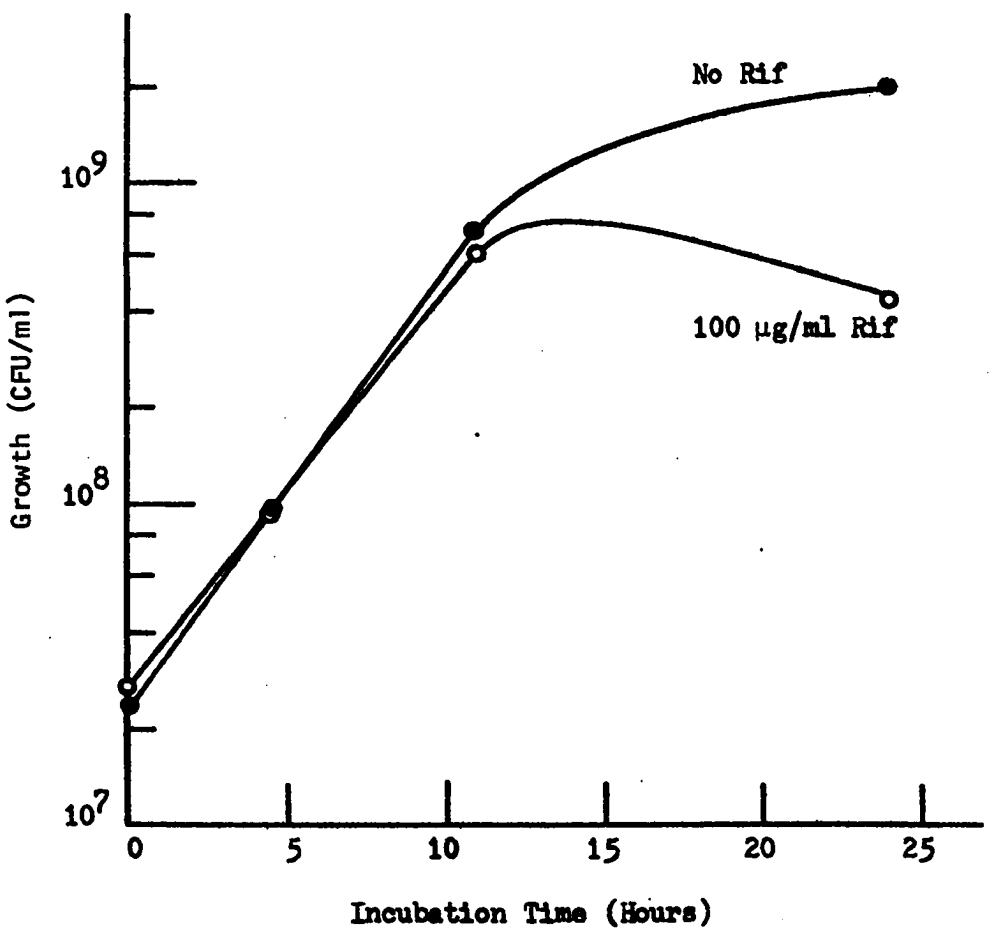


Figure 14. Retention by outgrown culture of rif-resistant phenotype after subculturing in serum-containing SP-YE medium in the absence of rifampin for 24 generations. Inoculum cells were in log phase and the rifampin was added as a DMSO solution. Viable counts were performed on PPLO agar. Plates were incubated for 4 days.

continued to increase between 12 and 24 hours of incubation. It was concluded from this experiment that the rifampin-resistance of outgrown cells is stable and heritable. However, upon approaching stationary phase the rifampin-grown culture appears to become vulnerable to rifampin. This experiment also reinforces the conclusion made based on the data in Table 2, that rifampin-resistant outgrowth cannot be attributed to changes in the medium or the rifampin during incubation.

Mutagen-Induced Rif-R Mutants

Mutagenic events are obvious possibilities for explaining the development of a rif-resistant population. Demonstration that common mutagens induce a frequency of rif-resistant variants that is significantly higher than the spontaneous frequency would provide evidence of the organism's capability for undergoing genotypic alterations. Frequencies of rif-resistant variants were therefore measured under spontaneous and mutagenic conditions (see Materials and Methods).

U.V.-induced mutants. The results of these experiments are reported in Table 11. The significant result of Table 11 is that, following U.V. irradiation that produced 70 percent killing, the frequency of rif-resistant variants increased significantly above spontaneous frequencies. When casitone agar lacking serum was used as the selective medium, the induction appeared more pronounced, with induced frequencies of 333 and 355 fold for resistance to 50 and 100 μg/ml rifampin, respectively. When serum is present in the selection plates, the lower induction, 80 and 84 fold for resistance to 50 and 100 μg/ml, respectively, appears due mostly to the higher frequencies of spontaneous variants. The spontaneous frequencies of rifampin-resistant variants on serum-containing selection medium are 5.5×10^{-7} and 5.1×10^{-7} for 50 and 100 μg/ml rifampin, respectively,

TABLE 11

INCREASED FREQUENCY OF RIF-RESISTANT VARIANTS OF *A. laidlawii*, A, FOLLOWING A 70 PERCENT REDUCTION IN CFU BY U.V. IRRADIATION

Mutagenic Conditions[a]	Supplements to Selective Agar		Total Number of CFU[b] Applied to Rif Agar	Rif-Resistant CFU[c]	Frequency of Rif-Resistant CFU[d]	Fold Increase of Rif-Resistant CFU Frequency[e]
	Serum (10 percent)	Rifampin (μg/ml)				
Induced	-	50	3.3×10^6	102	3.1×10^{-5}	333
Spontaneous	-	50	2.2×10^9	246	1.1×10^{-7}	
Induced	-	100	3.3×10^6	128	3.9×10^{-5}	355
Spontaneous	-	100	2.8×10^9	286	1.0×10^{-7}	
Induced	-	200	8.3×10^7	92	1.1×10^{-6}	-
Induced	+	50	8.3×10^6	366	4.4×10^{-5}	80
Spontaneous	+	50	1.7×10^9	923	5.4×10^{-7}	
Induced	+	100	8.3×10^6	356	4.3×10^{-5}	84
Spontaneous	+	100	2.8×10^9	1440	5.1×10^{-7}	

[a] Conditions for mutagenesis are described in Materials and Methods.

[b] The total number of CFU applied to the rif plates was determined by titering on serum-containing casitone agar without rifampin.

[c] Rif-resistant CFU are defined as CFU having a "fried egg" appearance after 4 days incubation on the selective medium indicated. Rifampin was added as a DMSO solution.

[d] The frequency of rif-resistant CFU was determined by dividing the number of rif-resistant CFU by the total number of CFU applied to rif plates.

[e] The fold increase of rif-resistant CFU frequency was calculated by dividing the induced frequency by the spontaneous frequency of rif-resistant CFU.

whereas the frequencies for the corresponding rifampin concentrations on non-serum casitone selection plates are 1.1×10^{-7} and 1.0×10^{-7}. Therefore, the spontaneous frequency of rifampin-resistant variants for both concnetrations of rifampin is higher in the presence than in the absence of serum by a factor of approximately five.

NTG-induced mutants. Table 12 shows that NTG, at the concentrations used, is not so effective as U.V. irradiation in inducing rifampin-resistant variants. An NTG concentration of 10 μg/ml elicits a 10 fold increase in the frequency of rifampin-resistant variants (5.9×10^{-6}) above that of the spontaneous frequency of 5.8×10^{-7}. However, since the frequency was not increased by raising the concentration to 20 μg/ml NTG (the frequency drops to 2.2×10^{-6}), NTG cannot be regarded as an effective inducer of rifampin-resistant variants of A. laidlawii, A, under these conditions.

Stability of Spontaneous and NTG-Induced Rif-R Mutants

As a test for the criterion used for the identification of rifampin-resistant variants, i.e., growth of a colony on rifampin-containing agar in 4 days (the time required for colony development on non-rifampin agar), growth curves were compared of two rifampin-resistant variants derived from the NTG mutagenesis conditions described in Table 11. One variant, RV4, arose spontaneously; and the other, RV8, was selected following treatment with 10 μg/ml NTG. The curves, represented by Figure 15, show the growth of the two variants subcultured in both the presence and absence of 5 μg/ml of rifampin after having been cultivated in the absence of rifampin for 42 hours from low inocula of thawed stock cultures (also depicted in Figure 15). Therefore, the two variants underwent over 14 doublings in the absence of rifampin before transfer into the rifampin-containing sub-

