ANTIBIOTICS AND *MYCOPLASMA*

I. MECHANISMS OF RESISTANCE

II. ANTIBIOTIC BIOSYNTHESIS

by JEFFREY L. SCHWARTZ

(Under the supervision of Professor D. Perlman)

The mechanisms of resistance to various antibiotics and the production of antimicrobial substances have been studied extensively in both bacterial and fungal systems. Until now however, little research on either of these two problems has been done with *Mycoplasma*. The present study examines the antibiotic resistance mechanisms in *Mycoplasma* and the production of antimicrobial substances by species of these organisms.

A study as to the sensitivity of *Mycoplasma* species to various antibiotics indicated that chloramphenicol, tetracycline, kanamycin, and gentamicin were effective in inhibiting *Mycoplasma* growth on agar at concentrations of 10 µg/ml. However, resistant strains of *Mycoplasma salivarium* and *Mycoplasma pharyngis* were observed. Erythromycin (10 µg/ml), streptomycin (20 µg/ml) and spectinomycin (20 µg/ml), were not effective in inhibiting the growth of the *Mycoplasma* species tested. The finding of antibiotic resistant strains of *Mycoplasma* prompted a study as to the mechanisms of antibiotic resistance of these organisms to streptomycin, chloramphenicol, and tetracycline.

An examination of streptomycin adenylylation (as determined by the method of Benveniste and coworkers) and chloramphenicol acetylation (as determined by the method of Shaw) indicated that no inactivation of either of these two antibiotics occurred by these methods. No chloramphenicol or streptomycin inactivation of any type could be demonstrated by incubating the antibiotics with growing cultures of Mycoplasma for varying lengths of time, indicating that resistance to both antibiotics was not due to any type of antibiotic degradation.

The negative results with enzymatic degradation studies led to experiments to study antibiotic uptake by Mycoplasma to determine if a lack of some type of accumulation mechanism was responsible for resistance. Accumulation of tetracycline, chloramphenicol, and dihydrostreptomycin was measured by centrifuging aliquots of a Mycoplasma culture incubated in the presence of radioactive antibiotic. The centrifuged cells were washed, resuspended in water, and assayed for radioactivity by counting an aliquot of the suspension in a scintillation apparatus using Bray's solution as the counting fluid. The protein concentration of the suspension was determined by Lowry's method. The results of the accumulation studies indicated that the resistance mechanisms to chloramphenicol, tetracycline, and dihydrostreptomycin by Mycoplasma are governed by a permeability/accumulation mechanism. Whether the difference in total antibiotic accumulation and absorption/efflux

patterns between resistant and sensitive Mycoplasma is due to an inhibition of the transport of the antibiotic into the cell, or the specific binding of the antibiotic to its active site, is still to be determined.

Mycoplasma cultures were also screened for the production of antimicrobial substances against the following test organisms: Bacillus subtilis, Escherichia coli, Sarcina lutea, Saccharomyces cerevisiae, and Mycoplasma laidlawii B. Broth cultures of 21 Mycoplasma cultures were grown for 5 days and lyophilized. The lyophilized material was resuspended in sterile water to yield a 20-25 fold concentration of the material. Filter paper discs capable of absorbing 0.1 ml of water were allowed to absorb samples of the suspended lyophilized material, and the discs placed on agar containing one of the test organisms. The test plates were incubated for 24 hours and observed for inhibition zones. Three cultures, M. laidlawii B, a Mycoplasma hominis I, and an unidentified human isolate, produced material capable of inhibiting B. subtilis, particularly spore cultures of the organism. The chemical nature of the three substances (as determined by the pH chromatographic method described by Betina) indicated that the substances were basic in nature as seen by their mobility in ethyl acetate at only basic pH. A preliminary characterization of the substance produced by M. laidlawii B showed it to be extractable from alkaline aqueous

solutions by n-butanol, and heat labile at acid and neutral pH.

APPROVED David Perlman

Professor D. Perlman

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ABBREVIATIONS USED IN THESIS

nm	nanometers
DNA	deoxyribonucleic acid
G+C	guanine plus cytosine
$NADH_2$	nicotinamide-adenine-dinucleotide reduced
$NADPH_2$	nicotinamide-adenine-dinucleotide phosphate reduced
NAD	nicotinamide-adenine-dinucleotide
FAD	flavin-adenine-dinucleotide
FMN	flavin-adenine-mononucleotide
FP	flavoprotein
MIC	minimum inhibitory concentration
C	centigrade
µg	micrograms
ml	milliliters
^{3}H	tritium
^{14}C	carbon 14
mg	milligrams
ATCC	American Type Culture Collection
R-factor	resistance factor
ATP	adenosine-tri-phosphate
Mg^{++}	magnesium ion
RTF	resistance transfer factor
PPLO	pleuro-pneumonia-like-organism
mCi	milli-curie
mM	milli-mole
U	uniformly labelled
sp. act.	specific activity
w/v	weight/volume
PPO	2,5-diphenyloxazole
M_2POPOP	1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene
n	normal
R	resistant
S	sensitive

ABBREVIATIONS USED IN THESIS - Cont.

$STM.H_2$	dihydrostreptomycin
L.H. 1	Lapinski Hominis culture #1
C-2	Crawford unidentified isolate #2

INTRODUCTION

A. Taxonomy and Metabolism of Mycoplasma

The members of the genus Mycoplasma represent the smallest free-living micro-organisms found in nature. A single reproductive unit may vary in size from 125 nm in diameter to 500 nm in diameter. The latter may be either spherical or highly branched (1). Mycoplasma contain no cell wall and for this reason are highly pleomorphic. All Mycoplasma, except for Mycoplasma laidlawii require exogenous sterol for growth (2).

In the 7th edition of "Bergey's Manual of Determinative Bacteriology" (1957) Mycoplasma were included in the 10th order of the class Schizomycetes. However, because of their lack of cell wall and non-identity to bacteria in both serologic and nucleic acid homology studies, Mycoplasma have been removed from the class Schizomycetes and placed in the new class Mollicutes (3).

Plant Kingdom

Division I. Protophyta Sachs, 1874, Emend Krassilnikov, 1949 (Primitive plants)

Class IV. Mollicutes Edward and Freundt, 1967

Order Mycoplasmatales Freundt, 1955

Family Mycoplasmataceae Freundt, 1955

Genus Mycoplasma Nowak, 1929

A relationship to protozoa is indicated by the presence of as much as 36% cholesterol in the lipid fraction of parasitic Mycoplasma membranes (4). However, Mycoplasma are prokaryotic and this argues strongly for their being included in the lower protista (5).

In the taxonomy of micro-organisms, a useful measure of relatedness between different micro-organisms is the nucleotide composition of their DNA. The figure of comparison generally used is the moles percent of guanine plus cytosine (G+C). Two organisms with similar G+C ratios are not necessarily closely related, however two organisms with G+C ratios substantially different suggest they belong to different species. Sueoka (6) has found that correlations exist between G+C component compositions of DNA and the amino acid compositions of the proteins of individual micro-organisms.

Studies of the G+C composition of micro-organisms has been carried out by Storck (7) and by Marmur, et al. (8). Storck (7) has found that the GC content of fungal DNA ranges from 38-63%. Within each class however, ranges are smaller-Zygomycetes G+C content are between 38-48%. Even smaller yet are the differences among members of the same genus--Mucor G+C compositions are between 38-40%.

Studies on micro-organisms (8) indicate a G+C range between 22-80%. Sarcina lutea and species of Streptomyces have G+C values of between 70-80% (8), while species of

Bacillus including B. cereus, B. circulans, B. alvei, B. subtilis, B. megaterium, and Lactobacillus have G+C values between 34-44% (8).

Species of Mycoplasma show a wide range of G+C values, 22-41% (9). M. pneumonia G+C ratios are between 39-41% (9), with the other Mycoplasma between 22-34% (9). M. laidlawii strains range from 31.7-34.4% G+C (9), M. arthritidis between 30.0-33.7% (9), and M. neurolyticum from 22.8-25.4% (9) guanine plus cytosine. The values in the low G+C range (M. neurolyticum) represent one of the lowest values found in any micro-organism. The ciliate protozoan Tetrahymena pyriformis (10) with a G+C ratio of 25% is one of the few organisms to approach such a low G+C value. On the other end of the Mycoplasma G+C scale, M. laidlawii and M. pneumonia have G+C values similar to species of Bacillus and Lactobacillus.

Metabolically, as their wide variance in DNA base composition suggests, Mycoplasma are a heterogeneous group of micro-organisms with a wide variation in the nature of their energy yielding systems and metabolic patterns. As a group, Mycoplasma can be broken down into fermentative and non-fermentative species. A summary of the metabolic characteristics of the fermenting and non-fermenting species is given in Table I (11). A summary of the respiratory activity of fermenting and non-fermenting species is given in Table II (12). Fermentative species

TABLE I

Metabolic Characteristics of Fermentative and Non-Fermentative Mycoplasma (11)

Pathway	Fermentative	Non-Fermentative
Embden-Meyerhof	+	0
Hexose monophosphate	+ or 0	partial
Tricarboxylic acid	0	+
Glyoxylate	?	+
Fatty acid oxidative	(0)	+
Pyruvic oxidase	+	(+)
Pyruvate dismutase	+	?

\+ indicates presence of pathway

0 indicates absence of pathway

() parentheses indicate probable presence or absence

TABLE II

A Summary of the Respiratory Characteristics of Fermentative and Non-Fermentative *Mycoplasma* (12)

Enzyme	Fermentative	Non-Fermentative
$NADH_2$ oxidase	+	+ (FAD)
$NADPH_2$ oxidase	+	+
Lactic dehydrogenase	+ (NAD)	+ (FP)
Succinic dehydrogenase	(+)	+ (FP)
Acyl CoA dehydrogenase	(0)	+ (FP)
Diaphorases	+	+ (FAD or FMN)
Quinone reductase	0	+
Cytochrome C reductase	0	+
Cytochrome oxidase	0	+
Catalase	0	+

\+ indicates presence

0 indicates absence

FAD
FMN
FP indicate cofactor specificity

derive their energy from substrate phosphorylation or flavin terminated respiration, while non-fermentative species derive their energy from fatty acid oxidation, the tricarboxylic acid cycle and cytochrome terminated electron transport (12).

Protein synthesis has been shown to be carried out on ribosomes of the 70 s type by Tourtellotte (13), thereby relating Mycoplasma to bacteria, whose ribosomes are also 70s. Protein synthesis in yeasts, protozoa, plants, and animals is carried out on 80s ribosomes (14). Therefore, except for their lack of cell wall and the presence of cholesterol in the Mycoplasma membrane, Mycoplasma most resemble bacteria, particularly Bacillus and Lactobacillus species.

B. Mycoplasma and Disease States

Diseases caused by Mycoplasma in animals include: chronic respiratory disease in chickens (M. gallisepticum); infectious synovitis in turkeys (M. synoviae); chronic pneumonia in swine (M. suipneumoniae); mastitis and arthritis in cattle (M. bovimastitidis); arthritis of rats (M. arthritidis); and rolling disease in mice and rats (M. neurolyticum)(15). Both M. gallisepticum and M. neurolyticum produce toxins which affect the central nervous system of their respective hosts (16).

In addition, there is growing evidence for the implication of Mycolplasma as causative agents in many plant and human diseases. In plants, electron microscopy has revealed Mycoplasma-like inclusions in the phloem elements of plants infected with what was initially thought to be a virus. Aster yellow, corn stunt disease, and several other leaf-hopper borne diseases are now thought to be caused by Mycoplasma, although these organisms have yet to be cultured on solid media (17).

In man, only one pathogenic Mycoplasma has been positively identified. M. pneumoniae (Eaton's agent) has been shown to be the cause of primary atypical pneumonia (18). However, there is evidence indicating that Mycoplasma may be associated with several other disease states of man. A causative agent for arthritis in cattle and rats has been identified as a Mycoplasma, and this has led to a search for Mycoplasma in human arthritics. Several reports have shown that Mycoplasma can be isolated from the joint fluid of human arthritics. Research on the relationship between Mycoplasma and arthritis is being carried out in several laboratories (19).

Non-gonococcal urethritis is now thought to be caused by Mycoplasma. Shepard (20) has isolated Mycoplasma which are designated "T- (tiny)-strain." This strain of Mycoplasma characteristically produces tiny colonies on solid media. "T-strain" Mycoplasma have been isolated in 50-80% of the patients with non-gonococcal urethritis by

Csonka, et al. (21) and Ford and DuVernet (22). These Mycoplasma are novel in that they possess a urease system and require urea for growth.

There have been reports of the isolation of Mycoplasma species (M. fermentans, M. laidlawii, M. orale, M. hyorhinis) from human malignant tissue and leukemic bone marrow (23-25). M. hyorhinis has been identified from solid tumors by Hayflick (26). This organism has also been isolated from nonmalignant tissue such as primary chick cells, primary green monkey kidney cells, and human diploid cell strains (26). The significance of the isolations from malignant tissue has yet to be evaluated, although there is no definitive proof of an etiologic relationship between Mycoplasma and cancer.

Research is also being done to determine the relationship between low birth-weight and spontaneous abortions and Mycoplasma infection. Harwick and coworkers (27) have reported isolation of an M. hominis strain from the liver and blood of an aborted fetus as well as the cervical discharge of the mother. They were able to demonstrate a sharp rise in antibody titer to M. hominis curing the convalescence of the mother. In a similar study, Jones (28) also reported isolating a strain of M. hominis from a number of aborted fetuses and the mothers of these fetuses. Jones reported a twofold increase in the percentage of mothers showing M. hominis

antibody formation in those women who have had spontaneous abortions, compared to a control group of normally pregnant women. Harwick later reported (29) that although a greater percentage of patients having spontaneous abortions were positive for M. hominis than normally pregnant mothers, an etiological relationship could not be proven from the data presented.

Kundsin, et al. have reported (30) isolation of a previously unidentified type of "T-strain" Mycoplasma from the membranes of an aborted fetus. The decidua showed extensive necrosis and the umbilical cord severe inflammation, presumably from a long standing infection. The same strain was isolated from the urethra of the mother. In follow-up studies (30), the same strain was also isolated from three out of six aborted fetuses and from the cervical culture of five out of ten women with a history of repeated spontaneous abortions. In other experiments, Kundsin and Driscoll (31) demonstrated a correlation between abortion and premature births, and the presence of "T-strain" Mycoplasma. In this study, "T-strains" were quite often associated with M. hominis. In another study (32), Kundsin and coworkers examined 38 fetal membranes, 25 from premature births and spontaneous abortions, 13 from normal, full-term babies and therapeutic abortions. They isolated Mycoplasma from 18/25 premature babies and spontaneous abortions, and from 1/13

normal babies and therapeutic abortions. The isolation of Mycoplasma from the normal group was from a mother with a history of repeated abortions. This was the first child she had carried to term. "T-strain" Mycoplasma and "T-strain" associated with M. hominis were the most commonly isolated Mycoplasma.