TABLE 12

FREQUENCY OF RIF-RESISTANT VARIANTS OF A. laidlawii, A, FOLLOWING TREATMENT WITH NTG

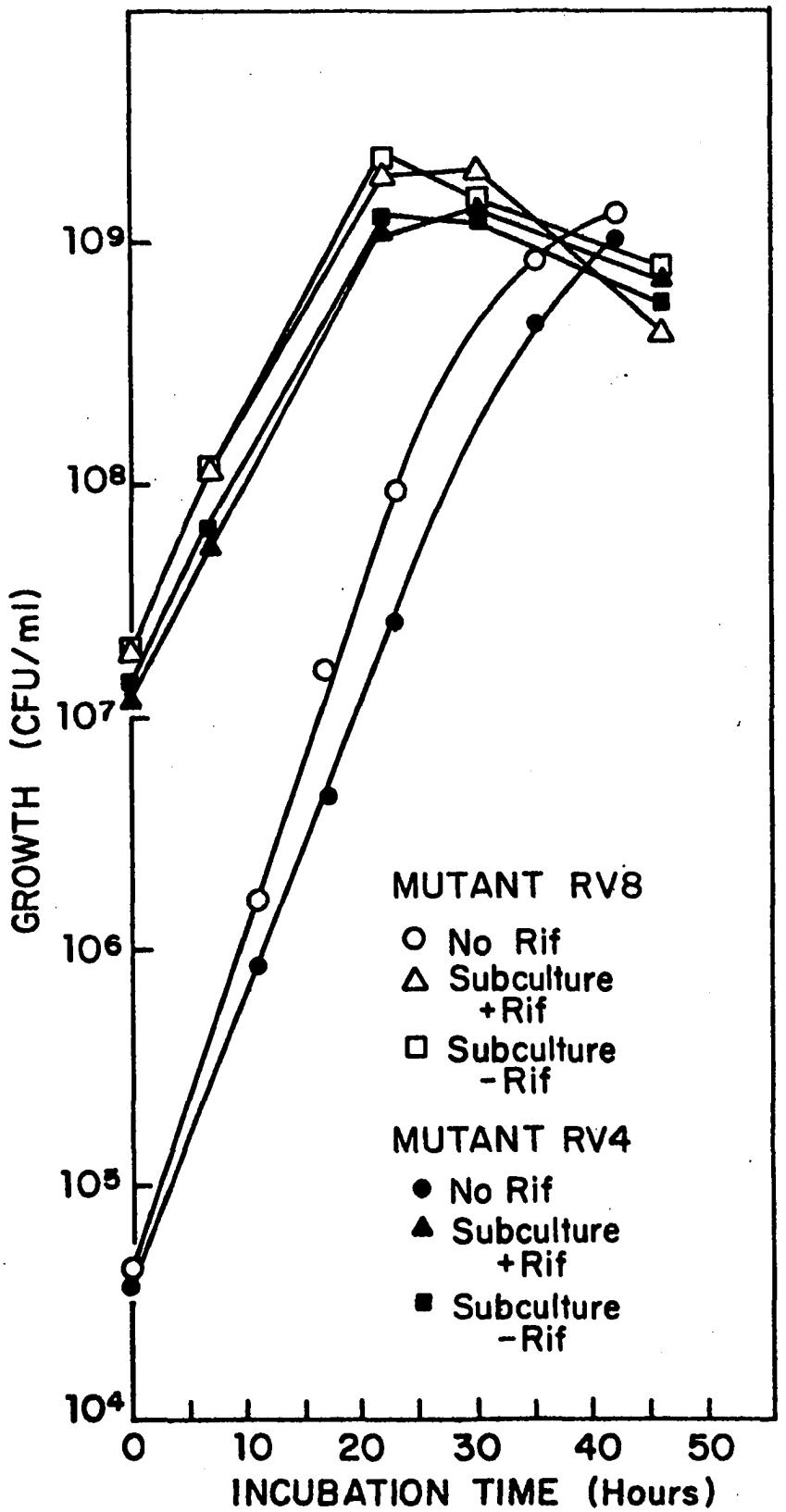
Concentration of NTG (μg/ml)	Total Number of CFU Applied to Rif Plates[a]	Number of Rif-Resistant CFU[b]	Frequency of Rif-Resistant CFU[c]
0	4.3×10^8	2.5×10^2	5.8×10^{-7}
5	2.9×10^8	2.1×10^2	7.2×10^{-7}
10	9.7×10^7	5.7×10^2	5.9×10^{-6}
20	1.9×10^8	4.2×10^2	2.2×10^{-6}

[a]The total number of CFU applied to the rif plates was determined by titering on PPLO agar without rifampin.

[b]Rif-resistant CFU are defined as the ability to produce a colony of "fried egg" appearance within 4 days on PPLO agar containing 100 μg/ml rifampin. Rifampin was added as an ethanol-medium solution.

[c]The frequency of rif-resistant CFU was calculated by dividing the number of rif-resistant CFU by the total number of CFU applied to the rif plates for a given concentration of NTG.

Figure 15. Stability of rif-resistant variants of A. laidlawii, A, in PPLO broth medium with and without 5 μg/ml rifampin. Rifampin was added as a DMSO solution. Open symbols designate variant RV8 and solid symbols designate RV4. The subcultures for both strains are represented by triangles when rif is present in the medium, by boxes when rif is absent. The growth of non-rif cultures serving as the sources of inocula for the subcultures are shown with circles; open circles for RV8 and solid circles for RV4. Viable counts were performed on PPLO agar. Plates were incubated for 4 days.


GROWTH (CFU/ml)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
0
10
20
30
40
50
INCUBATION TIME (Hours)
MUTANT RV8
No Rif
Subculture +Rif
Subculture -Rif
MUTANT RV4
No Rif
Subculture +Rif
Subculture -Rif

culture medium. Hence, no more than 3.0×10^{-5} percent of the original rif-resistant cells could have been present in the inoculum transferred to rif-containing broth. Prior to subculture, the doubling times for mutant RV4 and RV8 are 2.6 and 2.0 hours, respectively. The two rif-resistant variants resumed undelayed growth upon inoculation into the rifampin medium with growth rates very similar to those obtained in the absence of rifampin. The doubling time for both variants is approximately 3 hours. Another observation concerning the growth behavior of these variants is that upon reaching stationary phase, the titers in rifampin and non-rifampin cultures remained similar for both variants. It is concluded that the Rif-R property of variants of RV4 and RV8 is stable and heritable.

To further support the conclusion that the rif-resistant character of the variants is stable, samples of the two broth subcultures without rif (Fig. 15) were titered at 30 and 46 hours on PPLO agar containing 100 μg/ml rifampin. Variant RV8 showed 1.4×10^9 CFU/ml on the rif plates at 30 hours (compared to 1.8×10^9 CFU on non-rif plates) and a titer of 7.0×10^8 CFU/ml on both rif and non-rif containing plates at 46 hours. Similar results were observed for variant RV8. Hence, essentially all the cells in the population of each variant retain the ability to grow to normal size colonies in the presence of as much as 100 μg/ml rif even after extensive growth in its absence.

Effect of Various Inhibitors of RNA Synthesis on A. laidlawii, A

Effect on Growth

Effect of lomofungin, steffimycin, streptolydigin, streptovaricin and rifamycin SV on growth in PPLO broth. Inhibitors of RNA synthesis other than rifampin were added to log phase PPLO broth cultures of A. laidlawii, A,

and titers measured at various time intervals. The results are shown in Figure 16. Both lomofungin and steffimycin (100 μg/ml) kill A. laidlawii, A. Lomofungin has the more efficient cidal action of the two, reducing rapidly the initial population from 6×10^7 to 7×10^5 CFU/ml in 10 hours. This represents a kill of over 99 percent of the initial CFU. Steffimycin kills 40-50 percent in 10 hours. Additionally, streptolydigin results in static growth and streptovaricin appears to stimulate growth at these concentrations.

Table 13 shows that the cidal activity of both lomofungin and steffimycin reduce the titer below detectable levels after 24 and 72 hours, respectively, and that no outgrowth is observed even after 96 or 192 hours of incubation.

In another experiment, the results of which are presented in Figure 17, rifamycin SV was shown to inhibit the growth of A. laidlawii, A, when added to a log phase culture in PPLO broth. In a manner similar to rifampin (Fig. 4), rifamycin SV seems to cause a period of transient inhibition with a doubling time (DT) equal to 15.4 hours prior to a recovery phase of faster (DT = 6.9 hrs) but not normal (DT = 1.9 hrs) growth.

Effect of streptovaricin, lomofungin, steffimycin and streptolydigin on growth in casitone broth. Streptovaricin, lomofungin, steffimycin and streptolydigin were added to a late log phase A. laidlawii, A, culture in casitone broth without serum and titers were followed for 24 hours. Figure 18 shows that streptovaricin caused the most effective killing, reducing the titer from 1.5×10^8 CFU/ml to 6.0×10^4 CFU/ml (greater than 99.9% reduction) in 3 hours. CFU could not be detected in the culture after 6 and 24 hours incubation in the presence of this antibiotic. This action of streptovaricin in serum-less casitone broth is quite opposite

Figure 16. Growth responses of A. laidlawii, A, to various RNA synthesis inhibitors (100 μg/ml) in PPLO broth culture. Antibiotics were added as DMSO solutions. Abbreviations are as follows: stv, streptovaricin; stlg, streptolydigin; steff, steffimycin; lomo, lomofungin. All cultures were inoculated with log phase cells. Viable counts were performed on PPLO agar.