An association between low birth-weight and the presence of "T-strain" Mycoplasma has been demonstrated by Braun, et al. (33) although once again the data were insufficient to prove an etiologic relationship. However, the compiled data of the previous studies (27-33) does seem to indicate that "T-strain" Mycoplasma may be a genital tract pathogen during pregnancy.

The Mycoplasma species indigenous to man and their sites are listed in Table III (34). In general, these Mycoplasma are sensitive to protein synthesis inhibitors such as the tetracyclines and chloramphenicol, and resistant to cell-wall synthesis inhibitors such as the penicillins, cephalosporins, and bacitracin (35). However, there are reports of clinically isolated Mycoplasma resistant to specific antibiotics towards which they are normally sensitive (36). As with drug resistant bacteria and fungi, the development of antibiotic resistant Mycoplasma could be of clinical importance in the treatment of Mycoplasma infection, especially in light of the growing implication of Mycoplasma in human disease states.

TABLE III

Mycoplasma Species Indigenous to Man and Their Source of Isolation (34)

Mycoplasma Species	Source of Isolation
M. hominis I	Genitourinary tract oropharynx
M. hominis II (*M. arthritidis*)	Genitourinary tract
M. fermentans	Genitourinary tract
M. salivarium	Oropharynx
M. pneumoniae	Oropharynx
M. pharyngis (*M. orale* I)	Oropharynx
M. orale II	Oropharynx
("T-strain")	(Genitourinary tract)

The present study examines the mechanisms of resistance in Mycoplasma and other antibiotic associated problems including the production of antimicrobial agents by Mycoplasma, and the production of penicillinase from these organisms. The latter two are included to further elucidate the metabolic pathways present in Mycoplasma, and if possible, establish evolutionary relationships with other micro-organisms.

LITERATURE REVIEW

A. Absorption of Antibiotics by Fungi and Bacteria

The mechanisms and origins of antibiotic resistance in micro-organisms have been studied extensively in the last two decades. One aspect of study has been the elucidation of the mechanisms of resistance development. In 1960, Pollock (37), in summarizing the mechanisms known at that time, listed the following:

1) "A drug sensitive reaction essential for growth in susceptible strains may be either absent or supplemented by one that performs the same function and is unaffected by the drug."

2) Decreased drug accumulation
   a) cell impermeability to the drug.
   b) masking or absence of drug receptor.

3) Degradation or inactivation of the drug by the organism.

A review of some antibiotic absorption/binding and enzymatic inactivation studies by both fungal and bacterial species follows.

In 1959, Lampen and Arnow (38) found that absorption of the polyene antibiotic nystatin by cells was a critical factor in determining sensitivity to the antibiotic. Sensitive organisms Saccharomyces cerevisiae (MIC 3 µg/ml), Candida albicans (MIC 4 µg/ml) and Penicillium chrysogenum (MIC 5 µg/ml) absorbed 3-5 times more antibiotic than the insensitive bacteria Streptococcus faecalis and Escherichia coli, both resistant to more than 100 µg/ml of nystatin. Both resistant and sensitive organisms were assayed in the MIC range of the sensitive organisms (nystatin concentration 1.7-10 µg/ml). Uptake by the fungal species of nystatin did not occur at 4°C, was dependent on the antibiotic concentration, and the absorbed nystatin (as determined spectrophotometrically at 321 nm) was not removed by washing the cells with buffer, acetone, or by changing the pH of the medium. Studies on the accumulation of other polyene antibiotics such as amphotericin B, candidin, and filipin by Saccharomyces cerevisiae, Candida albicans, and Neurospora crassa also indicated that these sensitive species accumulated more antibiotic than resistant bacteria such as E. coli, B. subtilis, and Streptococcus faecalis (39-41).

Evidence points to the selective binding of polyene antibiotics to the sterols present in the membranes of fungi (42,43), Ergosterol, the main sterol in fungi, has been shown to be involved in the binding of polyene to the

fungal cell membrane by Kinsky (42) and Lampen (43). However, a mutant strain of Candida albicans resistant to nystatin absorbed 5-10 times less antibiotic than the sensitive parent strain (38). In another study Hebeka and Solotorovsky (44) developed a mutant of Candida albicans resistant to 75 µg/ml of candidin, representing a 150 fold increase in the level of resistance from the sensitive parent. This organism was cross-resistant to amphotericin B, showing an 11.5 fold increase in resistance (from 0.2 ug/ml to 2.3 µg/ml). A possible explanation of this resistance is a decreased permeability of the antibiotic by the cell, preventing the polyene from reaching the membrane sterols (45). There seems to be a direct correlation between an organism's sensitivity to the polyene antibiotics and its ability to accumulate the antibiotic.

El-Nakeeb and Lampen (46) reported on the accumulation of (4-methoxy-^{3}H)-griseofulvin, a spirolactone antibiotic, by a sensitive dermatophyte, Microsporum gypseum (MIC 0.5-1.5 µg/ml). A growing culture of the fungus accumulated griseofulvin intracellularly to a level 100 times the concentration of the drug in the media. Two phases of uptake were noted: first an instantaneous binding of the antibiotic, followed by a second, prolonged, temperature dependent absorption. Autoclaved cells of M. gypseum did not show the second phase of absorption.

Accumulation of the antibiotic was dependent on the concentration of drug in the media (at a range studied from 2.5-20 µg/ml).

Other studies (47) of micro-organisms indicated a relationship existed between griseofulvin sensitivity and the organisms ability to accumulate the drug, Candida albicans, Saccharomyces cerevisiae, and Escherichia coli, each insensitive to 10 µg/ml of griseofulvin, did not bind appreciable amounts of ^{3}H-griseofulvin at 10 µg/ml griseofulvin concentration. Slightly sensitive filamentous fungi Aspergillus niger and Neurospora crassa (10-20% inhibition with 10 µg/ml antibiotic) accumulated a considerable quantity of antibiotic, although most of the antibiotic was present in the water soluble pool, with little bound directly to cellular material. Sensitive organisms Microsporum gypseum, Trichophyton mentagraphytes and Trichophyton persicolor (complete inhibition at 10 µg/ml griseofulvin) also accumulated antibiotic. However, unlike the slightly sensitive fungi, over one-half the total of ^{3}H-griseofulvin from the sensitive organisms could only be recovered by hot trichloracetic acid and 1 N NaOH extraction of the cells. The nucleic acid and protein recovered by the 1 N NaOH and trichloroacetic acid treatment were found to contain the bound griseofulvin. No radioactivity was found to exist in the nucleic acid or protein itself. RNA preparations from M. gypseum (48)

were shown to form complexes with griseofulvin which were stable to dialysis and density-gradient centrifugation. Similar complexes were recovered from cultures of M. gypseum (48) which had been pretreated with griseofulvin. The authors concluded (47) that the difference between the slightly sensitive fungi (Aspergillus niger and Neurospora crassa) and the sensitive fungi was the greater affinity of the nucleic acids in the sensitive fungi for griseofulvin. They concluded that the resistant organisms were impermeable to griseofulvin.

The accumulation of cycloheximide, a glutarimide, has been studied with resistant and sensitive yeast (49-51). Westcott and Sisler (49) studied a strain of Saccharomyces fragilis, a cycloheximide resistant yeast (MIC greater than 1000 µg/ml), and Saccharomyces pastorianus, a sensitive yeast (MIC 1 µg/ml), and found that while S. fragilis did not concentrate cycloheximide from the extracellular fluid, S. pastorianus concentrated cycloheximide more than 10 times the external concentration at 0.1 or 1 µg/ml. The internal concentration in the resistant strain was essentially the same as that in the extracellular solution, suggesting to Westcott and Sisler that the lack of concentration of the antibiotic was due to a lack of binding sites.

Experiments by Cooper and coworkers (50) showed that in the cell-free incorporation of phenylalanine in the

presence of cycloheximide, using ribosomes and supernatant enzymes for protein synthesis derived from resistant (MIC 10 µg/ml) and sensitive (MIC less than 0.005 µg/ml) Saccharomyces cerevisiae, the ribosomes of the resistant organism conferred resistance in the cell-free incorporating system. Experiments by Siegel and Sisler (51) utilized cell-free protein synthesizing systems obtained from a cycloheximide sensitive yeast Saccharomyces pastorianus (MIC 0.17 µg/ml), and a cycloheximide resistant yeast, Saccharomyces fragilis (MIC greater than 1000 µg/ml). Various combinations of ribosomes and supernatant enzymes from the two yeasts showed that resistance or susceptibility of the protein synthesizing systems was determined by the ribosomes.

Thus, in these last two cases at least, antibiotic resistance could be attributed to the difference in affinity of the ribosomes of resistant and sensitive yeast to the cycloheximide.

Antibiotic uptake studies using chloramphenicol, the tetracyclines, erythromycin, and streptomycin have also been carried out with bacteria. In 1962, Hancock (52) demonstrated that streptomycin sensitivity (or resistance) in organisms such as Staphylococcus aureus, Streptococcus faecalis, Clostridium welchii, Bacillus subtilis, Escherichia coli, Clostridium sporogenes, Candida utilis, and Bacillus megaterium, was correlated to the uptake of

streptomycin by the particular organism. A resistant Streptococcus faecalis strain (MIC 50 µg/ml), accumulated one percent of the total accumulated by a sensitive Bacillus megaterium (MIC 0.5 µg/ml). A strain of Candida utilis (MIC greater than 1000 µg/ml) accumulated less than one-fifth the amount of antibiotic than did S. faecalis. In general, the amount of antibiotic absorbed was proportional to the sensitivity of the organism to streptomycin. Hancock (53) found that the uptake of streptomycin depended on the continued synthesis of cellular material. The quantity of streptomycin finally absorbed was proportional to the streptomycin concentration in the medium, and at the lowest growth inhibitory concentration, about 5×10^4 molecules per B. megaterium cell were absorbed.

Similar results with streptomycin were reported by Szybalski and Mashima (54) utilizing Escherichia coli resistant, sensitive, and dependent cultures. A maximum uptake of ^{14}C-streptomycin (prepared from uniformly labelled glucose as a precursor in biosynthesis) was 10^6 molecules/cell at 50 µg/ml streptomycin concentration with the E. coli K12 sensitive strain. Almost no uptake was recorded for the streptomycin resistant cells, while transient uptake for streptomycin dependent cells occurred. At 5 µg/ml of streptomycin, the uptake was one-tenth that at 50 µg/ml with the sensitive strain.

The 10^5 molecules per cell of streptomycin accumulated was enough to inhibit growth (as measured by turbidity and microscopic cell counts).

In 1960, Anand and coworkers (55) reported that cells of Escherichia coli sensitive to streptomycin accumulated ^{14}C-streptomycin (prepared from uniformly labelled glucose as a precursor in biosynthesis) in two phases. The first phase was extremely rapid and finished before the first time sample could be taken (1 minute). The second phase was slower and the amount of streptomycin absorbed leveled off in one hour, after a 3-4 fold increase in accumulated antibiotic. Resistant mutants exhibited the same initial absorption but no secondary rise. Treatment of the sensitive cells with toluene or polymyxin B before streptomycin exposure, increased the amount of streptomycin accumulated, presumably by damaging the cell membrane. The increased streptomycin absorption suggested to the authors that the site of action of streptomycin was the cell membrane and resistance was acquired by alteration of the cell membrane so as to prevent streptomycin damage. It has now been shown that streptomycin acts at the ribosome (56), and the results obtained by Anand, Davis and Armitage could also be explained in terms of impermeability of the resistant cells to streptomycin.

Vazquez (57,58) has studied the uptake of chloramphenicol by resistant and sensitive Escherichia coli B.

He reported a close relationship between the uptake of chloramphenicol (methylene-^{14}C) and antibiotic sensitivity of the resistant and sensitive E. coli B strains. The uptake of chloramphenicol by E. coli B (MIC 5 µg/ml) was four times higher than a resistant E. coli B #150 (MIC 300 µg/ml). In studies with spheroplasts prepared from the two organisms, the spheroplasts from the resistant organism accumulated much less antibiotic than the sensitive one (12.1 ng/mg dry weight of organism vs 0.6 ng/mg dry weight or 2.2×10^{13} molecules/mg compared to 1.1×10^{12} molecules/mg absorbed for the resistant culture).

The binding of chloramphenicol to resistant and sensitive ribosomes was also studied by Vazquez (58). Similar amounts of chloramphenicol were bound to ribosome preparations prepared from the resistant and sensitive E. coli strains, indicating resistance was not due to a difference in ribosome affinity for chloramphenicol.

Okamoto and Mizumo (59) also studied chloramphenicol activity on resistant and sensitive Escherichia coli B and K-12 strains. Chloramphenicol was found to inhibit equally cell-free protein synthesizing systems prepared from the ribosomes and supernatant enzymes of both a resistant (MIC 300 µg/ml) and sensitive (MIC 0.3 µg/ml) E. coli B. Incubation of chloramphenicol with supernatant enzymes of both strains indicated that no enzymatic degradation was taking place. Conclusions reached by

Vazquez (57) and Okamoto and Mizumo (59) were that resistance was due to a decreased permeability of the bacteria to chloramphenicol.

In 1963, Taubman and Corcoran (60) carried out studies on the bacterial absorption of erythromycin. Uptake by erythromycin resistant (MIC 3 µg/ml) and sensitive (MIC 0.04 µg/ml) Bacillus subtilis was measured by filtering aliquots of a growing culture incubated with ^{3}H-erythromycin (labelled in the macrolide ring using sodium 2,3-^{3}H-propionate as a precursor in biosynthesis) and measuring the cellular radioactivity in a scintillation spectrometer.

There was a significant difference (2-3 fold) in uptake between the sensitive and resistant Bacillus subtilis strains, the sensitive culture accumulating more antibiotic than the resistant. Similar differences were also noted in lysozyme spheroplasts from resistant and sensitive organisms. There was an initial rapid uptake of antibiotic from the media, then a slower release until an equilibrium level was attained. Uptake was complete within 20 minutes. Corcoran and Taubman concluded (60) that the 2-3 fold difference in uptake could not explain the 100 fold difference in sensitivity.

In the same study (60), Taubman and Corcoran found that erythromycin uptake in resistant and sensitive cells was proportional to the extracellular concentration of

the drug, even after multiplication of the sensitive strain ceased (7.5 µg erythromycin/l x 10^{11} bacteria). The erythromycin sensitive strain concentrated the antibiotic 60 times the extracellular concentration, while the resistant strain only 20 fold. At higher erythromycin concentrations, but below the MIC of the resistant strain, the amount of antibiotic accumulated in the resistant strain exceeded the amount causing inhibition in the sensitive strain. It was also demonstrated by the authors that cell-free protein synthesizing systems could be inhibited by erythromycin, while a similar system from the resistant strain could not. Chloroform extraction at pH 9.5, and diethyl ether extraction at neutral pH indicated that 90-100% of unchanged erythromycin (as calculated by isotopic dilution) could be recovered from the growing culture of the resistant B. subtilis incubated with erythromycin. The authors concluded that neither antibiotic destruction nor cellular impermeability could explain the resistance. The resistance mechanism of the resistant B. subtilis mutant was explained as being due to an increased tolerance to the antibiotic (60).

Mao and Putterman (61) also studied the accumulation of erythromycin by bacteria. Escherichia coli ATCC 11775 (MIC 15 µg/ml), Proteus vulgaris ATCC 7897 (MIC 38 µg/ml), Staphylococcus aureus 209 P (0.2 µg/ml), and Bacillus

subtilis ATCC 10707 (0.15 µg/ml) were the organisms studied. The sensitive Gram-positive S. aureus and B. subtilis accumulated 100 times more erythromycin (^{14}C-N-methyl) than the other two Gram-negative bacteria. Ether extraction of the Gram-negative fermented broth cultures recovered 93-97% unchanged erythromycin as determined by thin layer chromatography. The cell-free protein synthesizing preparations from both Gram-positive and Gram-negative organisms were equally susceptible to the antibiotic. Thus, neither destruction of erythromycin nor the absence of an erythromycin susceptible step played a role in the resistance mechanism of Gram-negative bacteria. Intracellular erythromycin concentration in the Gram-positive bacteria was 44-90 fold higher than the Gram-negative accumulation, indicating to the authors that the lack of accumulation of the antibiotic was responsible as the resistance mechanism.

The uptake of the tetracycline antibiotics by resistant and sensitive E. coli has also been investigated (62-67). In 1963, Arima and Izaki (62) found that at high oxytetracycline concentrations (100-400 µg/ml) an E. coli B accumulated large quantities of antibiotic. Heat killed cells (1 minute in boiling water) did not accumulate significant amounts. Uptake was measured spectrophotometrically at 340 or 260 nm to give the "relative" amounts of oxytetracycline accumulated.

In another study, Izaki and Arima (63) showed that a resistant E. coli K-12 (resistant to chloramphenicol, tetracycline, streptomycin and sulfonamide) accumulated 1/6 the amount of oxytetracycline than a sensitive E. coli K-12 at concentrations of 50 μg/ml. A decrease in uptake of oxytetracycline occurred concurrently with a rise in the level of resistance by the multiply drug resistant strain of E. coli. Izaki, Arima and Kiuchi (64) also found that resistant cells of E. coli K-12 grown in the presence of low concentrations of tetracycline, oxytetracycline, or chlortetracycline, accumulated less tetracycline when challenged against a higher concentration of antibiotic than cells not pre-exposed.

The conclusions reached by Arima, Izaki and Kiuchi (64) were that the tetracyclines neither penetrate nor accumulate in resistant cells; that accumulation and permeability were relevant to their bacteriacidal action; and that an induction mechanism was involved in the resistance mechanism in tetracycline resistant bacteria.

Franklin and coworkers (65-67) also examined tetracycline and chlortetracycline uptake in resistant and sensitive E. coli (ATCC 112299/198). The resistant E. coli strain (MIC 100 μg/ml) was shown to accumulate 10-30 times less chlortetracycline than the sensitive strain (MIC 0.5 μg/ml) at 10 μg/ml chlortetracycline concentration. A slightly lower difference in accumulation was noted for tetracycline. At 1 μg/ml the sensitive E. coli accumulated

1.01 µg tetracycline/mg protein while the resistant E. coli only 0.11 µg/mg of the antibiotic. Uptake of ^{14}C-(^{14}C-dimethylamino)-chlortetracycline and 7-^{3}H-tetracycline was measured by counting aliquots of the washed cells in a scintillation spectrometer. Cell-free systems for protein synthesis prepared by sonic oscillation from both resistant and sensitive organisms were inhibited equally by chlortetracycline.

Franklin and coworkers (66) demonstrated that the accumulation of tetracycline was linearly proportional to the concentration in the media up to a concentration of 400 µg/ml. At a concentration of 10 µg/ml, the net accumulation of tetracycline ceased after 7-10 minutes. When strains of Escherichia coli bearing transferable resistance factors (genetic factors for resistance to various antibiotics capable of being transferred from a resistant organism to a sensitive one among species of enterobacteria) to the tetracyclines were grown for 15-30 minutes in a sub-inhibitory (below MIC) concentration of oxytetracycline or tetracycline, a rapid increase in resistance occurred associated with a marked fall in the absorption of the drug by the resistant cells.

Recently, Franklin and Foster (67) have demonstrated that tetracycline resistance in an R-factor (transferable resistance factor) carrying E. coli could be drastically lowered by subjecting the cells to osmotic shock and

transiently lowered by treating the cells with 3×10^{3} M ethylenediamine tetracetic acid. Franklin and Foster concluded (67) that the mechanism of resistance of the resistant E. coli is an antagonism of the transport system whereby tetracycline is prevented from entering the cell. Similar conclusions on the impermeability of tetracycline to resistant E. coli were reached by Izaki, Arima, and Kiuchi (64).

In summary, a decreased antibiotic accumulation among the resistant bacteria and fungi reviewed seems to be a common factor. In Pollock's summary (37) of resistance mechanisms presented at the beginning of the Literature Review, a decreased drug accumulation was listed as one of the three possible mechanisms of resistance. In further categorizing the antibiotics discussed, resistance to nystatin (sterol binding), griseofulvin (nucleic acid binding), cycloheximide (ribosome binding), streptomycin (ribosome binding) and erythromycin (ribosome binding), can be attributed to a masking or absence of a drug receptor. Tetracycline and chloramphenicol resistance is due to a decreased cell permeability to the antibiotic.

B. Enzymatic Degradation and Inactivation of Antibiotics by Resistant Organisms

Besides decreased drug accumulation, Pollock also listed in his summary of resistance mechanisms (37), the

degradation or inactivation of an antibiotic as a mechanism of antibiotic resistance. Examples of such inactivation have been described for penicillin (69-75), streptomycin (76,77), spectinomycin (76), kanamycin (77-79), paromamine (79), and chloramphenicol (77,80).

Perhaps the classic example of antibiotic inactivation by enzymatic degradation is the hydrolysis of the β-lactam ring of penicillin and cephalosporin by micro-organisms producing β-lactamases (penicillinase, cephalosporinase).

Penicillinases are widely distributed among both Gram-positive and Gram-negative bacteria, and are capable of inactivating many penicillins. Penicillin resistant Staphylococcus aureus species inactivate penicillin primarily through the production of penicillinase, thereby reducing the clinical effectiveness of penicillin G. Molecular weights between 28,000 and 36,000 have been reported for penicillinases produced by Gram-positive bacteria such as Staphylococcus aureus (69), Bacillus cereus (70), and Bacillus licheniformis (71). These penicillinases are inducible enzymes (produced only in response to the substrate), and are secreted extracellularly. The penicillinases produced by non-R-factor carrying Gram-negative bacteria are constitutive enzymes (constantly produced whether enzyme substrate is present or not), and are not secreted extracellularly (72).

In 1965, Datta and Kontomichalou (73) reported that the genes responsible for synthesizing large amounts of

penicillinase in an enterobacteria were located on an R-factor. The penicillinase produced by Escherichia coli TEM (with an R-factor for penicillinase) was isolated and purified by Datta and Richmond (74). They reported the enzyme had a molecular weight of 16,700, a pH optimum between 5.8-7.0, and was both constitutive and periplasmic (produced between the plasma membrane and the cell wall of the bacteria). Penicillinase from another R-factor carrying E. coli isolated by Yamagishi (75) had properties closely related to the penicillinase isolated by Datta and coworkers. However a different E. coli carrying an R-factor for penicillinase was shown to possess different properties by Yamagishi (75). The protein was larger, and a pH optimum of 7.8, was basic in nature (the others being acidic), and was shown to be immunologically different. The results of Datta, Kontomichalou, and Yamagishi (73-75) suggest that the origins of penicillinase, even among Gram-negative bacteria are not the same. Compared to penicillinase produced by Gram-positive organisms, penicillinase produced by the R-factor resistant strains are similar to those produced by the Gram-negative organisms, indicating perhaps, a common origin. It may be possible to establish genetic relationships between micro-organisms by such a comparison of enzymatic properties. If a penicillinase enzyme could be isolated and characterized from Mycoplasma, it might be possible to relate the

Mycoplasma organism to another micro-organism producing a penicillinase with similar properties.

Enzymatic adenylylation of streptomycin and spectinomycin was demonstrated recently by Benveniste and coworkers (76). Cell-free enzyme preparations prepared by the osmotic shocking of various R-factor strains of E. coli resistant to both streptomycin and spectinomycin were shown to inactivate either antibiotic in the presence of ATP at 30°C.

Enzymatic inactivation of kanamycin was reported by Okamoto and Suzuki (77) in 1965 by R-factor carrying E. coli K-12. A cell-free system prepared by disrupting the cells with a French pressure cell inactivated kanamycin in the presence of Coenzyme A, acetate, and ATP. The authors postulated that the inactivated product was an acetylated kanamycin derivative. In 1967, Umezawa and coworkers (78), using the same organism as Okamoto and Suzuki, succeeded in isolating, purifying and identifying the inactivated product as mono-N-acetylkanamycin.

In 1967, Umezawa (79) and coworkers reported the inactivation of kanamycin, paromamine, and dihydrostreptomycin by a resistant Escherichia coli K-12 ML 1629. The three antibiotics were shown to be inactivated by phosphorylation by chemical analysis and treatment with alkaline phosphatase (the active antibiotic being re-generated after the enzyme treatment). The antibiotics were inactivated by an enzyme solution (prepared by the

disruption of the E. coli cells with a French pressure cell apparatus) in the presence of Mg^{++} and ATP. The purified products were identified as being phosphorylated on the 3-hydroxyl group of the 6-amino-6-deoxy-D-glucose moiety of kanamycin, or the 3-hydroxyl group of the glucosamine moiety of paromamine.

Another example of enzymatic inactivation of an antibiotic was reported by Shaw (80). Chloramphenicol was inactivated by enzymatic acetylation using an R-factor resistant strain of Escherichia coli. Cell-free extracts prepared by sonic oscillation inactivated chloramphenicol in the presence of acetyl-coenzyme A. The purified products were shown to be 3-acetoxy and 1,3-diacetoxy chloramphenicol, both lacking antibacterial activity.

C. R-Factors and Their Role in Antibiotic Resistance

Shigella strains of bacteria with multiple-drug-resistance patterns have been isolated in Japan with increasing frequency since 1957 (81,82). The dysentery caused by Shigella was then, and still is, a serious medical problem in Japan (82,83). In the late 1950's, 80,000 cases were reported (83). In spite of improved sanitary conditions and prophylactic inoculations, the incidence of infection caused by Shigella strains has not decreased (82).

Various derivatives of sulfonamide were introduced into Japan at the end of World War II for the treatment of dysentery. Reported infections decreased from 95,000 (83) in 1946 to 15,000 (83) in 1948. However sulfonamide resistant Shigella began appearing in 1949 (85), and by 1952 over 100,000 cases of dysentery were reported (83). Between 80-90% of all Shigella strains isolated were resistant to the sulfonamide antibiotics by 1952 (82).

In 1950, chloramphenicol and tetracycline were first introduced into Japan from the U.S. Production of the tetracyclines started in 1953, and chloramphenicol in 1954. Production of streptomycin began in Japan in 1950. However in 1952, a Shigella simultaneously resistant to tetracycline, streptomycin and sulfonamide was recovered from a dysenteric patient by Suzuki and coworkers (84). In 1955 a strain simultaneously resistant to tetracycline, streptomycin, chloramphenicol and the sulfonamides was isolated by Kitamoto and coworkers (85). After 1957, the number of multiply resistant Shigella strains isolated increased markedly (81). The frequency of such strains was estimated over 70% in 1967 (70% of all Shigella isolated were resistant to two or more drugs). Moreover, other enteric bacteria were found to be developing resistance to the same antibiotics. In a survey compiled by Mitsuhashi (87) from 1965-1967, 1306 strains of bacteria were examined. Of these, 71.2% of the Escherichia coli

(775 strains examined), 60% of the E. freundii (40 strains examined), 63% of the Klebsiella (195 strains examined), 62.3% of the Aerobacter (60 strains examined) and 35.1% of the Proteus strains (236 strains examined) were resistant to one or more of the following antibiotics: tetracycline, chloramphenicol, streptomycin, and sulfonamide derivatives. Between 30-40% of these species except Proteus were simultaneously resistant to all four antibiotics.

The problem of multiple drug resistance is not restricted to Japan. In Greece, 95.8% of Shigella isolated between 1965 and 1967 contained R-factors (88,89). In the U.S., 75% of the Klebsiella (90), 71% of the Salmonella (91) and over 70% of the E. coli (90,92) examined between 1965 and 1967 also contained R-factors.

In 1959, Akiba and Kimura (93) and Ochiai (94) noted that resistant and sensitive strains of Shigella of the same serological type could be isolated from different patients during outbreaks of dysentery. Matsuyama and coworkers (95) isolated multiple drug resistant E. coli strains during a similar dysenteric outbreak. Ochiai, et al. (96) and Akiba, et al. (97) independently reported that multiple resistance could be transferred between drug resistant E. coli and sensitive Shigella strains by mixed cultivation. It has now been demonstrated (98) that multiple resistance factors can be transferred to every genus of enteric bacteria and to other genera including

the Vibrio group and Pasteurella pestis by non-pathogenic bacteria such as E. coli.

In 1960, Mitsuhashi (99) proposed the term "R" factor for the property of transmissible drug-resistance. Watanabe (100) has proposed the term RTF or "Resistance Transfer Factor" for that part of the genetic material responsible for autonomous replication, specific pilus formation, and transfer by conjugation. The capability of the R-factor to be transmissible by bacterial conjugation, to be eliminated by acridine dye, and to replicate faster than the host chromosome has given proof of the extra-chromosomal or episome nature of the R-factor (101). Besides the four antibiotics previously discussed as being carried on the R-factor, kanamycin (97), paromomycin (79), and ampicillin (73-75) have been shown to be included on some R-factors. With the exception of the sulfonamide antibiotics, the biochemical mechanisms of R-factor resistance have previously been discussed.

MATERIALS AND METHODS

I. ANTIBIOTIC RESISTANCE IN MYCOPLASMA

A. Materials

Mycoplasma cultures. We are indebted to Dr. S. Madoff, Massachusetts General Hospital, Boston, to the late Mr. Y. E. Crawford, Mycoplasma Research Division, Naval Medical Research Unit No. 4, Department of the Navy, and to Dr. E. Lapinski, Veterans Administration Hospital, Madison, Wisconsin, for the clinical isolates of Mycoplasma used in these studies. The Mycoplasma laidlawii strain B was obtained from Professor H. E. Morton, University of Pennsylvania. The avian Mycoplasma were obtained from Dr. A. H. Hamdy of The Upjohn Company, Dr. E. Aycardi, University of Wisconsin, Veterinary Science Department (who is now in Bogota, Colombia), and Dr. E. Lapinski.