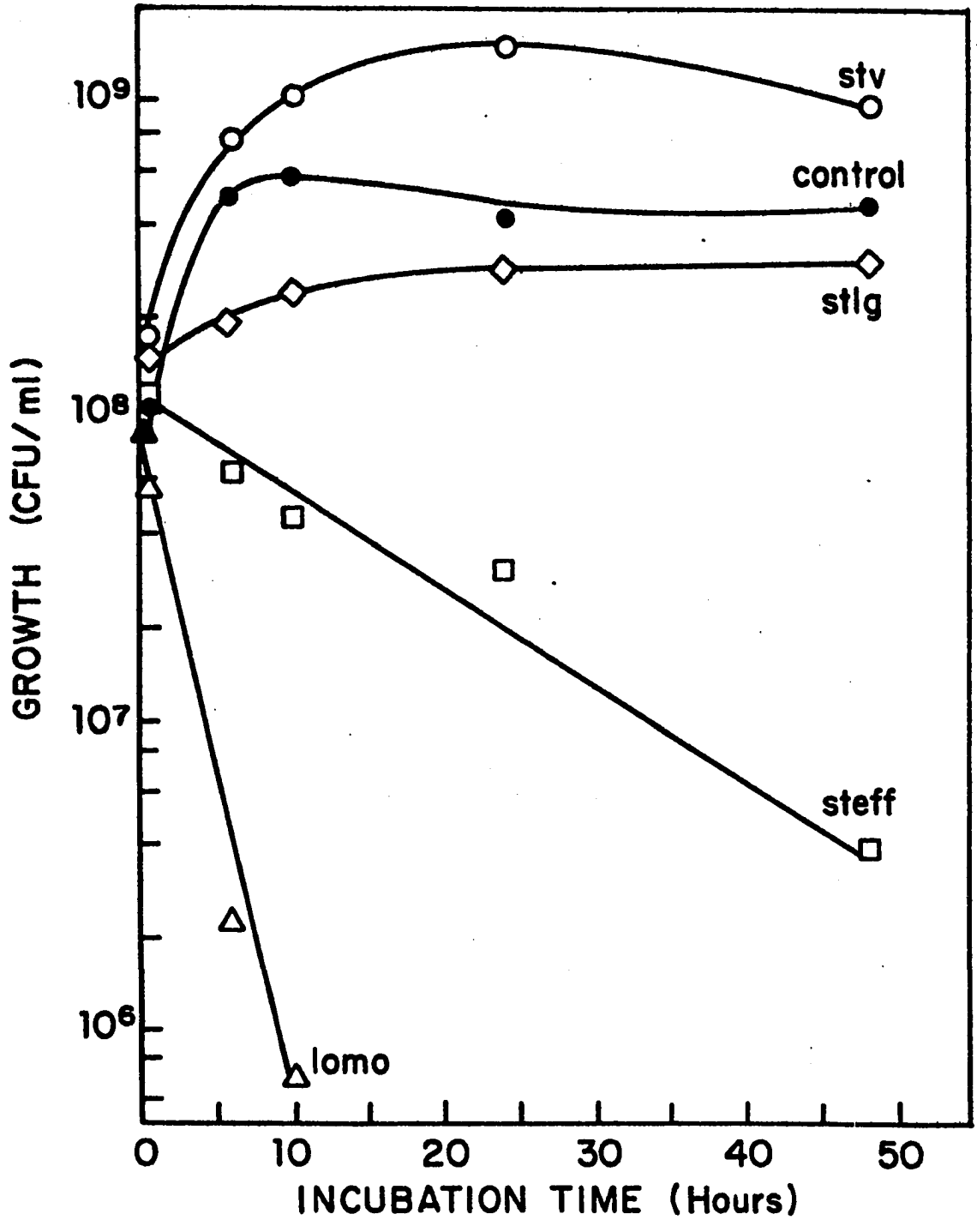

stv
control
stlg
steff
lomo
GROWTH (CFU/ml)
10⁹
10⁸
10⁷
10⁶
0
10
20
30
40
50
INCUBATION TIME (Hours)

TABLE 13

MYCOPLASMACIDAL ACTIVITY OF STEFFIMYCIN AND LOMOFUNGIN IN PPLO BROTH THROUGHOUT EXTENDED INCUBATION PERIODS

Antibiotic (100 μg/ml)	Survival (CFU/ml) Incubation Time (hours)					
	Zero Time	24	48	72	96	192
Steffimycin	8.6×10^7	3.0×10^7	3.8×10^6	$<5 \times 10^3$	$<5 \times 10^3$	$<5 \times 10^1$
Lomofungin	8.6×10^7	$<5 \times 10^3$	$<5 \times 10^3$	$<5 \times 10^3$	$<5 \times 10^3$	$<5 \times 10^1$

Inocula were log phase cells, antibiotics were added as DMSO solutions, and viable counts were performed on PPLO agar plates.

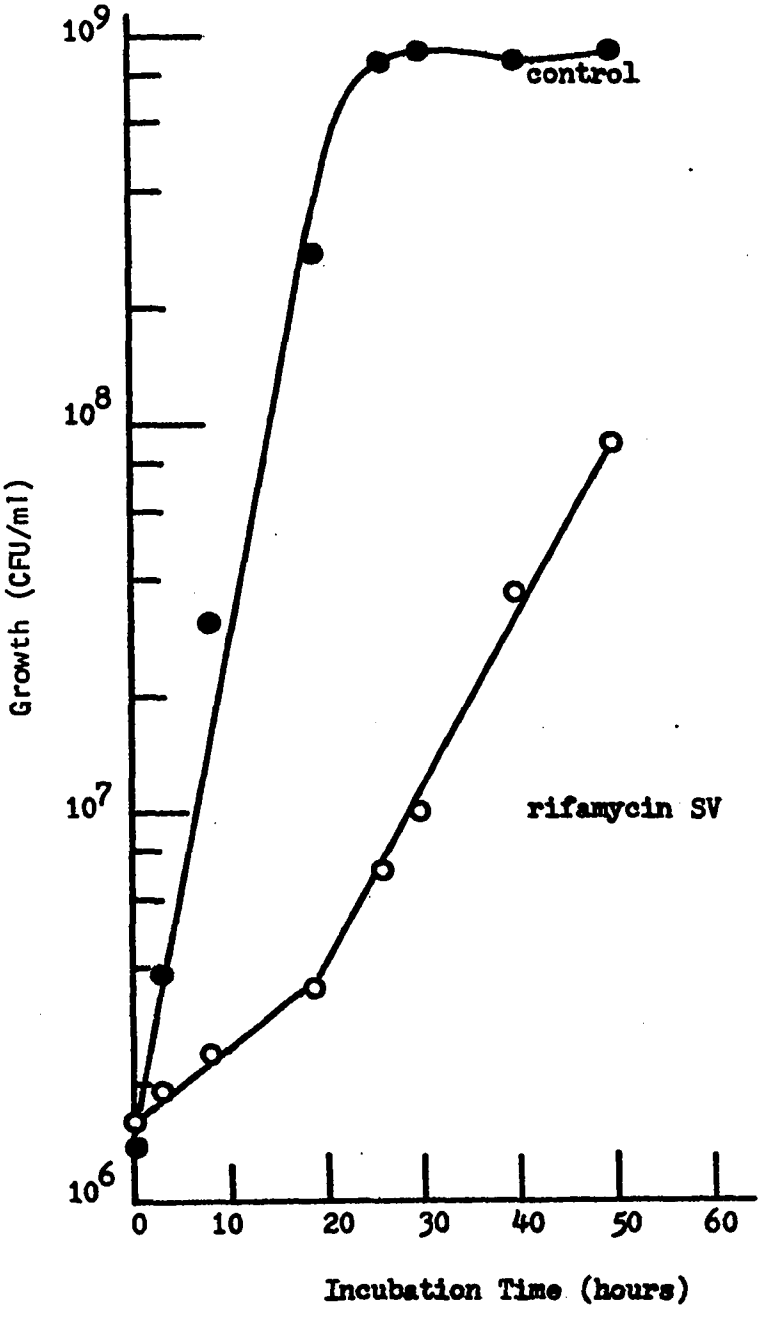
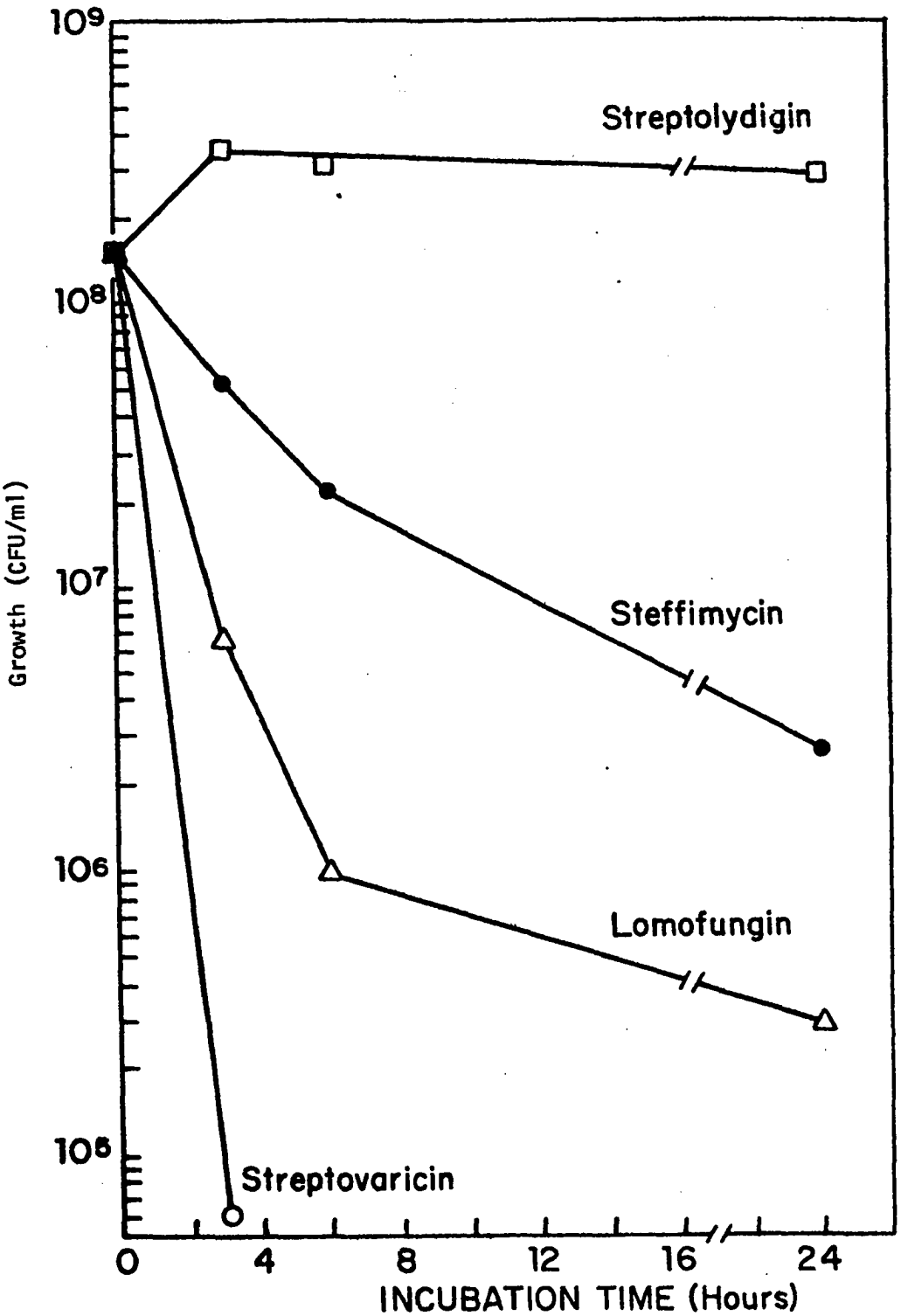