Bacterial cultures. The Salmonella typhimurium S-1, Klebsiella #24, E. coli #8, and E. coli #15 cultures were obtained from Dr. E. Grunberg, Hoffmann-La Roche, Inc.

Media. All Mycoplasma were grown in Difco PPLO broth supplemented with 0.5% yeast extract, 0.1% sodium acetate, and either 10% heat inactivated human serum (serum heated to 55°C for 45 minutes), or 2% PPLO serum fraction (Bacto). Difco PPLO agar supplemented with 10% heat inactivated

human serum or 2% Bacto PPLO serum fraction was used for solidified media. Nutrient broth and nutrient agar were used for the growth of bacterial cultures.

Antibiotics. The tetracycline-HCl, erythromycin, chloramphenicol, gentamicin·SO_4, kanamycin·SO_4, streptomycin·SO_4, dihydrostreptomycin·SO_4, and paromomycin used in these studies were commercial grade materials. The spectinomycin was a gift from Dr. G. B. Whitfield, Jr., The Upjohn Company. The potassium penicillin V salt was obtained from Dr. R. Morin, University of Wisconsin, School of Pharmacy. The mannosidostreptomycin was obtained from Dr. E. Inamine, Merck, Sharp and Dohme Research Laboratories. All antibiotics were used as received without further purification.

Radioactive antibiotics. Tetracycline-7-T hydrochloride (sp. Act. 800 mCi/mM) was obtained from Schwarz BioResearch, Inc. The dihydrostreptomycin-T-sesquisulfate (sp. act. 3 Ci/mM) and chloramphenicol (methylene-^{14}C sp. act. 10.1 mCi/mM) were obtained from Amersham/Searle Corporation. All were used without further purification and identification.

Radioactive chemicals. Adenosine-^{14}C(U)-5'-triphosphate (sp. act. 568 mCi/mM) was obtained from Amersham/Searle. Adenosine-8-^{14}C-5'-triphosphate (sp. act. 41.9 mCi/mM) was obtained from New England Nuclear Corporation.

B. Maintenance and Growth of Mycoplasma

Mycoplasma cultures stored for long periods of time (one year or more) were kept in sealed glass vials in a liquid nitrogen freezer. Growth was re-established by plating the thawed culture on PPLO agar and incubating the agar culture at 37°C in a 5% CO_2/95% air atmosphere. This incubation procedure, that is, 37°C in a 5% CO_2/95% air atmosphere, should be regarded as the standard method of incubation whenever incubation of Mycoplasma is mentioned. Broth cultures were prepared by transferring an agar block containing Mycoplasma colonies (verified by microscopic examination) to five or ten ml of broth containing PPLO serum fraction and incubating for 3-5 days. This culture was then utilized for the inoculum for various experiments.

In some instances, clinical isolates were received already cultured on agar plates. In such cases, the broth inoculum was prepared directly from these plates. Mycoplasma cultures contained on agar plates were found to remain viable if stored at 4°C, as long as the plates were sealed against agar drying. Sealing the plates with masking tape and wrapping them in aluminum foil before refrigeration proved satisfactory.

C. Centrifugation of Mycoplasma

Because certain experiments necessitated the harvesting of Mycoplasma from broth culture by centrifugation, the effects of high and low speed centrifugation on Mycoplasma was studied. Mycoplasma laidlawii B and an avian Mycoplasma, designated "POY-PH"(isolated from a pheasant in Poynette, Wisconsin) obtained from Dr. E. Aycardi, were used in this study. (The probable serotype of the avian specie was "G".)

To 200 ml of PPLO broth in a 500 ml Erlenmeyer flask, 20 ml of a 48 hour old culture of Mycoplasma were added. After 48 hours of incubation, 100 ml portions of each culture were centrifuged at 4°C as follows:

2,000 x g for 60 minutes
12,000 x g for 20 minutes.

The centrifuge was a SorVal refrigerated centrifuge with the GSA head. The harvested cells were resuspended in 1 ml of PPLO broth and the titer determined by diluting the suspension in PPLO broth and pipetting 0.1 ml of a series of dilutions on PPLO agar with 2% PPLO serum fraction. The broth culture was spread across the surface of the agar with a sterilized glass rod. Colonies were counted after 2 days incubation and again at 5 days and the titers calculated. The titer of the initial broth culture was also determined without centrifugation. The results of a study on the effects on viability of two Mycoplasma

cultures centrifuged at high and low speeds are presented in Table IV.

Centrifuging both cultures at 2000 x g for 60 minutes did not significantly lower the titer of the culture flask when compared to the titer obtained before centrifugation. However, centrifugation at 12,000 x g for 20 minutes considerably lowered the titer. This loss in viability at the higher speed virtually eliminated high speed centrifugation of Mycoplasma as a method of harvesting viable Mycoplasma cells.

D. Determination of the Antibiotic Sensitivity of Mycoplasma

Sensitivity of Mycoplasma to various antibiotics was determined by streaking loopfuls of 72 hour old broth cultures of various Mycoplasma species on agar plates containing the antibiotic. The plates were then incubated for up to 10 days and were observed by microscopic examination periodically for growth. An agar diffusion assay described by D. Perlman and J. L. Schwartz (102) was also employed as an alternate method of determining Mycoplasma susceptibility to the various antibiotics.

TABLE IV

Effects of Centrifugation on the Viability of Mycoplasma Cultures

	Colony Forming Units/ml	
	Mycoplasma laidlawii B	Avian Mycoplasma POY-PH Serotype G
0-time	$2.3 \pm 1 \times 10^{10}$	$1.0 \pm 0.5 \times 10^{9}$
2000 x g 60 minutes	$2.2 \pm 1 \times 10^{10}$	$2.0 \pm 1 \times 10^{9}$
12000 x g 20 minutes	$8.0 \pm 2 \times 10^{8}$	$5.0 \pm 1 \times 10^{6}$
% decrease in titer at 12000 x g for 60 minutes	greater than 90	greater than 99

E. Cross-Resistance of a Gentamicin Resistant Mycoplasma laidlawii B to Kanamycin Sulfate and Paromomycin

A gentamicin resistant M. laidlawii B was developed by culturing 1 ml of a 48 hour old broth suspension of M. laidlawii B on agar containing 3 µg/ml of gentamicin sulfate. After 4 days of incubation, an agar block containing a resistant colony was transferred to fresh broth lacking the antibiotic and incubated for 48 hours. One ml of this suspension was transferred to fresh broth containing 10 µg/ml of gentamicin and incubated for 3 days. One ml of this suspension was then transferred to fresh broth containing 30 µg/ml of gentamicin. After 48 hours of incubation, growth at this concentration of antibiotic was excellent as observed by plating an aliquot of the culture on agar containing 30 µg/ml of gentamicin. Colony formation was observed within 48 hours by low power microscopic examination.

The minimum inhibitory concentrations (MIC) of the normally sensitive M. laidlawii B and of the resistant M. laidlawii B to gentamicin, kanamycin, and paromomycin were determined by the culture streaking technique on agar containing the antibiotic. Cross-resistance of the gentamicin-resistant mutant with kanamycin and paromomycin was also determined by the agar diffusion method. Filter paper discs 6.35 mm in diameter capable of absorbing

0.03 ml of water were allowed to absorb solutions of the three antibiotics at concentrations of 1000, 500, and 100 µg/ml. The discs were placed on assay plates containing the resistant or sensitive M. laidlawii, and incubation and dye staining carried out as previously described (102). Pyridinethione sodium (OMADINE®) 100 µg/ml, was also used as an antimicrobial test standard on all assay plates.

F. Detection of Penicillinase Production by Mycoplasma

The assay procedure utilized for the detection of penicillinase production was based on a procedure by Perret, et al. (103). Klebsiella #24 (as a standard for known penicillinase production), Sarcina lutea (as a standard for lack of penicillinase production), and various Mycoplasma cultures were cultured on separate agar plates containing 0.2% soluble starch. The Klebsiella and S. lutea organisms were cultured as a small colony in the center of the Petri dish by dipping the tip of a sterile platinum wire into a broth culture of the organism and touching it to the agar. Separate drops of Mycoplasma culture were left to absorb into the PPLO agar to give areas of heavy Mycoplasma growth visible to the eye. The cultures were incubated for 24-48 hours, then flooded with phosphate buffer (0.05 M, pH 6.0) containing 3 mg/ml iodine,

15 mg/ml potassium iodide, and 30 mg/ml potassium penicillin V. The plates were left to stand for five minutes, drained of solution, then placed back in the incubator and examined over the next few hours for a reduction in the blue-black color of the starch-iodine complex surrounding the organisms.

An alternative procedure based on that described by Novick (104) was also employed for the detection of penicillinase. The reagents used were: starch solution 2% w/v, iodine solution (0.08 M iodine in 3.2 M potassium iodide), 0.05 M phosphate buffer (pH 5.8), and potassium penicillin V (0.01-0.1 mM). The assay mixture consisted of 1.0 ml of starch-iodide solution (27 ml phosphate buffer, 3 ml starch solution, and 0.045 ml iodine solution), 1.0 ml of potassium penicillin V solution, 1.0 ml of phosphate buffer, 0.2 ml of 2% starch, and 1.0 ml of washed cells as enzyme source. Twenty-four hour old cultures of Klebsiella #24 and various Mycoplasma species were harvested by centrifugation and washed twice in the phosphate buffer. Cell dilutions of Klebsiella #24 from 0.109-10.9 mg/ml were used to find the appropriate amount of cells to add as an enzyme source. The assay tubes were incubated at 37°C in a water bath and viewed for color reduction over a period of three hours. Boiled cell controls (1 minute in boiling water bath) and controls containing 2.0 ml of buffer and no cells were also included.

G. Antibiotic Inactivation Studies

1. Streptomycin adenylylation. Benveniste, et al. (76) have found that bacterial species possessing resistance to both streptomycin and spectinomycin inactivated both antibiotics by adenylylation. Since a number of Mycoplasma species studied were resistant to both streptomycin and spectinomycin, the possibility that these Mycoplasma possessed an adenylylation enzyme was investigated. At first, however, several bacterial species were examined for an adenylylating enzyme so that the techniques in enzyme handling and assay procedure could be mastered. Cultures of E. coli (strains #8 and #15), Klebsiella strain #24, and Salmonella typhimurium (strain S-1) were assayed for the adenylylating enzyme by the procedure of Benveniste, et al. (76). The cells were grown in nutrient broth with 0.5% dextrose at 37°C on a shaker. They were harvested in late logarithmic phase of growth and osmotically shocked using a procedure based on one described by Nossal and Heppel (105). After centrifugation the cells were washed with Tris buffer (0.033 M, pH 7.8) and recentrifuged. The cells were then suspended in 36 volumes of a buffer containing 20% sucrose, 3×10^{-3} M ethylenediamine tetracetic acid, and 0.033 M Tris-chloride (pH 7.8), stirred for about 20 minutes at room temperature, and centrifuged at 15,000 x g for 20 minutes. The pellet was then suspended in 24 volumes of cold 5×10^{-4} M $MgCl_2$ and

stirred for 20 minutes at 4°C. The suspension was centrifuged for 30 minutes at 16,000 x g at 4°C to give the osmotic shockate. The osmotic shockate was made 0.03 M in Tris-chloride (pH 8.0) and concentrated by ammonium sulfate fractionation from 25-55% saturation. The protein precipitate was resuspended in 0.005 M Tris-chloride (pH 8.0), 10^{-2} M $MgCl_2$, and 5 x 10^{-4} β-mercaptoethanol (buffer #1) and dialyzed overnight against the same buffer.

Further purification of the S. typhimurium adenylylating enzyme was accomplished by lyophilizing the dialyzed protein and resuspending the powder in one-tenth of the previous volume of buffer #1. Denatured protein was removed by centrifugation at 10,000 x g at 4°C for 15 minutes. The adenylylating enzyme proved to be stable to lyophilization. The concentrated enzyme was placed on a Sephadex G-100 column and eluted with buffer #1.

The assay for adenylylation consisted of a reaction mixture containing 9 nmoles of streptomycin or mannosidostreptomycin, 13 nmoles of $^{14}C(8)$-adenosine-5'-triphosphate, 0.15 μmole of $MgCl_2$, and 0.25 μmole of β-mercaptoethanol in a total volume of 30 microliters. Either 15 or 30 microliters of enzyme solution was added to the reaction mixture, the solution then incubated at 34°C for various periods of time. Ten microliter aliquots were transferred at different time intervals to a 1 cm square of

phosphocellulose paper (Whatman P-81). The squares were allowed to dry for 15 seconds and then immersed in hot distilled water (70-80°C) for 1 minute to stop the reaction. The squares were then washed three times with distilled water and dried under a heat lamp, then counted in a Tri-Carb scintillation spectrometer (model 2002), Packard Instrument Co., Inc., Downers Grove, Ill. The scintillation fluid consisted of 4 grams of 2,5-diphenyl-oxazole (PPO) and 0.3 grams of 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene (M_2POPOP) in one liter of toluene.

One unit of enzymatic activity is defined as that amount of enzyme producing 1 nmole of adenylylated streptomycin or mannosidostreptomycin per minute at 34°C under the above assay conditions.

In testing resistant Mycoplasma for the presence of the adenylylating enzyme, various methods of breaking the cells were attempted including sonication, grinding with alumina and disruption by a French Pressure apparatus. The assay procedure was carried out as described for the bacterial cultures.

2. Chloramphenicol acetylation. Chloramphenicol acetylation was determined by a modification of Shaw's method (80). Klebsiella #24 was used as a control organism producing the chloramphenicol acetylase. One liter of culture containing 10 µg/ml of chloramphenicol was grown for 48 hours on a shaker at 37°C. The cells were

collected by centrifugation and the supernatant extracted with 3 x 100 ml portions of ethyl acetate. The solvent was removed in a flash evaporator and the resultant precipitate dissolved in 1 ml of ethyl acetate. The solution was chromatographed on silica gel H thin layer plates in a solvent system of chloroform:methanol (95:5). Suitable controls including ethyl acetate extracts of culture media plus chloramphenicol, culture media lacking antibiotic and growth organism, culture media grown for 48 hours with Klebsiella #24 but lacking chloramphenicol, and a chloramphenicol standard were also chromatographed.

Cultures of chloramphenicol resistant Mycoplasma were assayed in the same manner as Klebsiella #24. ^{14}C-chloramphenicol was added to the media before incubation to aid in detecting less than microgram amounts of any metabolites. When viewed under ultraviolet light, the chloramphenicol and chloramphenicol metabolites emitted a deep red fluorescence. Positions of the chromatographed material were confirmed by iodinating the aromatic portion of chloramphenicol. The thin layer plates were exposed to iodine vapor in a glass tank containing crystals of iodine, until the iodinated compounds were visible as brown spots. Co-spotted material from the Klebsiella #24 and Mycoplasma extractions were scraped off the glass plate, eluted with 0.1 ml of ethyl acetate, and counted in a Tri-Carb (model 2002)

spectrometer. The scintillation fluid was the same as described in the adenylyl-streptomycin assay. The R_f of the various metabolites were calculated and compared to the values obtained by Shaw (80).

H. Protein Determination

The protein content of washed Mycoplasma cells was determined by suspending an aliquot of cells in Lowry's reagent (106). Crystalline bovine serum albumin was the reference standard. Repeated determinations showed that 1×10^{11} cells contained 1 mg of protein.

I. Antibiotic Absorption

Absorption of chloramphenicol, dihydrostreptomycin, and tetracycline by various strains of Mycoplasma was carried out as follows: each culture was grown in 50 ml of broth media for 24 hours. Ten ml of this culture were then transferred to 100 ml of fresh broth in a cotton plugged 250 ml Erlenmeyer flask and incubated for 18 hours. Radioactive antibiotic was then added, an initial sample removed, and the flask replaced in the incubator. The initial time sample (and later samples) was centrifuged for 40 minutes at 3500 x g on a SorVal type SP centrifuge at 4°C, and the pellet and the centrifuge tube surface rinsed with non-radioactive antibiotic at the same concentration to remove surface absorbed antibiotic.

The pellet was then suspended in 50 microliters of water and aliquots of the suspension analyzed for radioactivity and for protein. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (model 2002) with Bray's (107) solution as counting fluid.

II. SCREENING MYCOPLASMA FOR PRODUCTION OF ANTIMICROBIAL SUBSTANCES

A. Materials

Source of Mycoplasma cultures. Obtained as mentioned on page 35.

Test organisms. Organisms used as test organisms in the screening of Mycoplasma cultures for the production of antimicrobial substances included Sarcina lutea and Bacillus subtilis (both obtained from the Department of Bacteriology, University of Wisconsin), Saccharomyces cerevisiae L-24, Escherichia coli B and Mycoplasma laidlawii B.

Media. Except for the avian Mycoplasma cultures, the Mycoplasma were grown in Difco PPLO broth fortified with 0.5% Bacto yeast extract, 0.1% sodium acetate, and 10% heat inactivated horse serum (serum heated to 55°C for 45 minutes). Avian serotypes J and D, both glucose fermentors, were grown in Phenol Red broth (Difco) supplemented with 1% yeast extract, 0.5% glucose, and

10% heat inactivated horse serum. Avian cultures, serotypes B and E, non-glucose fermentors, were grown in Albimi Mycoplasma broth supplemented with 0.5% yeast extract and 10% heat inactivated horse serum. Difco PPLO Agar supplemented with 2% Bacto PPLO serum fraction was used for solidified media.

Bacterial cultures were grown in Difco nutrient broth. For Bacillus subtilis and Saccharomyces cerevisiae, 2% glucose was added to the broth before fermentation. Nutrient agar was used for solidified media.

Standard. Sodium pyridinethione (OMADINE® solution), 100 µg/ml was used as an antimicrobial standard on all assay plates.

B. Fermentation of Antimicrobial Substances by Mycoplasma Cultures

Broth cultures of Mycoplasma were prepared by culturing species of Mycoplasma from samples stored in liquid nitrogen on agar and incubating for 3-5 days. Incubation conditions for Mycoplasma were kept constant throughout the experiment and should be taken as standard whenever Mycoplasma incubation is mentioned: 37°C in a 5% CO_2/95% air atmosphere. Newly received isolates were also transferred to agar and incubated in the standard manner. An agar block of growing culture (as verified by

microscopic examination) was transferred to a cotton stoppered 0.5 by 6 inch test tube containing 5 ml of PPLO broth containing 2% PPLO serum fraction and incubated for 5 days. Three ml of this culture were then transferred to 100 ml of broth in a 250 ml cotton stoppered Erlenmeyer flask. All flasks were incubated for 5 days, and were checked at day three for Mycoplasma growth or possible contamination by transferring 0.3 ml of the growing culture to PPLO agar and incubating for 2 days. The contents of each flask were lyophilized at the end of the five day incubation period, the powder being reconstituted with 4 ml of sterile water and kept in the freezer when not in use.

C. Assay for Antimicrobial Substances

The resuspended lyophilized material was tested for antibiotic activity against the test organisms as follows: 1.5 ml of test organism (24-48 hours old culture) was added to 150 ml of melted agar kept at 50°C in a water bath. (Nutrient agar was used for bacteria and yeast, and PPLO agar for M. laidlawii.) The agar was poured into previously sterilized 3 quart baking dishes, and allowed to harden. Filter paper discs (12.7 mm in diameter) were allowed to absorb a quantity of test material (0.1 ml) and then placed on the agar. Each test material was assayed in duplicate. The plates containing B. subtilis, E. coli,

and M. laidlawii B were incubated at 37°C overnight, the M. laidlawii B in an atmosphere of 5% CO_2/95% air. The S. cerevisiae was incubated at 30°C. Filter paper discs (6.35 mm in diameter) were dipped in Omadine® solutions, 100 µg/ml, and placed on each plate as a standard. After 24 hours incubation, all plates were examined for antibiotic activity. Inhibition of an organism could be seen as a clear circular zone around a disc containing an inhibitory substance. The test plates containing Mycoplasma laidlawii B were stained with 2,6-dichlorophenolindophenol as described elsewhere (107) to aid in the viewing of inhibitory zones.

D. pH Chromatography of the Active Material

In order to obtain some information on the chemical nature of the inhibitory substances, pH chromatography as described by Betina (108) was carried out. Whatman #1 filter paper cut into 19 x 15 cm strips were soaked in buffer solutions at either pH 3, pH 5, pH 7, or pH 9 (0.2 M phosphate buffer and 0.2 M phosphate/0.1 M citrate buffer). The papers were dried overnight at room temperature, then spotted with 10-15 microliters of active sample. The chromatograms were developed in an ascending manner using as solvent system water saturated ethyl acetate. After chromatogram development, the

papers were dried and the antibiotic activity located by placing the chromatographed paper in a 3 quart baking dish containing 150 ml of nutrient agar seeded with 1.5 ml of a 48 hour old culture of Bacillus subtilis. Penicillin V (0.1 μg) was spotted on each paper as a control. Another control consisting of lyophilized media incubated with no Mycoplasma was also spotted on the chromatograms before development. The plates were incubated at 4°C for 1 hour, the papers removed, and the baking dish incubated at 37°C until inhibitory zones could be detected.

It was found that Bacillus subtilis cells soon overgrew the inhibitory zones at 37°C. To overcome this effect, a spore suspension of B. subtilis was prepared by incubating 100 ml of a broth suspension of the organism at 37°C on a shaker for 14 days. The flask was then heated to 65°C for 1 hour to kill all remaining vegetative cells, and the spores collected by centrifugation. The spores were then washed with distilled water, recentrifuged, and resuspended in 10 ml of distilled water. The suspension contained 2.5×10^8 spores/ml. The spore suspension was used for all subsequent experiments and was stored at 4°C when not in use. It was also found that incubating the B. subtilis assay plates at 30°C also reduced the overgrowing of inhibitory zones. The pH chromatography described above was reported using the

spore suspension and an incubation temperature of 30°C.

E. Extraction of the Inhibitory Substance

pH chromatography of the active samples showed the basic nature of the antimicrobial substances. One gram of Mycoplasma laidlawii B lyophilized material was suspended in one ml of phosphate buffer (0.2 M, pH 8.5), and extracted twice with 2 ml portions of n-butanol. The butanol was removed in a flash evaporator and the precipitate redissolved in distilled water (0.5 ml), and neutralized with 0.1 N HCl (Hydrion pH paper was used to measure pH). The extracted material was assayed for antimicrobial activity by dipping filter paper discs (12.7 and 6.35 mm in diameter) in the redissolved extract and placing the discs in Petri dishes containing 5 ml of nutrient agar seeded with 0.05 ml of the spore suspension. The plates were incubated at 30°C and examined over a 24 hour period for inhibitory zones.

F. Sensitivity of Bacillus subtilis to Various Concentrations of the Inhibitory Substance Produced by M. laidlawii B

The effect of varying the concentration of the Mycoplasma laidlawii B extracted material on B. subtilis was tested. The butanol extracted material was diluted

with 0.2 M phosphate buffer (pH 7.0) to 1/2, 1/4, and 1/8 the concentration of the original sample. Filter paper discs (6.35 mm in diameter) were allowed to absorb samples of each dilution including the undiluted material, placed on Petri dishes containing 5 ml of nutrient agar seeded with 0.025 ml of B. subtilis spore suspension, and incubated for 6 hours at 4°C, then overnight at 30°C. Zones of inhibition were measured the following day, and a dose-response curve drawn on semi-log paper.

G. Heat Stability of the Antimicrobial Substances

Four samples (0.1 ml) of active substance obtained from the M. laidlawii fermentation by n-butanol extraction were buffered to pH 3, pH 5, pH 7 and pH 9, respectively, with the 0.2 M phosphate and 0.2 M phosphate/0.1 M citrate buffers previously described. The test tubes containing the samples were placed in a beaker of boiling water for 15 minutes. Evaporation of the samples was minimized by placing a glass marble over the top of each test tube. After the heat treatment, the samples were cooled, adjusted to pH 7 with 1 N NaOH or 1 N HCl (using Hydrion pH paper as the indicator), and assayed for bioactivity as described above for the dose-response curve.

RESULTS AND DISCUSSION

I. ANTIBIOTIC RESISTANCE IN MYCOPLASMA

A. Antibiotic Sensitivity of Mycoplasma

An examination of the antibiotic sensitivity of clinical isolates of Mycoplasma is summaried in Table V. Although most strains examined were sensitive to 10 µg/ml of chloramphenicol, gentamicin, kanamycin, and tetracycline, resistant strains of Mycoplasma hominis, Mycoplasma pharyngis, and Mycoplasma salivarium were noted with each antibiotic. As far as these species are concerned, many strains appeared to be resistant to erythromycin, spectinomycin, and streptomycin. In general, M. pharyngis was more often resistant to the antibiotics tested than M. salivarium.

A survey of the reports from other laboratories on the antibiotic sensitivity patterns of various species of Mycoplasma (109-116) shows that M. fermentans, M. hominis, M. pneumoniae, and M. salivarium are generally sensitive to less than 6.25 µg/ml of chloramphenicol, gentamicin, kanamycin, and tetracycline, although resistant strains (resistant to at least 10 µg/ml) are not uncommon. For example, in 84 strains of M. salivarium examined, Stewart and coworkers (36) noted 28 resistant to chloramphenicol,

TABLE V

Antibiotic Sensitivity Patterns in *Mycoplasma*

Species	No. Strains Tested	Tetracycline		Erythromycin		Chloramphenicol	
		R	S	R	S	R	S
M. pharyngis	22	5	17	13	9	4	18
M. salivarium	22	1	21	9	13	2	20
M. hominis	3	0	3	2	1	0	3
M. fermentans	1	0	1	0	1	0	1
M. orale II	1	0	1	1	0	0	1

Species	No. Strains Tested	Spectinomycin		Streptomycin		Kanamycin		Gentamycin	
		R	S	R	S	R	S	R	S
M. pharyngis	22	8	14	15	7	6	16	4	18
M. salivarium	22	10	12	9	13	5	17	4	18
M. hominis	3	2	1	2	1	0	3	0	3
M. fermentans	1	0	1	0	1	0	1	0	1
M. orale II	1	1	0	0	1	0	1	0	1

Abbreviations:

R - resistant. As measured by growth on agar plates containing 10 µg/ml of tetracycline, erythromycin, chloramphenicol, kanamycin, and gentamicin; and 20 µg/ml of spectinomycin and streptomycin

S - sensitive.

30 resistant to kanamycin, and 26 resistant to tetracycline. Erythromycin and streptomycin are generally found to be ineffective in inhibiting Mycoplasma with the exception of erythromycin on M. pneumoniae, which is effective at concentrations between 0.1-1 µg/ml.

The results presented here seem to agree with the findings in other laboratories on the antibiotic sensitivity of Mycoplasma and the not uncommon isolation of resistant species.

B. Cross-Resistance of a Gentamicin-Resistant Mycoplasma laidlawii B with Kanamycin Sulfate and Paromomycin

The results of a study on the cross-resistance of a gentamicin-resistant mutant of Mycoplasma laidlawii B with kanamycin sulfate and paromomycin are listed in Tables VI and VII. In Table VI the minimum inhibitory concentrations of the resistant and sensitive M. laidlawii B to the three antibiotics are shown. A 10-15 fold increase in resistance is seen toward gentamicin, while a 5-10 fold increase is noted with the two other related antibiotics.

Table VII gives further indication of the cross-resistance of the gentamicin resistant M. laidlawii B with kanamycin sulfate and paromomycin. Zone sizes obtained for the resistant M. laidlawii are significantly reduced compared to the normally sensitive organism.

TABLE VI

Sensitivity of *Mycoplasma laidlawii* B to Amino-Glycoside Antibiotics

Antibiotic	Minimum Inhibitory Concentration - µg/ml	
	Sensitive Parent M. laidlawii B	Resistant Mutant M. laidlawii B
Gentamicin sulfate	1 - 5	25 - 50
Kanamycin sulfate	5 - 10	25 - 50
Paromomycin	10 - 25	50 - 75

TABLE VII

Cross-Resistance of a Gentamicin-Resistant *M. laidlawii* B to Kanamycin and Paromomycin by the Agar Diffusion Assay

Antibiotic	Antibiotic per Disc (µg)	*Mycoplasma laidlawii* B Inhibition Zones (mm)	
		Resistant	Sensitive
Gentamicin·SO_4	3	0,0	20,20
	15	8,8	27,28
	30	10,10	32,34
Kanamycin·SO_4	3	0,0	15,15
	15	7.5,trace	22,23
	30	8,8	25,28
Paromomycin	3	0,0	8,9
	15	0,0	18,18
	30	0,0	22,24

C. Detection of Penicillinase Production by Mycoplasma

In examining the penicillinase production of Klebsiella #24, a sharp colorless zone surrounding the colony of the Klebsiella organism was apparent within ten minutes after the drainage of the assay solution from the plate. The colorless zone was due to the reduction of iodine to iodide by the penicilloic acid liberated by the penicillinase hydrolysis of the β-lactam ring of penicillin. The reduction of the iodine decolorized the starch-iodine complex, initially blue black in color. Zones reached a maximum size of 18 mm in diameter after 2 hours. The color and zones could be re-established by reflooding the plates with the test iodide solution. The colony size of the Klebsiella #24 was less than 2 mm in diameter. No dye reduction was noted on plates containing the penicillin sensitive Sarcina lutea. Five species of Mycoplasma salivarium, three of Mycoplasma pharyngis, two of Mycoplasma orale, two of Mycoplasma hominis I, five of avian Mycoplasma (serotype unidentified), and one of an unidentified human isolate were assayed for penicillinase production as described. All Mycoplasma cultures were resistant to more than 1000 ug/ml of penicillin. No zone formation, even when the Mycoplasma were observed under a microscope, could be observed, indicating that none of the Mycoplasma tested produced penicillinase.