Figure 17. Inhibition by rifamycin SV of growth of A. laidlawii, A, in PPLO broth. Inoculum cells were in log phase. The final concentration of rifamycin SV was 10 mg/ml. The antibiotic was added from a DMSO stock solution. Viable counts were performed on PPLO agar.

Figure 18. Growth responses of A. laidlawii, A, to various RNA synthesis inhibitors (100 μg/ml each) in casitone serum-less broth culture. Antibiotics were added as DMSO solutions. All cultures were inoculated with log phase cells. Viable counts were performed on PPLO agar.


10^9
10^8
10^7
10^6
10^5
Growth (CFU/ml)
Streptolydigin
Steffimycin
Lomofungin
Streptovaricin
0
4
8
12
16
24
INCUBATION TIME (Hours)

the observation in Figure 16, where, in PPLO broth culture, streptovaricin may have stimulated an increase in titer. Lomofungin and steffimycin appear to be effective in casitone broth to a similar degree as in PPLO broth (Figure 16), killing 99 percent of the CFU in approximately 5 hours and greater than 24 hours, respectively. Streptolydigin had detectable effect on growth in these experiments.

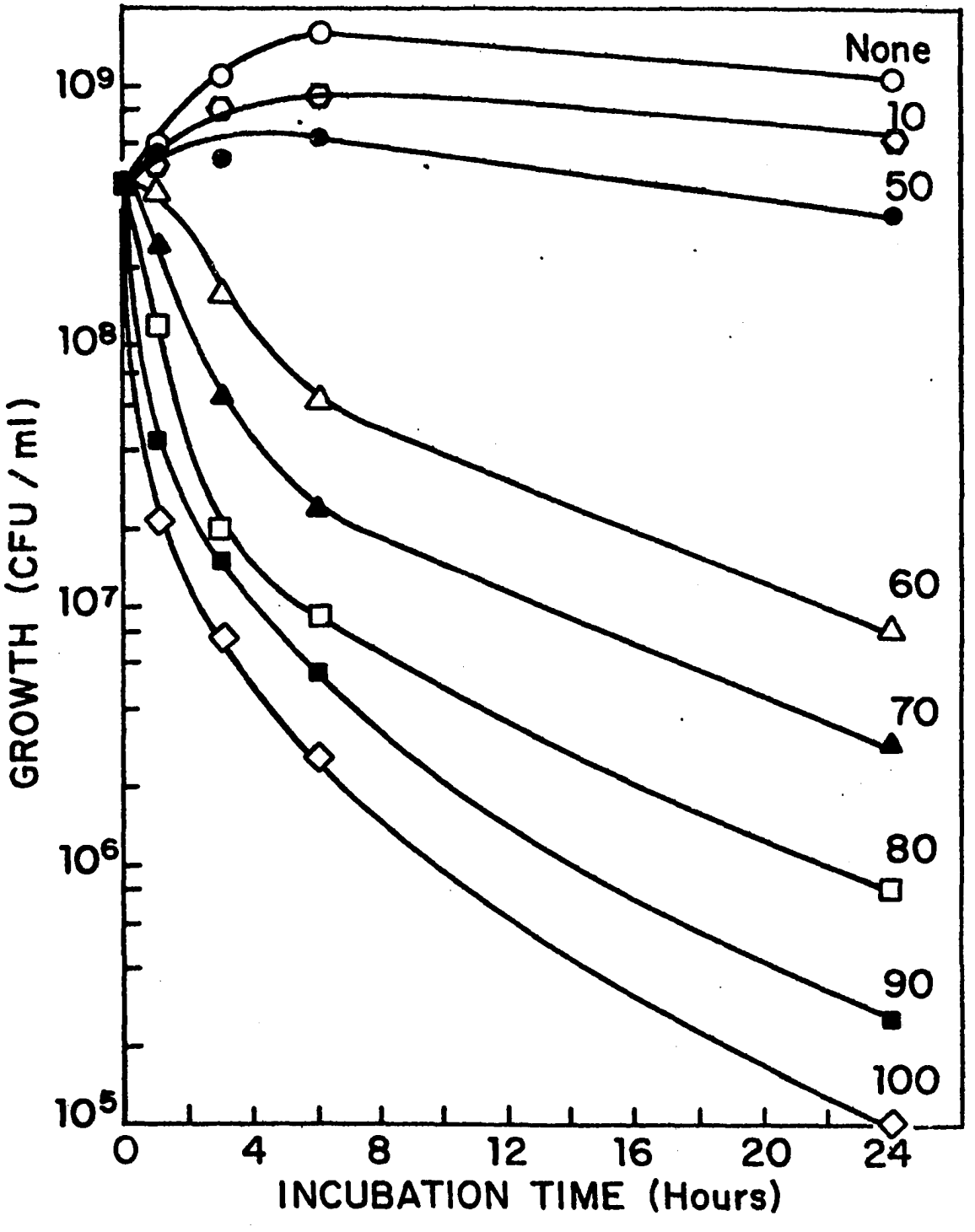
Survival in PPLO broth as a function of lomofungin concentration. Since lomofungin, at a concentration of 100 μg/ml, appeared to be such an effective mycoplasmacidal agent, we investigated the influence of different concentrations of this antibiotic on the survival of A. laidlawii, A. Measurements consisted of performing viable counts at various time intervals. Results of the experiment are represented in Figure 19. The outstanding finding is that under these conditions the organisms reveal a resistance to the cidal activity of the lomofungin until a threshold concentration level is reached, after which killing occurs. This threshold level appears to be between concentrations of 50 and 60 μg/ml of lomofungin.

At concentrations below 50 μg/ml, lomofungin shows no killing below the initial inoculum size of 4×10^8 CFU/ml. At concentrations above 60 μg/ml, the antibiotic is mycoplasmacidal. During the first 6 hours, the initial rate of killing is a function of lomofungin concentration. After 6 hours, the rate of killing is relatively constant and apparently independent of lomofungin concentration. Therefore, we conclude that there is an effective threshold level of lomofungin between 50 and 60 μg/ml.

Effect on In Vivo RNA Synthesis

Inhibition by rifampin, lomofungin and steffimycin of RNA synthesis. The basis for the difference in PPLO broth between the static growth response elicited by rifampin and the mycoplasmacidal action of

Figure 19. Survival of A. laidlawii, A, in PPLO broth as a function of lomofungin concentration. Inocula were log phase cells. The numbers associated with each curve represents μg/ml of lomofungin in the culture. Lomofungin was added from a DMSO stock solution. Viable counts were performed on PPLO agar. Plates were incubated for 4 days.