When utilizing the procedure of Novick (104), optimum color reduction of the starch-iodine complex was observed when the cell-weight of Klebsiella #24 added was between 1.1-11 mg/ml, and the penicillin concentration between 0.05 mM-0.1 mM. At 11 mg Klebsiella #24 cells/ml, complete color reduction was observed within 20 minutes. At lower Klebsiella cell concentrations, the time of complete color reduction could be varied from 30 minutes to 3 hours by varying the penicillin concentration. Three avian Mycoplasma (serotypes J, L and I) were assayed for penicillinase by this procedure. Various concentrations of penicillin (0.05 mM-0.1 mM) and Mycoplasma cells were utilized in the assay system. In no case was any color reduction observed, again indicating the lack of penicillinase production.

Thus, in all the cultures of Mycoplasma assayed, no penicillinase activity could be demonstrated even though all the cultures possessed an absolute resistance to penicillin. The natural resistance of Mycoplasma to penicillin can be explained by virtue of their lack of cell wall. Penicillin, being a cell wall inhibitor, would not be expected to inhibit Mycoplasma. However, if Mycoplasma were initially derived from Bacterial L-forms (bacteria that have lost their cell wall and grow as spheroplasts), the possibility that a gene for penicillinase production might still be present in Mycoplasma was

examined. The results presented here do not suggest any connection between Mycoplasma and Bacterial L-forms.

D. Antibiotic Inactivation

1. Streptomycin adenylylation. The adenylylation of streptomycin, mannosidostreptomycin, and dihydrostreptomycin by adenylylating enzymes isolated from four different Gram-negative bacteria have been studied. As is shown in Table VIII, mannosidostreptomycin is adenylylated at between 10 and 20% of the rate of streptomycin and dihydrostreptomycin.

In Figure 1 is shown the protein concentration and enzymatic activity of the fractions collected from the Sephadex G-100 column chromatography of the S. typhimurium enzyme. Fractions 22 and 23 represent an average activity of 21 units per mg compared to a value of 0.45 units/mg for the osmotic shockete (starting preparation), a 47 fold purification. The recovery of 6.4 units for fractions 22 and 23 represents an enzyme recovery of 11.6%.

No evidence of streptomycin adenylylation could be demonstrated with 8 cultures of Mycoplasma tested (Mycoplasma hominis, strains PG 27, 41Tc, and No. 7; Mycoplasma salivarium, strains No. 6 and No. 9; Mycoplasma pharyngis, strains No. 13 and No. 21; and avian Mycoplasma T-40, probable serotype F), using methods giving positive results with the bacterial species carrying an R-factor for streptomycin adenylylation. Protein concentrations of

Table VIII

Substrate Specificity of Adenylylating Enzymes from Gram-negative Bacteria

Culture	Specific Activity (units/mg) Streptomycin	Dihydrostreptomycin	Mannosidostreptomycin
Escherichia coli #8	0.16 ±0.01	0.10 ±0.007	0.037±0.005
Escherichia coli #15	0.12 ±0.01	0.11 ±0.01	0.034±0.008
Klebsiella species #24	0.075±0.01	0.048±0.005	0.016±0.003
Salmonella typhimurium species S-1	1.4 ±0.15	1.3 ±0.10	0.13 ±0.01

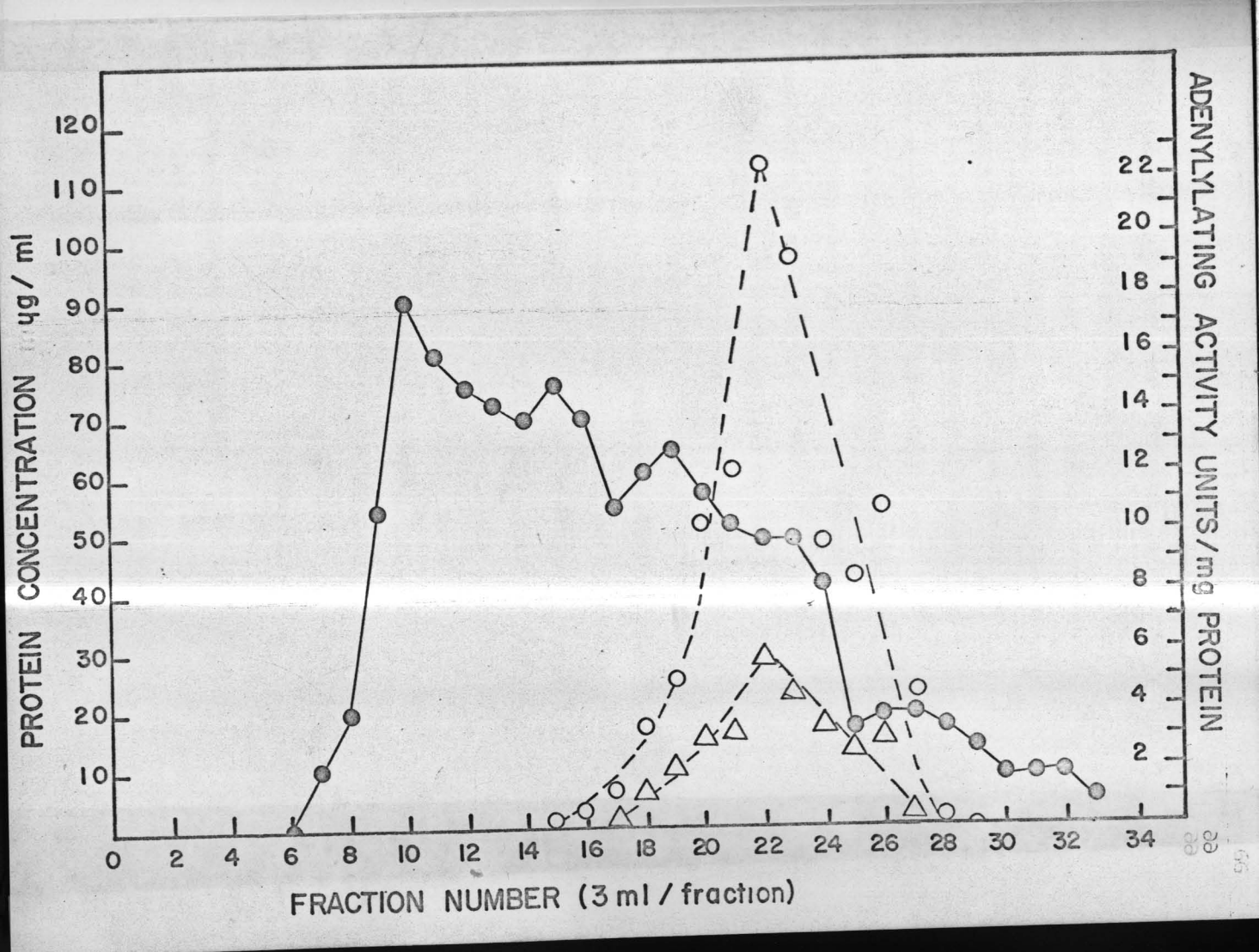
Figure 1. Enzymatic Activity of the Fractions from Column Chromatography of the *Salmonella typhimurium* Adenylylating Enzyme Preparation.

● - Protein concentration, μg/ml

○ - Streptomycin adenylylating enzyme activity, units/ml

△ - Mannosidostreptomycin adenylylating activity, units/mg

7.0 mg of enzyme protein (28.2 units) were placed on a Sephadex G-100 column (42 cm x 2 cm) and eluted with buffer #1 at 4 C. The flow rate of the column was 0.4 ml/minute. Buffer #1 - 0.005 M Tris-chloride (pH 8.0), 10^{-2} M $MgCl_2$, and 5 x 10^{-4} β-mercapto-ethanol.


PROTEIN CONCENTRATION µg / ml
ADENYLYLATING ACTIVITY UNITS / mg PROTEIN
FRACTION NUMBER (3 ml / fraction)
120
110
100
90
80
70
60
50
40
30
20
10
22
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

the PPLO extracts ranged between 7-20 mg/ml, about twice that used in the studies with bacterial preparations. No inactivation of any type, either by chemical assay or by bioassay could be demonstrated by incubating streptomycin with Mycoplasma cultures for varying lengths of time.

The results indicate that the resistance of the Mycoplasma species tested in this study is not due to streptomycin adenylylation, even though the species were resistant to both spectinomycin and streptomycin. That no inactivation of streptomycin occurred when incubated with the Mycoplasma cultures (assayed by bioassay) would seem to indicate that the mechanism of streptomycin resistance was not dependent on streptomycin degradation.

This study has also shown that mannosidostreptomycin is a suitable substrate for the bacterial adenylylating enzymes. The poor rate of adenylylation compared to streptomycin and dihydrostreptomycin (10-20%) indicates that mannosidostreptomycin may be a more desirable antimicrobial agent in treating infections, especially those carrying RTF for streptomycin than originally supposed (117,118). Sephadex G-100 purification of the S. typhimurium enzyme indicates that the same enzyme is responsible for adenylylating streptomycin and mannosidostreptomycin.

2. Chloramphenicol acetylation. Thin layer chromatography of the ethyl acetate extracts from media fermented by Klebsiella #24 for 48 hours in the presence

of chloramphenicol, indicated material at R_f 0.18, 0.34, 0.48, 0.59, and 0.68. Media incubated for 48 hours without organism or antibiotic gave only one spot at R_f 0.34. Chloramphenicol standard gave a single spot at Rf 0.18. Chloramphenicol dissolved in the basal medium and incubated for 48 hours without any micro-organisms and extracted as described gave material at R_f 0.18 and 0.35. Mycoplasma pharyngis No. 24 and Mycoplasma salivarium No. 6 could not be shown to acetylate ^{14}C-chloramphenicol. Only one spot other than the chloramphenicol spot at R_f 0.18 could be seen, that at R_f 0.35. When the extracted material from both Mycoplasma cultures were co-spotted with the Klebsiella #24 (incubated with non-labelled chloramphenicol) extract, and all spots analyzed for radioactivity, only R_f 0.18 was found to be radioactive. No trace of radioactivity could be found at R_f 0.35, 0.48, 0.59, or 0.68, indicating that no acetylated chloramphenicol, nor any other detectable chloramphenicol metabolite was produced. In Shaw's TLC system, material at R_f 0.27, 0.54, 0.61, and 0.79 were identified as chloramphenicol, 1-acetoxychloramphenicol, 3-acetoxychloramphenicol, and 1,3-diacetoxychloramphenicol, respectively.

The results show that a process other than chloramphenicol inactivation is responsible for the resistance of the two Mycoplasma species tested to chloramphenicol.

E. Antibiotic Absorption

Results of experiments on the absorption by Mycoplasma species of dihydrostreptomycin are summarized in Table IX. Sensitive cultures of Mycoplasma laidlawii B and Mycoplasma fermentans show similar patterns of initial absorption and later efflux of antibiotic. Mycoplasma fermentans shows a slight rise in absorption after 4 hours, while a very slight decrease in absorption is observed in the same time period with M. laidlawii. After 4 hours, efflux of antibiotic is observed to levels of $1 - 2.5 \times 10^4$ molecules/cell. A continual pattern of antibiotic absorption is observed with Mycoplasma salivarium No. 19. However, initial absorption values are lower with this culture (6-10 times less), and its absorption pattern probably reflects its poorer initial absorption, as final values are closer to the other cultures (3-4 times less).

A consistent decrease in absorbed antibiotic with time occurred with the dihydrostreptomycin resistant cultures. Absorption levels of antibiotic per cell were 5-10 fold lower than with those seen with the antibiotic sensitive cells. The efflux rate observed with Mycoplasma orale II was quite a bit lower than the other resistant cultures, but again this can be attributed to a lower initial absorption. Final values of all resistant cultures are almost the same at $1\text{-}2 \times 10^3$ molecules of antibiotic absorbed per cell.

TABLE IX

Dihydrostreptomycin Absorption by *Mycoplasma*

	Exposure Time	Mg. Protein/ 100 ml Culture	CPM Absorbed/ mg. Protein	Molecules $STM \cdot H_2$ Absorbed/ mg. Protein
A. Sensitive Cultures				
M. fermentans Pg 18	10 min.	0.29	7965	1.9×10^{15}
	4 hrs.	0.38	10473	2.4×10^{15}
	8 hrs.	0.18	4778	1.0×10^{15}
	24 hrs.	0.15	6233	1.3×10^{15}
M. laidlawii B	10 min.	0.33	17850	3.8×10^{15}
	4 hrs.	0.31	16903	3.6×10^{15}
	8 hrs.	0.37	7270	1.6×10^{15}
	24 hrs.	0.31	10130	2.2×10^{15}
M. salivarium 19	10 min.	0.27	1593	3.4×10^{14}
	4 hrs.	0.52	1702	3.7×10^{14}
	24 hrs.	0.72	2000	4.6×10^{14}
B. Resistant Cultures				
M. hominis 7	10 min.	0.56	1607	3.5×10^{14}
	4 hrs.	1.1	659	1.5×10^{14}
	24 hrs.	2.1	540	1.2×10^{14}
M. salivarium 9	10 min.	0.6	2333	5.1×10^{14}
	4 hrs.	1.2	1054	2.4×10^{14}
	24 hrs.	2.1	467	1.0×10^{14}
M. pharyngis 21	10 min.	0.28	3000	6.4×10^{14}
	4 hrs.	0.30	953	2.1×10^{14}
	24 hrs.	0.60	667	1.4×10^{14}
M. pharyngis 6	10 min.	0.34	2676	5.7×10^{14}
	4 hrs.	0.63	706	1.7×10^{14}
	24 hrs.	1.1	727	1.7×10^{14}
M. orale II	10 min.	0.83	1277	2.9×10^{14}
	4 hrs.	0.99	919	2.1×10^{14}
	8 hrs.	1.1	936	2.2×10^{14}
	24 hrs.	2.1	629	1.5×10^{14}

Dihydrostreptomycin ($STM \cdot H_2$) was added to each flask at a concentration of 20 µg/ml.

1 mg of protein is equivalent to 1.0×10^{11} *Mycoplasma* cells.

The results of a study of tetracycline absorption by Mycoplasma are collected in Table X. At concentrations ten to fifty times higher than the minimum inhibitory concentrations (MIC) of these organisms, a pattern of rapid uptake followed by a sharp decrease in absorbed tetracycline occurred. At levels closer to the MIC of each culture, varying patterns of absorption are seen. Mycoplasma laidlawii B (MIC of 0.5 µg/ml) at a tetracycline concentration of 0.6 µg/ml shows a large increase at 4 hours of accumulated tetracycline. By eight hours, efflux of tetracycline has occurred and a slight absorption increase takes place through the final sampling at 24 hours. Much slower rates of efflux are observed at 2 µg/ml of tetracycline with Mycoplasma pharyngis No. 25 (compared with rates at 10 µg/ml with the other cultures). After 4 hours, a rise in absorption is observed. Such a rise in final absorption is also observed with the avian Mycoplasma U-10 (Figure 2), serotype D, at a concentration just at its MIC of 0.2 µg/ml.

A resistant laboratory developed mutant of Mycoplasma laidlawii B* showed a strikingly different pattern of tetracycline absorption and efflux than its sensitive counterpart. At concentrations of 10 µg/ml and 0.6 µg/ml

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*This culture selected by Mr. Carl Fraterrigo had an MIC greater than 60 µg/ml. The sensitive parent had an MIC of 0.5 µg/ml.

TABLE X

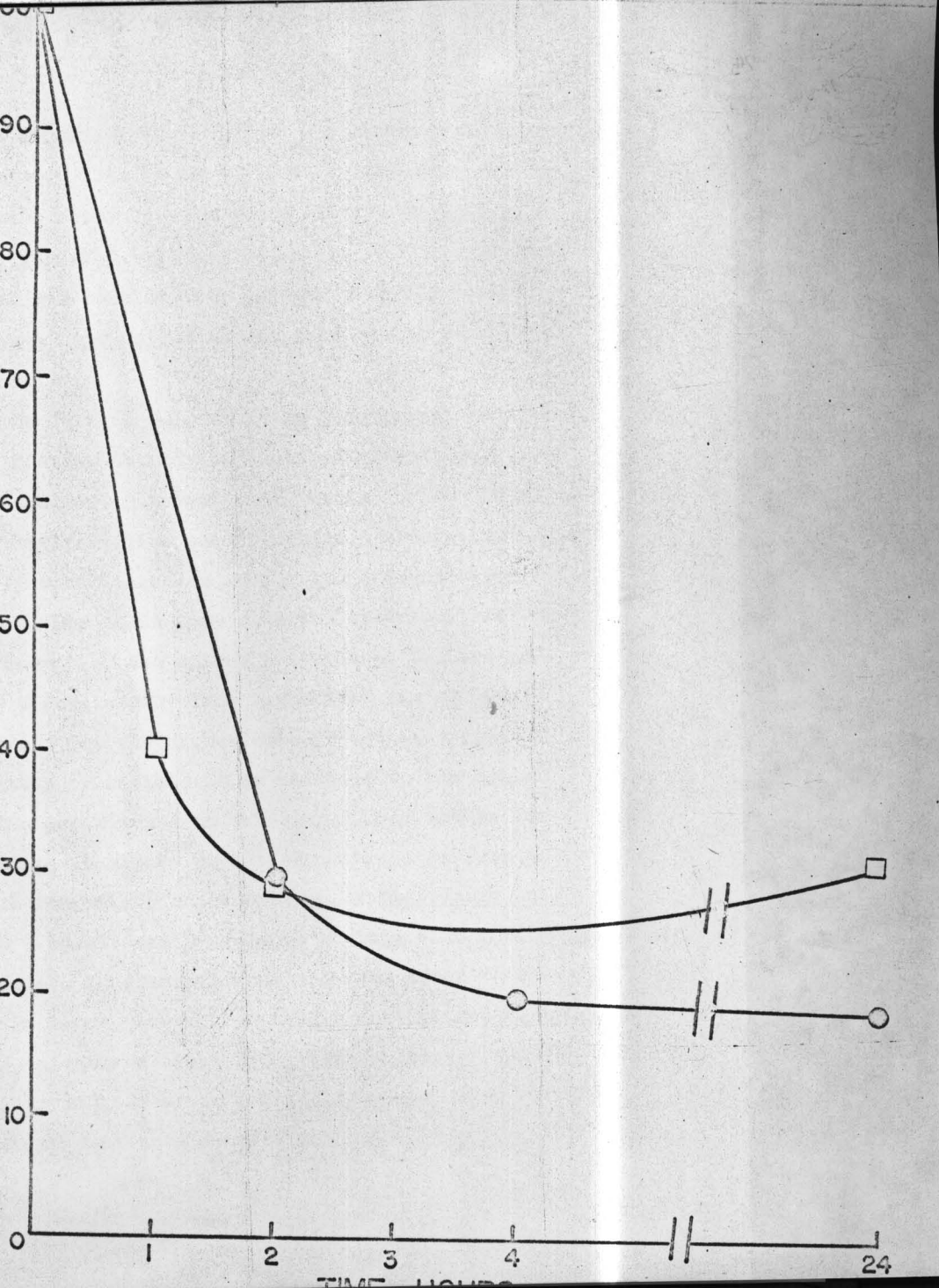
Tetracycline Absorption by *Mycoplasma*

	Concentration µg/ml	Exposure Time	Mg. Protein/ 100 ml Culture	CPM Absorbed/ mg. Protein	Molecules Tetracycline Absorbed/mg Protein
A. Sensitive Cultures					
M. salivarium 19	10	10 min.	0.55	89820	2.6×10^{16}
		4 hrs.	0.40	27500	8.3×10^{15}
		8 hrs.	0.42	14905	4.6×10^{15}
M. salivarium 20	10	10 min.	0.31	148387	2.2×10^{16}
		4 hrs.	0.33	22575	3.3×10^{15}
		10 hrs.	0.29	10500	1.6×10^{15}
		24 hrs.	0.46	24111	3.7×10^{15}
M. hominis 41Tc	10	10 min.	0.9	70333	8.4×10^{15}
		4 hrs.	1.3	21385	2.7×10^{15}
		10 hrs.	1.2	16500	1.9×10^{15}
		24 hrs.	1.6	10375	1.2×10^{15}
M. salivarium 17	10	10 min.	0.42	52860	8.0×10^{15}
		4 hrs.	0.68	18675	2.8×10^{15}
		10 hrs.	0.78	13400	2.0×10^{15}
		24 hrs.	0.82	9900	1.5×10^{15}
M. laidlawii B	10	10 min.	0.34	78820	1.2×10^{16}
		4 hrs.	0.37	27430	4.2×10^{15}
		8 hrs.	0.30	11930	1.8×10^{15}
		24 hrs.	0.56	9600	1.6×10^{15}
M. laidlawii B	0.6	10 min.	0.39	85720	7.6×10^{14}
		4 hrs.	0.36	237500	2.1×10^{15}
		8 hrs.	0.39	28461	2.7×10^{14}
		24 hrs.	0.40	50500	4.6×10^{14}
M. pharyngis 25	2.0	10 min.	0.29	37931	1.1×10^{15}
		2 hrs.	0.27	21480	6.5×10^{14}
		4 hrs.	0.26	11460	3.5×10^{14}
		24 hrs.	0.11	15000	4.7×10^{14}
B. Resistant Cultures					
M. laidlawii B	10	10 min.	0.25	124600	1.9×10^{16}
		2 hrs.	0.26	16000	2.4×10^{15}
		4 hrs.	0.30	14970	2.3×10^{15}
		24 hrs.	0.41	15585	2.4×10^{15}
M. laidlawii B	0.6	10 min.	0.36	116940	1.0×10^{15}
		4 hrs.	0.50	36400	3.2×10^{14}
		8 hrs.	0.61	30000	2.7×10^{14}
		24 hrs.	0.99	16670	1.5×10^{14}

Figure 2. Absorption and Efflux of Tetracycline from Avian *Mycoplasma* Culture U-10.

□ - tetracycline concentration - 0.2 µg/ml; initial absorption - 2.5×10^3 molecules/cell

◍ - tetracycline concentration - 1.5 µg/ml; initial absorption - 2.5×10^4 molecules/cell


90
80
70
60
50
40
30
20
10
0
1
2
3
4
24

a rapid efflux of tetracycline was observed after initial absorption. This is in contrast to the large rise in absorption observed with the sensitive Mycoplasma laidlawii B at 0.6 µg/ml. Even the rate of efflux at 10 µg/ml was considerably greater with the resistant Mycoplasma laidlawii B as compared to the sensitive organism.

With culture U-1, an avian Mycoplasma serotype J, four different tetracycline concentrations were used in uptake studies. In Figure 3, initial uptake is plotted against tetracycline concentration. Absorption increases linearly with increased antibiotic concentration. In general, this holds true with the other cultures as well.

Absorption and efflux patterns of U-1 are seen in Figure 4 for tetracycline concentrations of 100 and 0.2 µg/ml. At the higher concentration, a greater percentage of antibiotic is retained in the cell.

The experiments on chloramphenicol uptake are summarized in Figure 5. At concentrations just above the minimum inhibitory concentration of Mycoplasma laidlawii B (MIC 8 µg/ml) and Mycoplasma hominis 41Tc (MIC 2 µg/ml) an increase in absorption is observed after 4 hours. After 24 hours, absorption levels have decreased to about 70% of initial values. Mycoplasma pharyngis No. 25 (MIC 2 µg/ml), after an initial decrease, also shows a pattern of increased absorption. With Mycoplasma

Figure 3. Initial Absorption of Tetracycline by Avian *Mycoplasma* U-1.

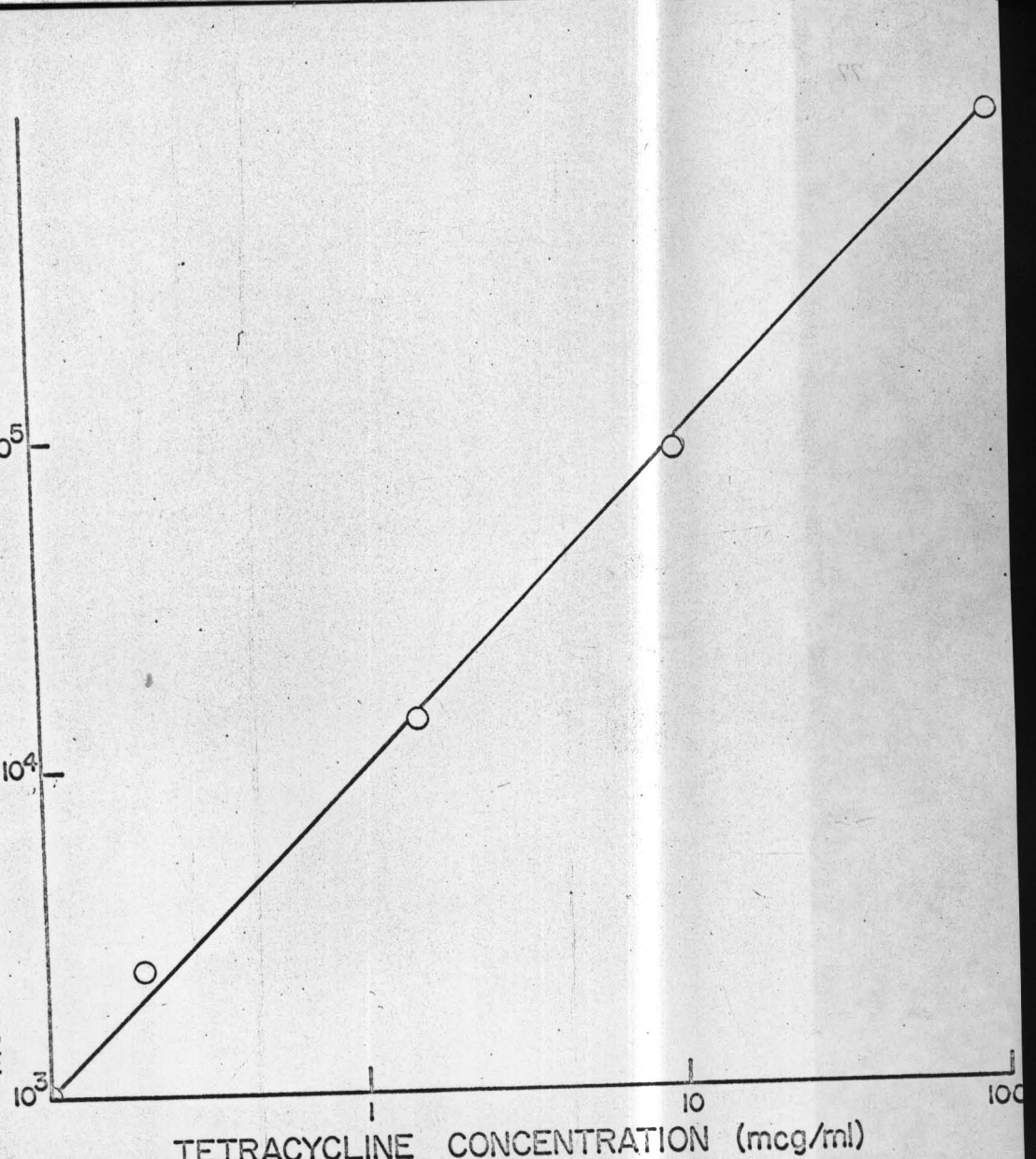

10^5
10^4
10^3
1
10
100
TETRACYCLINE CONCENTRATION (mcg/ml)

Figure 4. Absorption and Efflux of Tetracycline from Avian Mycoplasma U-1.

● - tetracycline concentration - 100 µg/ml; initial absorption - 9.1×10^5 molecules/cell

○ - tetracycline concentration - 0.2 µg/ml; initial absorption 2.4×10^3 molecules/cell

% TETRACYCLINE REMAINING BOUND TO CELLS
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
24
TIME HOURS

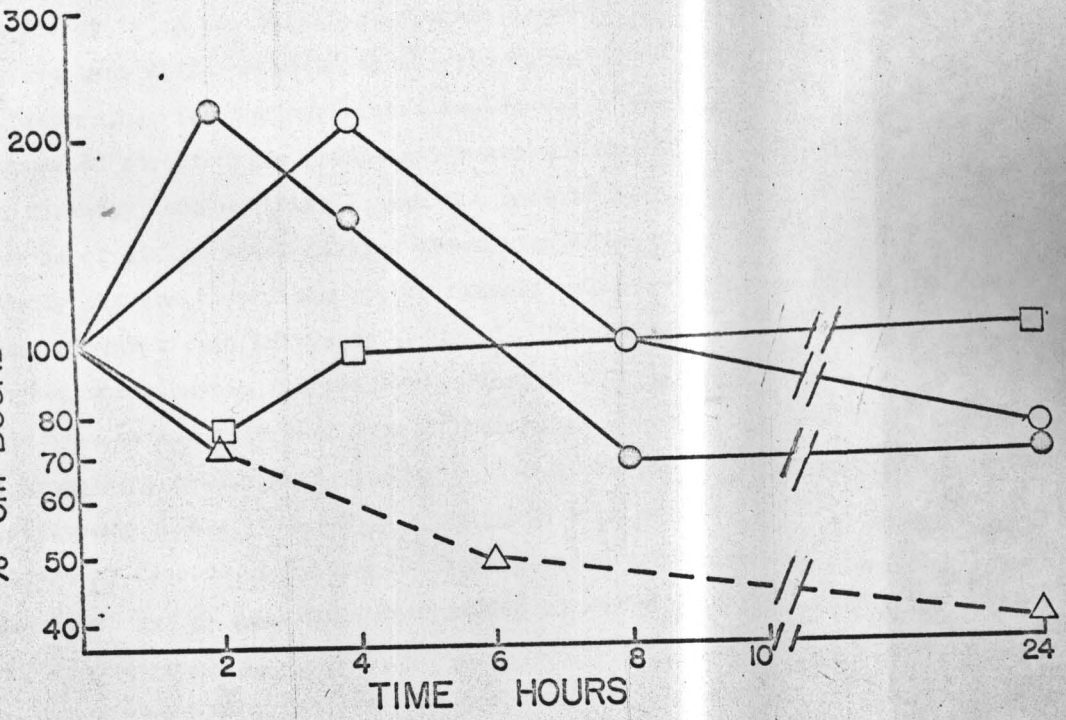
Figure 5. Absorption and Efflux of Chloramphenicol from *Mycoplasma* Cultures.