None
10
50
60
70
80
90
100
10^9
10^8
10^7
10^6
10^5
GROWTH (CFU / ml)
0
4
8
12
16
20
24
INCUBATION TIME (Hours)

lomofungin and steffimycin was sought by measuring the response to these antibiotics of in vivo RNA synthesis. Table 14 shows the inhibition by 100 μg/ml of the three antibiotics of uridine 5-^{3}H incorporation into acid precipitable material. Uninhibited incorporation for 2 minutes amounted to 862 cpm above the zero time incorporation and was set equal to 100 percent. The three antibiotics, when incubated 30 minutes with the culture prior to the 2 minute pulse, allowed 0 percent, 6 percent and 4 percent incorporation for rifampin, lomofungin and steffimycin, respectively. Rifampin, the antibiotic which does not kill mycoplasma, and lomofungin, the most cidal of the three tested, were equally effective as inhibitors of uridine incorporation. Moreover, in the case of rifampin, RNA synthesis appeared to be completely inhibited by 100 μg/ml, although this concentration (Figure 11) allows growth of log phase cells in PPLO broth.

It is difficult to reconcile the effectiveness of rifampin (at high concentrations) in completely inhibiting RNA synthesis (Table 14) with its lack of killing (Fig. 10). However, the suggestion of an eventual release from rifampin inhibition of RNA synthesis (Table 5) should be taken into account. A "reversal" mechanism may provide the basis for explaining the lack of rifampin killing, and may well be associated with recovery and outgrowth.

In considering the mycoplasmastatic effect of rifampin as compared to the mycoplasmacidal action of lomofungin and steffimycin, it should be noted that the mechanisms of action of these antibiotics are known to differ. Rifampin binds reversibly to the beta subunit of bacterial RNA polymerase, inhibits polymerase activity, and prevents initiation of transcription (124a). Lomofungin inhibits RNA polymerase and prevents chain elongation rather than initiation (9). Steffimycin binds to the

TABLE 14

INHIBITION BY SELECTED RNA SYNTHESIS ANTIBIOTICS OF URIDINE-5-H^3 INCORPORATION IN A. laidlawii, A, IN PPLO BROTH

Antibiotic[a] (100 μg/ml)	Incorporation[b] (percent)
None	100
Rifampin	0
Lomofungin	6
Steffimycin	4

[a]The antibiotics were added as solutions in DMSO, and incubated 30 minutes with the culture prior to pulsing for 2 min.

[b]The zero time incorporation (Chapter II) was equal to 257 cpm; uninhibited incorporation was 1,119 cpm; therefore uninhibited incorporation was 862 cpm above zero time value of 257 cpm.

DNA template and inhibits RNA synthesis (92).

Inhibition of RNA synthesis as a function of lomofungin concentration. Because mycoplasmacidal activity requires a certain threshold concentration of lomofungin, and because this threshold level is relatively high (50 - 60 μg/ml), it was of interest to know whether inhibition of RNA synthesis would follow a similar pattern. The inhibition by a wide range of lomofungin concentrations of uridine-5-^{3}H incorporation into acid precipitable material was measured. The results are shown in Figure 20, which is a composite of several experiments. The cpm values were normalized by setting the uninhibited value equal to 100 percent incorporation. The incubation time of lomofungin with the cultures prior to pulsing was 30 minutes. Figure 20 shows that a threshold concentration of between 50 and 60 μg/ml is required before inhibition of uridine incorporation occurs.

We conclude that inhibition of RNA synthesis in cultures of A. laidlawii, A, in PPLO broth requires threshold concentrations of between 50 and 60 μg/ml. This concentration range correlates closely with the concentration threshold of lomofungin required for mycoplasmacidal activity.

Correlation between inhibition by lomofungin of RNA synthesis and of growth. We then asked whether the extensive mycoplasmacidal effect of lomofungin could be associated with its known activity as an inhibitor of RNA synthesis.

The results shown in Figure 21 are expressed as percent survival and percent uridine incorporation as a function of lomofungin concentration. Percent survival in 6 hours (Figure 19) and percent uridine incorporation (Figure 20) were calculated by setting the corresponding values obtained in the absence of lomofungin equal to 100 percent. The initial inoculum, depicted by the broken line, represents 40 percent of the final

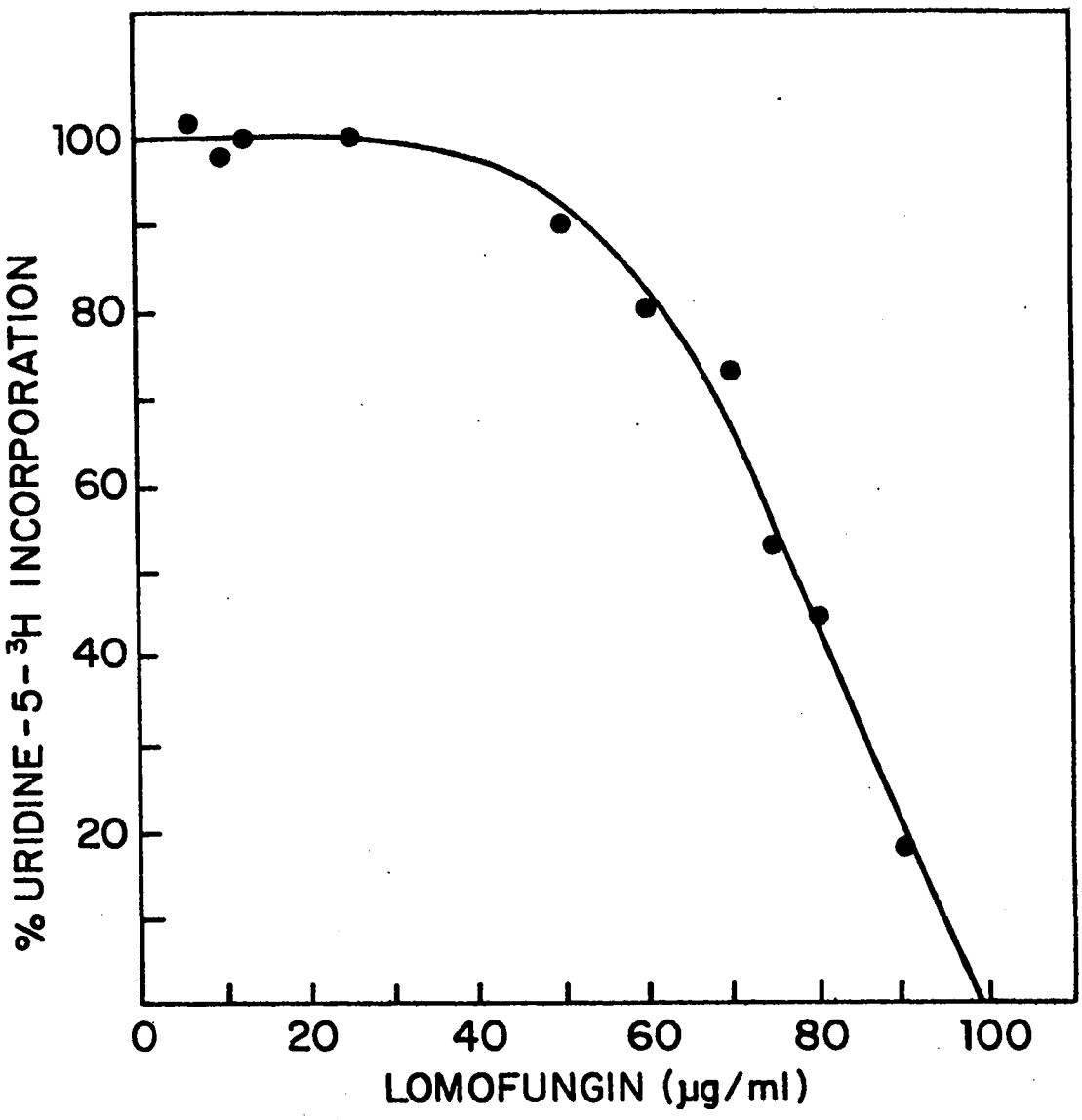
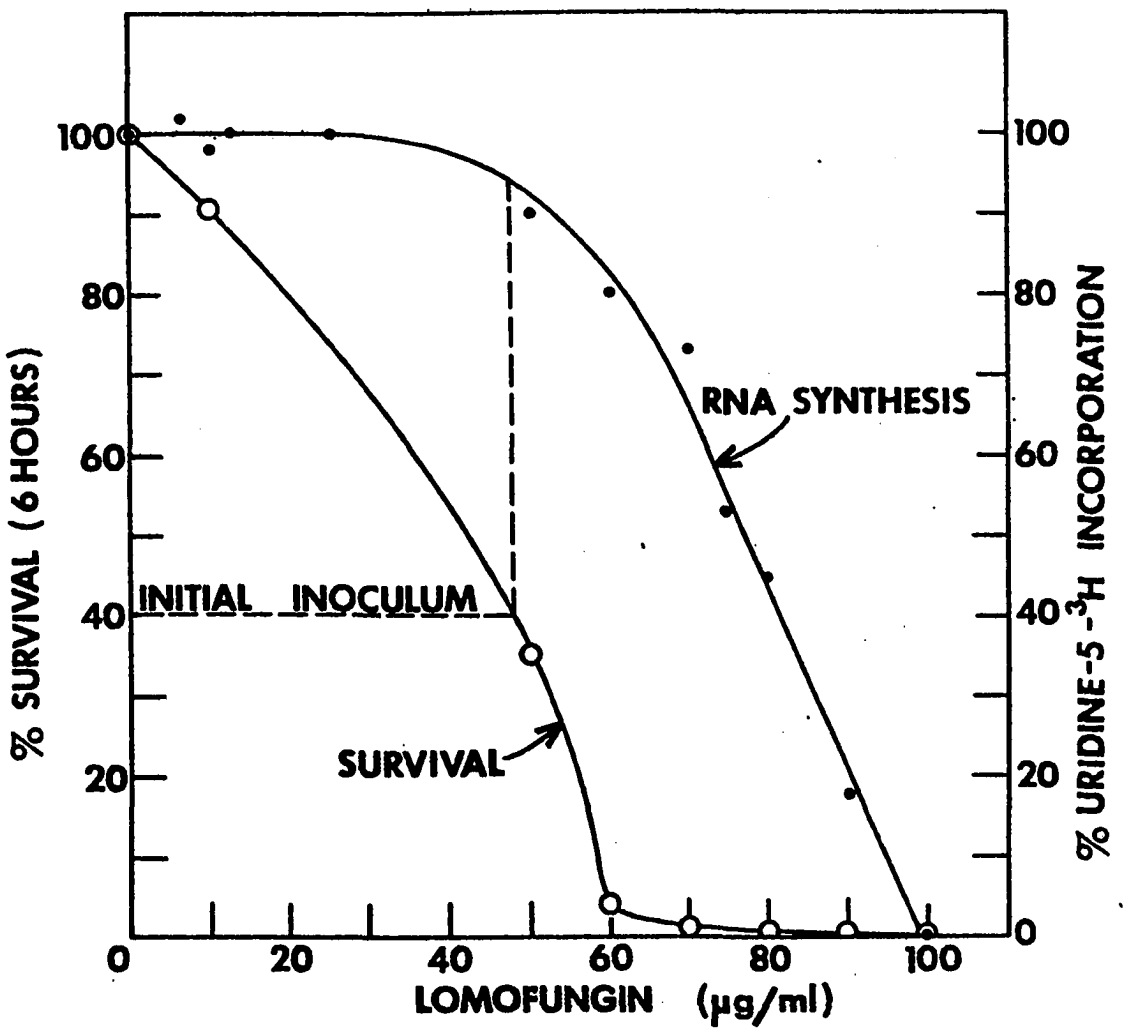