● - *M. hominis* 41TC; chloramphenicol concentration - 3 µg/ml; initial absorption - 3.7×10^4 molecules/cell

○ - *M. laidlawii* B; chloramphenicol concentration - 10 µg/ml; initial absorption - 2.1×10^5 molecules/cell

□ - *M. pharyngis* 25; chloramphenicol concentration - 4 µg/ml; initial absorption - 5.7×10^4 molecules/cell

△ - *M. salivarium* 6; chloramphenicol resistant; chloramphenicol concentration - 10 µg/ml; initial absorption - 6.8×10^4 molecules/cell


300
200
100
80
70
60
50
40
2
4
6
8
10
24
TIME HOURS

salivarium No. 6, a culture resistant to more than 10 μg/ml of chloramphenicol, the amount of antibiotic absorbed decreased with increased incubation.

The study on accumulation of antibiotics by Mycoplasma suggests that differences in antibiotic uptake-permeability are responsible for the antibiotic resistance noted. In the case of streptomycin, resistant organisms absorb significantly less antibiotic than the sensitive cultures. Patterns of efflux after initial absorption are also markedly different, and seem to be closely related to the amount of antibiotic initially absorbed. Final values of absorbed streptomycin for the three sensitive Mycoplasma range between 4.6×10^3 - 2.2×10^4 molecules streptomycin per Mycoplasma cell. This contrasts with 5×10^4 molecules of streptomycin absorbed per B. megaterium cell as reported by Hancock (53), and 10^5 molecules of streptomycin accumulated per E. coli cell as reported by Szybalski and Mashima (54).

The results with tetracycline are similar to those found by Franklin, et al. (65,66) with tetracycline absorption studies on Escherichia coli. Franklin found that at 10 μg/ml, accumulation of drug ceased after 10 minutes, and that initial drug absorption was linearly proportional to the concentration of tetracycline in the media. The finding that much higher tetracycline concentrations (100 μg/ml) efflux and absorption reach

equilibrium at a higher level than at lower concentration is also similar to Franklin's results (66). Franklin (65) also demonstrated that a resistant E. coli accumulated significantly less tetracycline than a sensitive culture. In the one resistant organism studied here, the accumulation patterns at MIC tetracycline levels of the sensitive M. laidlawii B clearly indicate a substantial difference in the amount of antibiotic absorbed compared to the resistant M. laidlawii.

In comparing the two avian cultures at the same low concentration of 0.2 μg/ml of tetracycline, it will be noted that U-10 (MIC of 0.2 μg/ml) absorption patterns indicate an equilibration between efflux and absorption, with the final absorption value of tetracycline being 7.9×10^2 molecules/cell, while U-1 (MIC 1.0 μg/ml) shows a continuous decrease in efflux throughout the sampling period (final value of 2.9×10^2 molecules/cell). The levels of absorption and efflux may be indicative of their differences in sensitivity. This pattern is similar to that observed by Corcoran, et al. (60), where it was noted that at the same concentration, a resistant bacterial culture will accumulate less erythromycin than a sensitive culture.

The same patterns of higher drug accumulation in sensitive cultures and differential efflux patterns between resistant and sensitive cultures are also observed in the

absorption studies with chloramphenicol. The only direct comparison can be made between M. laidlawii B and M. salivarium No. 6, both assayed at 10 µg/ml of chloramphenicol. A final accumulation of 1.6×10^5 molecules per cell was noted for the sensitive M. laidlawii culture, while that of the resistant M. salivarium was 2.7×10^4 molecules per cell. The general pattern of an increase in absorption somewhere in the sampling period for the sensitive cultures, and a continuous decrease for the resistant culture was also the same as the other antibiotics studied.

The conclusions then, that resistance of Mycoplasma to dihydrostreptomycin, tetracycline, and chloramphenicol is related to some permeability-accumulation mechanisms is supported by the data presented. Whether the mechanism of resistance is related to drug impermeability due to an inhibition of the drug-transport mechanism, or to a change in binding characteristics at the drug's active site has not been determined.

II. SCREENING MYCOPLASMA FOR THE PRODUCTION OF ANTIMICROBIAL SUBSTANCES

A. Fermentation and Assay for Antimicrobial Substances

The results of screening Mycoplasma cultures for the production of antimicrobial substances are summarized in Table XI. Three cultures, a Mycoplasma hominis obtained from Dr. Lapinski (designated L. H. 1), an unidentified human isolate obtained from Mr. Crawford (designated C-2), and Mycoplasma laidlawii B, were found to produce antimicrobial substances when assayed against Bacillus subtilis. Antimicrobial activity was repeatedly demonstrated against B. subtilis in several fermentations, however difficulties were encountered in standardizing the assay procedure. When the Bacillus subtilis test plates were incubated at 37°C, only transitory inhibitory zones were observed and the B. subtilis cells soon overcame the inhibitory effects of the antibiotic substance (within 6-8 hours). The use of the standardized spore culture and incubation of the assay plates at 30°C (as described in the Methods section) overcame this difficulty and gave more reproducible responses.

B. pH Chromatography of the Active Material

In Table XII are listed the responses of Bacillus subtilis to the pH chromatograms of the antimicrobial

TABLE XI

Antimicrobial Activity of the Lyophilized *Mycoplasma* Cultures

Mycoplasma Culture	Test Organisms*				
	B. subtilis	E. coli	S. cerevisiae	S. lutea	M. laidlawii
M. salivarium (Crawford)					
2	0,0	0,0	0,0	0,0	0,0
7H	0,0	0,0	0,0	0,0	0,0
M. hominis (Crawford)					
II	0,0	0,0	0,0	0,0	0,0
M. hominis (Lapinski)					
1	15,15	0,0	0,0	0,0	0,0
2	0,0	0,0	0,0	0,0	0,0
3	0,0	0,0	0,0	0,0	0,0
M. hominis (Madoff)					
41TC	0,0	0,0	0,0	0,0	0,0
Avian (Hamdy) Serotype					
J	0,0	0,0	0,0	0,0	0,0
D	0,0	0,0	0,0	0,0	0,0
B	0,0	0,0	0,0	0,0	0,0

(Cont.)

TABLE XI - Cont.

Mycoplasma Culture	Test Organisms* B. subtilis	E. coli	S. cerevisiae	S. lutea	M. laidlawii
Unidentified (Lapinski)					
1	0,0	0,0	0,0	0,0	0,0
2	0,0	0,0	0,0	0,0	0,0
3	0,0	0,0	0,0	0,0	0,0
Unidentified (Crawford)					
1	0,0	0,0	0,0	0,0	0,0
2	16,17	0,0	0,0	0,0	0,0
3	0,0	0,0	0,0	0,0	0,0
4	0,0	0,0	0,0	0,0	0,0
5	0,0	0,0	0,0	0,0	0,0
6	0,0	0,0	0,0	0,0	0,0
7	0,0	0,0	0,0	0,0	0,0
M. laidlawii	15,16	0,0	0,0	0,0	0,0
Media Control	0,0	0,0	0,0	0,0	0,0
Omadine® Average	15,15	15,15	15,15	16,16	18,18

*zone sizes in mm.

TABLE XII

Responses of *Bacillus subtilis* to the pH Chromatograms of the Antimicrobial Substances Produced by 3 *Mycoplasma* Cultures

Test Material	R_f of the Antimicrobial Activity (zone sizes)			
	pH 3	pH 5	pH 7	pH 9
M. laidlawii extract	-	-	0 (20 mm)	0.27 (15 mm)
C-2 extract	-	-	-	0.81 (12 mm)
L.H. 1 extract	-	-	-	0.62 (9 mm)
Media control	-	-	-	-
Penicillin V (0.1 µg)	1 (25 mm)	0.5 (15 mm)	0.2 (15 mm)	0 (25 mm)

substances. The inhibitory substances obtained from all 3 cultures were found to be basic in nature as they showed mobility in the organic solvent (ethyl acetate) only at basic pH. That no inhibitory zones were observed in the acid pH range may be due to lack of antibiotic activity or inactivation (degradation) of the antibiotic material at pH 5 or below.

C. Sensitivity of Bacillus subtilis to Various Concentrations of the Inhibitory Substance Biosynthesized by M. laidlawii B

The sensitivity of Bacillus subtilis to various concentrations of the n-butanol extractable material produced by M. laidlawii B is shown in Figure 6. Doubling the antibiotic concentration results in an increase of 3 mm in diameter of the inhibitory zones, e.g., a slope of 3.

D. Heat Stability of the Antimicrobial Substance Biosynthesized by M. laidlawii B

Heat stability (100°C for 15 minutes) of the antimicrobial agent(s) biosynthesized by M. laidlawii B indicated that at pH 3, pH 5, and pH 7, complete inactivation of the inhibitory material occurred. No inhibitory zones were observed when the heat treated

Figure 6. Sensitivity of Bacillus subtilis to Various Concentrations of the Inhibitory Substance Biosynthesized by M. laidlawii B.

LOG DILUTION OF ACTIVE SUBSTANCE
1
1/2
1/4
1/8
6
7
8
9
10
11
12
13
14
15
16
17
ZONE SIZE (mm)

material was assayed against B. subtilis. At pH 9, the inhibitory zones were 10 mm in diameter, compared to an untreated control of 14 mm in diameter, representing a loss of 60% activity at this pH.

In summary, the antimicrobial substances produced by the three Mycoplasma cultures are basic in nature and effective against Bacillus subtilis. The substance(s) produced by M. laidlawii is extractable from alkaline aqueous solution with n-butanol, and is inactivated at neutral and acid pH by heating in boiling water for 15 minutes.

In 1968, Berdy and Magyar surveyed reports on the occurrence of antibiotics. They reported that of the 1,862 antibiotics known in 1965, 1,266 were produced by species of Actinomycetales, 374 by species of fungi, and 222 by bacterial species. A further characterization of the bacterial species that produce antibiotics is listed in Table XIII. More than 50% of the reported antibiotics produced by bacteria are synthesized by species of Bacillus and Lactobacillus.

In view of the similarity between species of Mycoplasma and Bacillus mentioned earlier, it is not too surprising that species of Mycoplasma are capable of producing antimicrobial substances. The major difficulty encountered in the screening program for the detection of

TABLE XIII

Distribution of Antibiotics Produced by Bacterial Species

Organism	Number of Antibiotics Produced	
Pseudomonales		39
Pseudomonas	39	
Eubacteriales		
Rhizobiaceae	4	
Achrobacteraceae	2	
Enterobacteraceae	15	
Brucellaceae	1	
Micrococcaceae	6	179
Neisseriaceae	1	
Lactobacillaceae	21	
Propionibacteriaceae	3	
Corynebacteriaceae	2	
Bacillaceae (Bacillus)	124	
Myxobacteriales		3
Spirochetales		1
		222

an inhibitory agent from Mycoplasma, is obtaining a sufficient concentration of metabolites to detect biological activity. In the early days of antibiotic fermentation, 20 grams/liter of fermenting organisms (molds) were capable of yielding 10-20 mg of antibiotic. Using this as a standard, a Mycoplasma fermentation might be expected to yield between 100 and 200 µg of antibiotic substance from its total cell weight of 200 mg/liter. Lyophilization of the fermented media was used to overcome this low yield and effectively concentrated the biosynthesized metabolites and allowed detection of the inhibitory substances.

SUMMARY

An examination of the antibiotic sensitivity of 49 clinical isolates of Mycoplasma including 22 strains of Mycoplasma pharyngis, 22 strains of Mycoplasma salivarium, 3 strains of Mycoplasma hominis, and one strain each of Mycoplasma fermentans and Mycoplasma orale II showed a general sensitivity of Mycoplasma to protein synthesis inhibitors including tetracycline, chloramphenicol, gentamicin, and kanamycin, at concentrations below 10 µg/ml of antibiotic. However, Mycoplasma species resistant to these antibiotics were not uncommon. More than 50% of the cultures tested were resistant to 20 µg/ml of streptomycin and spectinomycin, and 10 µg/ml of erythromycin.

The similarity in antimicrobial activity of the amino-glycoside antibiotics gentamicin, paromomycin, and kanamycin was demonstrated in the cross-resistance of a gentamicin-resistant Mycoplasma laidlawii B with paromomycin and kanamycin.

There was no indication in experiments using whole cells of resistant Mycoplasma or cell-free extracts of these strains of any enzymatic inactivation or degradation of antibiotics by resistant Mycoplasma by the following mechanisms: chloramphenicol acetylation, streptomycin adenylylation, and penicillin hydrolysis by penicillinase.

Studies in the accumulation by Mycoplasma of streptomycin, tetracycline, and chloramphenicol, indicated that resistance mechanisms of Mycoplasma are governed by a permeability/accumulation mechanism. Whether the difference in total antibiotic accumulation and absorption/efflux patterns between resistant and sensitive Mycoplasma is due to an inhibition of the transport of the antibiotic into the cell, or the specific binding of the antibiotic to its active site is still to be determined.

A study on the metabolism of 21 cultures of Mycoplasma including two species of Mycoplasma salivarium, 5 species of Mycoplasma hominis, 3 avian Mycoplasma, one Mycoplasma laidlawii B and 10 unidentified clinically isolated Mycoplasma showed that 3 cultures (M. laidlawii B, M. hominis L.H. 1, and an unidentified human isolate, C-2) are capable of synthesizing antibiotic substances inhibiting the growth of Bacillus subtilis. A preliminary characterization of the substance produced by M. laidlawii B showed it to be a basic compound extractable from alkaline aqueous solutions by n-butanol. The substance(s) was heat labile at acid and neutral pH.

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