Figure 20. Inhibition of uridine-5-^{3}H incorporation into A. laidlawii, A, by lomofungin. The average zero time incorporation (Chapter II) was equal to 674 cpm, and uninhibited incorporation was 1524 cpm. Further description of the experiment is included in the text.



CORRELATION BETWEEN INHIBITION BY LOMOFUNGIN OF RNA SYNTHESIS AND OF GROWTH

Figure 21.

number of CFU attained by the control culture without lomofungin. Killing is defined as a reduction in the number of CFU/ml below that present in the initial inoculum. Therefore, above the broken line there is inhibition of growth but no killing. Below the broken line there is death.

The data show that no detectable inhibition of RNA synthesis and no death occur until the lomofungin concentration exceeds the threshold level of approximately 50 μg/ml. At concentrations above this threshold, there appears to be a parallel reduction in RNA synthesis and in the number of survivors.

We conclude that there is an apparent correlation between the inhibition by lomofungin of RNA synthesis and its mycoplasmacidal action.

CHAPTER IV

DISCUSSION

Response of Mycoplasma to Rifampin

General Consideration

In order to provide a relevant context in which the results of this research can be presented, a discussion follows of two areas of possible influence on the conclusions to be drawn.

Mechanism of rifampin action in bacteria. Rifampin is a semi-synthetic antibiotic of the rifamycin group which was isolated from the fermentation broth of Streptomyces mediterranei (103). Rifampin is also known as rifampicin and rifamycin AMP. Chemically, the rifamycins are ansa compounds consisting of a chromophoric naphthohydroquinone ring which is spanned by a long aliphatic bridge (124). Rifampin activity is directed against the RNA polymerase of prokaryotes and binds to the β subunit of the enzyme (124a), thereby preventing the initiation of RNA synthesis (123). The action of this antibiotic is considered to be bacteriocidal (45,123,59). Lancini et al., (59) based their conclusion that RNA polymerase inhibition is the rifampin activity causing cell death on the close correlation between concentrations of the antibiotic which block in vivo incorporation of labeled uracil and concentrations which are bacteriocidal. Rifampin (0.5 µg/ml) reduced the viable count of E. coli in broth culture by over 4 logs within one hour. There is

evidence that the loss of viability of the cells immediately follows the onset of inhibition of RNA synthesis.

Mindlin et al., (72a) studied the basis for rifampin resistance in spontaneous mutants of E. coli. Merodiploid strains mutated spontaneously to rifampin resistance at a frequency of 10^{-9} whereas haploid strains showed a frequency of 10^{-5} to 10^{-6}. These mutants, plus 60 other spontaneously-derived rif-resistant mutants, all mapped within the RNA polymerase region of the E. coli chromosome, at minute 78 between argH and thi. Moreover, Mindlin et al. state that all the rifampin-resistant mutants isolated in different laboratories map in the RNA polymerase region. Since many efforts have failed to isolate rifampin-resistant mutants that map outside the RNA polymerase region, it seems unlikely that permeability mutants would give rise to high level rifampin resistance in E. coli. However, mutants with increased permeability to rifampin have been obtained (78,101). Mindlin et al., (72a) obtained, among their spontaneous mutants to rifampin resistance, many that were resistant to 50-100 μg/ml of rifampin only when plated on rich agar; the mutants were sensitive to these same concentrations of rifampin when plated on minimal medium. A possible explanation for this is that, on minimal medium, altered RNA polymerase molecules slightly inhibited by the presence of rifampin would not be able to synthesize enough messenger RNA to provide the cell with the proteins necessary for growth (53). Lancini et al., (59) showed, however, that growth is not necessary for rifampin to exert its cidal effect on E. coli. In other studies concerning the nature of the alleles for RNA polymerase in E. coli, it

was observed that merodiploid cells heterozygous for resistance to rifampin ceased RNA synthesis and growth in the presence of rifampin. This finding demonstrates that rifampin sensitivity is dominant (55a). The mechanism for this dominance was suggested to be the ability of the sensitive enzyme in the heterozygous merodiploid to compete for promoter sites and thereby block transcription when rifampin was present.

Features of mycoplasma growth and RNA synthesis. The forms of mycoplasma thus far observed consist of the following morphologies (69): 1) coccoid cells; 2) coccoid cells with membrane tubules; 3) filamentous cells, sometimes showing branching; 4) filamentous cells with terminal structures; and 5) pear-shaped cells with terminal structures. Both A. laidlawii and M. orale belong to the class with filamentous morphology that sometimes shows branching (69). Mycoplasma of the filamentous type reproduce by both binary divisions and by elongation and constriction at several points, giving rise to several viable filamentous cells (8). Survival curves of A. laidlawii, A following U.V. irradiation show multitarget kinetics; i.e., there is an initial shoulder followed by an exponential decrease in CFU. This indicates that the cells are multinucleate, and that the number of genomes per cell increases as stationary phase is approached (27). However, this approach cannot distinguish between a multinucleate and a multicellular viable unit. When stationary cells of M. gallisepticum are transferred to fresh medium, there is an exponential increase in cell number but a lag in corresponding DNA synthesis resulting in a decrease in the amount of DNA per cell during early stages of growth. Exponential DNA synthesis begins in mid-log phase

of the growth curve and continues into stationary phase to produce a relative increase in the amount of DNA per cell. Smith (110) has shown that DNA replication in A. laidlawii is semiconservative and proceeds unidirectionally from, at most, a few growing points. A small amount of non-conservative repair replication has been found in normally growing A. laidlawii and is also believed to occur after thymine starvation (111). It appears that DNA replication continues throughout the cell cycle (112). A. laidlawii was shown to have both light and dark repair mechanisms. The photo repair mechanism reaches its maximal capacity in middle to late exponential-phase cells (27). Dark repair occurs by an excision-repair mechanism (27) and excises the region, estimated to be about 150-600 nucleotides in length (111), on either side of the U.V.-induced pyrimidine dimer. Das et al., (27) found that 60 percent of the breaks were repaired within 2 hours after irradiation.

Ribonucleic acid comprises 8-17 percent of the cell mass (87). For M. gallisepticum, the RNA:DNA ratio is 4.5 during log phase growth, and 2.0 for cells in early stationary growth (57,74). About 86 percent of the total RNA of M. gallisepticum is ribosomal RNA (57). RNA species found in mycoplasma include 22s, 16s and 5s rRNA, 4s tRNA and unstable mRNA (69). Ribosomal RNA compositions are similar to each other and to those of E. coli (69). Mycoplasma tRNA has been shown to sediment at 4s, as does E. coli tRNA, and to have a thermal denaturation curve similar to that of E. coli tRNA, indicating similar secondary structures (46). Kirk (56) reported that the messenger RNA of M. gallisepticum represents about 4.5 percent of the total RNA,

and that its chemical half-life equals 2 minutes. Tourtellotte (122) showed that 20 μg/ml of Actinomycin D provided immediate cessation of all RNA synthesis in the seven mycoplasma strains studied (A. laidlawii, A, was included). He was also able to determine that the functional half-life of the messenger RNA was less than 4 minutes by measuring the rate of protein synthesis following RNA synthesis inhibition by Actinomycin D. Tourtellotte's studies also showed that 20 μg/ml of Actinomycin D had little effect on incorporation of ^{14}C-thymidine into DNA. Additionally, the studies helped demonstrate that mycoplasmas synthesize RNA from a DNA-dependent RNA polymerase (122).

The membranes of mycoplasma are composed of 50 to 59 percent protein, 32 to 40 percent lipid, and 0.5 to 2 percent carbohydrate. A. laidlawii membranes contain about 5 percent glucosamine and galactosamine (69). Members of the genus Mycoplasma require cholesterol for growth and incorporate it into the membrane where it composes 12 to 30 percent of the total lipid. Acholeplasma species, when grown in the presence of cholesterol, are able to incorporate it into the membrane only to the extent of 3 to 4 percent of the total membrane lipid (113). A thermal phase transition in membranes of A. laidlawii has been described whereby the lipids in the mycoplasma membrane undergo a transition from a highly ordered configuration of fatty acids to a liquid paraffin-like state (118). The temperature at which this transition occurs is a function of the lipid composition of the membranes which, in turn, can be varied with the growth temperature of the mycoplasma culture (71).

Lysis and death of M. mycoides, strain V5, occurs following

inhibition of lipid synthesis by glycerol, cholesterol or fatty acid deprivation. Since lysis does not occur when protein and RNA synthesis are prevented by addition of chloramphenicol during uracil deprivation, it has been suggested that glycerol-less death and lysis are the results of unbalanced growth, in which, cytoplasmic synthesis continues in the absence of membrane synthesis. Thus, it could be concluded that membrane and cytoplasmic synthesis are uncoordinated (95). In another strain, the goat pathogen strain Y, glycerol deprivation prevents lipid synthesis, but cytoplasmic and DNA synthesis continues. When DNA synthesis is prevented by thymine deprivation, lipid and protein synthesis continue, and some cells produce large numbers of small enucleate forms, possibly analagous to minicells (96). Thus, lipid synthesis is not necessary for DNA replication, and DNA synthesis is not required for continuing protein and lipid synthesis or for cell fragmentation.

The initial qualitative observations (Tables 1 and 2) of the growth response of A. laidlawii, A, to rifampin - that the organism is sensitive to the antibiotic, and that outgrowth eventually occurs - was reinforced and defined more clearly by quantitative measurements. The mycoplasmastatic, rather than mycoplasmacidal, activity of rifampin is apparent from results of several experiments. Results depicted in Figures 4, 5 and 6 show that although growth is extremely sensitive to rifampin, a reduction in CFU cannot be attained even by high concentrations of rifampin. The most definitive argument for the static action of rifampin, however, is presented in Table 4, where the evidence clearly illustrates that individual CFU have the capacity to grow

in the presence of rifampin. The small proportion (8-12 percent) of the total population of log phase cells of A. laidlawii, A, that were not able to develop colonies on rifampin-containing agar is not very significant and may represent unhealthy cells. Such results argue against explanations for static growth that invoke an equilibrium between dying cells and emerging resistant cells. The reduction in the proportion of cells able to form colonies on rifampin agar as the culture ages suggests that changes in susceptibility to rifampin occur. Increased susceptibility may reflect either a change in membrane structure and function, or altered metabolism. As pointed out above, A. laidlawii, A, is thought to increase the amount of DNA per cell as cultures approach stationary phase growth. Moreover, if there is a lack of coordination between RNA synthesis and other cellular functions, inhibition of RNA synthesis by rifampin may not result in cell death even if the antibiotic were able to inhibit RNA polymerase activity as effectively in mycoplasma as it does in E. coli.

Since a state of polyploidy can probably exist in A. laidlawii, A, there is the possibility that the organism is capable of assuming an alternate mode of reproduction when confronted with environmental stress, in this case, the presence of rifampin. The biphasic pattern of growth response to rifampin by A. laidlawii cells, as shown in Figure 5, would suggest such an adjustment phenomenon. Another possible mode of adjustment might occur at the membrane.

The fact that mycoplasma are able to alter the configuration of lipids in their membrane by regulation of the lipid components in response to temperature suggests an adaptive mechanism. The biphasic

response of growth to rifampin suggests that cells are very sensitive to rifampin upon initial contact, then become less sensitive, although they are still affected by the presence of rifampin as seen by their slower growth rate and reduced cell yield (Figures 2 and 3).

Another attempt to explain the growth responses to rifampin depicted in Figures 2, 3 and 4 would suggest another type of membrane affect. Possibly, rifampin molecules, unable to penetrate the mycoplasmal membrane, simply bind to it, thereby inhibiting its optimum function. The long aliphatic component of the rifampin molecule adds to the credibility of this suggestion.

It must be remembered, however, that the sensitivity of uridine incorporation into acid-insoluble material (Figure 9) suggests that rifampin indeed gets inside the cell and inhibits RNA synthesis very effectively. Table 5 shows that 3 μg/ml rifampin causes 70-80 percent inhibition of uridine incorporation for up to 20 minutes after which rifampin inhibition may be released. Therefore, there is possibly a period of transient inhibition for RNA synthesis as there is for growth. That growth does occur in the presence of rifampin under conditions very similar to those causing inhibition of RNA synthesis is verified in Figure 10 which shows that the culture grows in the presence of 100 μg/ml.

In considering the possible explanations for the contrast seen in the responses of growth and uridine incorporation to rifampin, a most mundane explanation presents itself. If rifampin alters membrane function so as to exclude labeled uridine, incorporation of isotope into RNA would certainly be reduced, but RNA synthesis

could actually be unaffected by the direct action of rifampin. The transient phase of growth inhibition could be due to the unavailability of required nutrients due to the impaired membrane function, the resumption of growth could be the result of induction of pathways or enzymes able to utilize alternate but available metabolites, and the reduction in growth rate and cell yield might reflect the inefficiency of the induced mechanism(s). If rifampin inhibited uridine uptake the cells would need to switch to an alternate pool for the RNA precursor. The supply might be derived from other pyrimidine bases in the medium, since A. laidlawii, A has a minimal requirement for nucleic acids of adenosine, guanosine and cytidine (83). Adenine, or one of the mononucleotide phosphates of adenine, can replace adenosine; guanosine and cytidine can be replaced by their respective 5' mononucleotides. A. laidlawii grows suboptimally in the presence of the free bases together with ribose and deoxyribose.

The foregoing mechanism is speculative, and it should not be assumed that inhibition of labeled uridine incorporation is due to anything other than inhibition of RNA synthesis. Indeed, an argument against the involvement of such an uptake mechanism would seem to be the 12 percent residual uridine incorporation in the presence of rifampin concentrations of 5-20 μg/ml (Figure 9). Although increasing rifampin concentrations in this range do not increase inhibition of uridine incorporation, they do cause increased inhibition of growth as seen in Figures 6 and 7.

The initial increase in titer observed occasionally (Figures 7 and 10) has been reported for the growth response of M. pneumoniae to the presence of tetracycline (60). The basis for this rise in CFU

immediately after contact with the antibiotic is not clear. It might be due to an initial stimulation of synthetic machinery before the involved mechanisms become inactivated, or, possibly, a lack of coordination between cell division and the site of action of the antibiotic. Another possibility is that the antibiotic alters membrane surface charges and thereby causes clumps of cells to disaggregate. The results shown in Figure 10 argue against the latter explanation since the titer increased for two measurements, 30 minutes and one hour after contact with rifampin. Therefore, this increase would appear to be due to growth since disaggregation by a charge effect might be expected to occur immediately upon introduction of the antibiotic.

If impermeability to rifampin were critical in the static rather than cidal effect of rifampin on A. laidlawii, A, such impermeability is not changed by a growth temperature of 30 C (the usual incubation temperature is 37 C) which might allow for a slight change in the configuration of membrane lipids (Figure 12).

The basis for the protection from cidal activity of rifampin afforded A. laidlawii, A by serum in casitone (Table 7) broth probably involves many factors. Among them could be stabilization of the cell membrane by lipoproteins in the serum, osmotic and buffering action, possible binding of the antibiotic by serum proteins or other unknown possibilities. The fact that various NaCl concentrations in the medium do not appear to alter cidal activity (Table 6) argues against ionic strength playing a decisive role. Since soy peptone medium also protects against the cidal activity of rifampin (Table 8) a role is suggested for the types of protein present in the medium. Soy peptone

is known to possess fermentable carbohydrates which may also be a factor.

The outgrowth culture shown to possess a stable rifampin resistant property in Figure 14, nevertheless becomes vulnerable to rifampin action upon reaching stationary phase. This finding coincides with the results of Table 4, which shows a decrease in the proportion of rifampin resistant CFU, and Table 6, which shows marked cidal activity by rifampin in populations as stationary phase approaches. These observations, plus the results of experiments showing that membrane active agents can potentiate rifampin's cidal activity (Table 9) present a strong case for membrane involvement in the resistance by A. laidalwii, A, to a mycoplasmacidal activity of rifampin. M. orale, a mycoplasma possessing a membrane that requires cholesterol, shows a similar response to rifampin (Figure 11) as does A. laidlawii, A, thus the difference in membrane structure between these two organisms is not a critical factor in their sensitivity to rifampin in PPLO broth. However, since carotenoids are synthesized by acholeplasmas when grown in the absence of cholesterol (84) (as in serum-free casitone broth) they may play a role in the different responses of A. laidlawii, A, to the presence of streptovaricin. Streptovaricin was markedly cidal in the serum-less medium (Fig. 18), but not at all cidal in PPLO broth which contained 10 percent serum (Fig. 16).

The various inhibitors, other than rifamycin, that were used in this study represent antibiotics that inhibit RNA synthesis by various mechanisms. Some of them, steffimycin and lomofungin, have not, as yet, received detailed analysis as to their modes of action.

Lomofungin is an antibiotic from Streptomyces lomodensis that

inhibits the growth of bacteria, yeasts and fungi (55) and has been shown to inhibit three DNA-dependent RNA polymerases in cell-free extracts of S. cerevisiae (9). Lomofungin selectively inhibits the synthesis of rRNA and polydisperse RNA, while having no effect on the synthesis of tRNA or 5s RNA in the yeast Schizosaccharomyces pombe (40).

The streptovaricin used in the studies reported in this paper was a complex mixture of related structures. The mechanism of action of this antibiotic is similar to rifampin in that it binds to the beta subunit of the RNA polymerase molecule to prevent the initiation of RNA synthesis. Streptovaricin is active also against certain viruses (10).

Streptolydigin is a relatively strong enol acid of a quite different structure from either rifampin or streptovaricin. It inhibits RNA polymerase to reduce the rate of RNA synthesis by acting on the catalytic function of the enzyme. This antibiotic inhibits phosphodiester bond formation, decreases the affinity of the enzyme for UTP and CTP, and also affects the pyrophosphate-exchange reaction catalyzed by RNA polymerase (107). The evidence that streptolydigin stabilizes both the enzyme-DNA complex and the RNA-enzyme-DNA complex led Cassani et al. (11) to suggest that the mechanism of inhibition is a tightening of the interaction between the enzyme and the DNA which results in an inability of the enzyme to form phosphodiester bonds and carry on pyrophosphate exchange. Concomitantly, the affinity of the enzyme for UTP and CTP is slightly affected.

Steffimycin inhibits RNA synthesis by binding to the DNA template (92). However, in vitro RNA synthesis could not be completely

inhibited even with high concentrations (up to 200 μg/ml) of steffimycin in the reaction mixture. Therefore, it has been suggested that the antibiotic acts with specificity toward the secondary structure of the primer DNA (92).

The responses of A. laidlawii, A, to the above antibiotics showed that only lomofungin and steffimycin are cidal in PPLO broth cultures (Figures 16 and 19). The dose effects of lomofungin on survival and RNA synthesis suggest that the antibiotic is unable to enter the cell until a threshold concentration of 50-60 μg/ml is reached. If small amounts of lomofungin were able to enter the cell, some inhibition of RNA synthesis might be expected. This opinion is based upon the assumption that the polymerase is sensitive to lomofungin, which has not been shown directly. However, reports of the sensitivity of lomofungin of RNA polymerases from other sources suggest that concentrations lower than the threshold levels (50-60 μg/ml) should inhibit RNA synthesis, provided the antibiotic were able to establish contact with the enzyme. Cano et al. (9) report that, in vitro, 5 μg/ml of lomofungin inhibits E. coli RNA polymerase by 45 percent and that 20 μg/ml gives 90 to 100 percent inhibition of the RNA polymerase of the yeast Saccharomyces, strain 1016. From these considerations, it follows that the high threshold concentration of lomofungin required to inhibit RNA synthesis in A. laidlawii, A, is possibly a membrane permeability phenomenon. The observation that inhibition of labeled uridine incorporation is closely correlated with loss of viability (Figure 21), suggests that death is associated with inhibition of RNA synthesis. However, it is possible that the cidal

effect of lomofungin is due to the accumulation of the drug on the membrane resulting in its rupture and the consequent lysis of the cells. The fact that the threshold levels of lomofungin that cause killing still allow 40-50 percent incorporation of uridine-5-H^3 indicates that a fraction of the RNA that is essential to the survival of the cell is being eliminated (Figure 21). This contrasts with the 88 percent inhibition by 5-20 μg/ml of rifampin of incorporation of uridine-5-H^3 that nevertheless allows the mycoplasma to continue to grow (Figures 7 and 9).

CHAPTER V

SUMMARY

The growth of A. laidlawii, A, in serum-containing broth culture is quite sensitive to rifampin. From an inoculum of 2.2×10^7 CFU/ml, the titer attained in 24 hours was reduced by 90 percent with 5 μg/ml rifampin. However, increasing the rifampin concentration to 100 μg/ml allowed no significant killing. Therefore, rifampin is mycoplasmastatic, rather than mycoplasma cidal, under these conditions. Outgrowth of the culture occurred in the presence of 100-200 μg/ml of rifampin in the medium when the static culture was allowed to incubate for prolonged periods. The time required for outgrowth to occur is dependent upon whether the cells are in exponential growth phase when they are exposed to rifampin, upon the inoculum size, and upon the concentration of rifampin in the culture.

The initial response of the culture to rifampin is a transient period of inhibition, after which growth resumes, but at a slower rate than that of control cultures without rifampin. The slower growth rate may be accompanied by a reduction in the optimum yield of cells attained by the culture.

Individual colony forming units possess the capacity to resist rifampin killing and to adapt and grow in its presence since outgrowth of A. laidlawii cultures unexposed to rifampin occurs on agar plates

containing rifampin. Such outgrowth requires approximately two weeks, and the titer represents over 90 percent of the CFU determined on non-rifampin plates.

Uridine-5-H^3 incorporation by log phase A. laidlawii, A, cultures was inhibited by 50 percent by 0.25 μg/ml of rifampin. However, the percent inhibition could not be increased beyond 87 percent by concentrations of rifampin of 5-20 μg/ml. The transient inhibition of growth by rifampin is consistant with the significant inhibition of incorporation by 3 μg/ml rifampin which appeared to be relaxed after 30 to 45 minutes incubation with the antibiotic.

From the reduction in cell yield, in growth rate and in uridine incorporation for extended periods, it was concluded that rifampin continues to be active against A. laidlawii, A, beyond the period of static growth.

The effect of rifampin upon the growth of Mycoplasma orale is similar to that upon A. laidlawii, A, since the growth rate and peak titers are reduced by rifampin.

Casitone broth medium lacking serum allowed rifampin to exert a potent mycoplasmacidal effect on A. laidlawii, A, and this effect was antagonized when 10 percent serum was added to the medium. Soy peptone broth lacking serum did not allow a cidal effect by rifampin.

The impermeability of the mycoplasmal membrane to rifampin in soy-peptone broth cultures is suggested by the observation that some agents known to act on membranes (gramicidins J and S, Tween 80 and mellitin) and DMSO, in which rifampin is very soluble, are able to potentiate the cidal action of rifampin against A. laidlawii, A.

A genetic involvement in resistance to antibiotics was demonstrated

by the observation that a culture of A. laidlawii, A, in the absence of rifampin contains a small proportion of cells that are resistant to rifampin. This subpopulation is rapidly selected when rifampin is added to the culture, and, after prolonged incubation, predominate in the total cell population. These cells appear to arise in a non-rifampin-containing culture as a result of random spontaneous events, since the variance in their occurrence was very large according to the Luria-Delbruck fluctuation test.

The capability of A. laidlawii, A, to undergo mutagenic events leading to rifampin-resistant variants was demonstrated by the increased frequency of variants following treatment of cells with U.V. irradiation. Exposure to U.V. rays resulted in an 80-84 fold increase in the frequency of variants above the spontaneous frequency. A 10-fold increase was observed following treatment with NTG. Two variants (spontaneously-occurring and NTG-induced), selected for rifampin resistance, were stable when re-exposed to rifampin after subculturing in the absence of rifampin.

Among the RNA synthesis inhibitors (rifampin, rifamycin SV, streptolydigin, streptovaricin, steffimycin and lomofungin), only steffimycin and lomofungin were able to produce a cidal activity against A. laidlawii, A, in serum-containing broth culture. Streptovaricin, however, showed potent mycoplasmacidal activity in non-serum-containing broth cultures.

Lomofungin dose curves indicate that both viability and RNA synthesis of A. laidlawii, A, are resistant to this antibiotic until threshold concentrations of 50-60 μg/ml are attained. When higher concentrations of lomofungin are present, A. laidlawii, A, cultures decrease in titer and incorporation of uridine-5-H^3 is concomitantly inhibited.

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