ANTIBIOTICS AND ACHOLEPLASMA LAIDLAWII B

I. MECHANISM OF TETRACYCLINE RESISTANCE IN ACHOLEPLASMA LAIDLAWII B

II. CHARACTERISTICS OF ANTIMICROBIAL AGENTS PRODUCED BY ACHOLEPLASMA LAIDLAWII B (TETRACYCLINE RESISTANT)

by

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ABBREVIATIONS USED IN THESIS

DNA	deoxyribonucleic acid
GC	guanine:cytosine ratio
MIC	minimum inhibitory concentration
C	centigrade
mcg	micrograms
ml	milliliters
^{3}H	tritium
^{14}C	carbon 14
mg	milligrams
R-factor	resistance factor
ATP	adenosine triphosphate
GTP	guanosine triphosphate
Mg^{++}	magnesium ion
PPLO	pleuro-pneumonia-like-organism
μC	micro-curie
μM	micro-mole
U	uniformly labelled
sp. act.	specific activity
n	normal
nm	nanometers
RNA	ribonucleic acid
Poly U	polyuridylic acid
TCA	trichloroacetic acid
Poly A	polyadenylic acid

ABBREVIATIONS USED IN THESIS - Cont.

ppt.	precipitate
PEP	phosphoenolpyruvate
PEP Kinase	phosphoenolpyruvate kinase
t-RNA	transfer ribonucleic acid
R_f	mobility relative to solvent front
S-30	30,000 x g supernatant
mRNA	messenger ribonucleic acid
mm	millimeters
L.	liter
L-	absolute configuration
NAD	nicotinamide-adenine-dinucleotide
$NADH_2$	nicotinamide-adenine-dinucleotide reduced
NADP	nicotinamide-adenine-dinucleotide phosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
^{32}P	phosphorous 32
$NADPH_2$	nicotinamide-adenine-dinucleotide phosphate reduced
K^+	potassium ion
NH_4^+	ammonium ion
AA	amino acid
gm	gram

INTRODUCTION

A. Taxonomy, Metabolic Activities, and Epizoology of Mycoplasma

Mycoplasmas are a group of small procaryotic cells bounded by a single lipoprotein ("unit") membrane (1). They contain the smallest free living cells known and the smallest known viable cells are about 330 nm in diameter (2). Although most Mycoplasmas replicate by a binary division (i.e., one cell forms two daughter cells), some filamentous species are probably able to break into a small number of viable filamentous daughter cells (1). Because these organisms lack a rigid cell wall, they are pleomorphic, highly susceptible to osmotic shock and resistant to penicillin (3). All Mycoplasma, except Acholeplasma laidlawii have an exogenous sterol requirement for growth (4).

The organisms of the pleuropneumonia group are now classified under the proposed new class Mollicutes (derived from the Latin adjective mollis meaning soft or pliable and the Latin noun cutis meaning skin). Under this class the order Mycoplasmatalis has been established. Only one family, Mycoplasmataceae, is presently recognized. Two genera, Mycoplasma (organisms which require sterol) and Acholeplasma (organisms which do not require sterol), have been designated (1,5).

Mycoplasma occur as parasites or commensals in most animals: man, cattle, sheep, goats, swine, rodents, fowl, cats, dogs, guinea pigs, horses, and monkeys (6,7). These organisms have also been isolated from contaminated cultured tissue cells, sewage, compost, leaves and insects. Recently several reports have been published proposing that Mycoplasma may be implicated etiologically in several important plant diseases (8).

Mycoplasmas, like other organisms, are taxonomically defined and identified by a great variety of different biological properties, which can be summarized under the headings of morphology, ultrastructure, biochemical and physiological activities, antigenic structure, habitat, and pathogenicity. The relative importance of these properties in taxonomy depends on whether they are determined by the molecular structure of the nucleic acids of the microorganisms (9).

Although Mycoplasma have similar colonial and cellular morphology, they exhibit great diversity with respect to nutritional requirements, metabolic activities, DNA composition, protein components, and ubiquity of their occurrence. The wide variation in base compositions of DNA suggests that Mycoplasma are heterogeneous. The guanine cytosine (GC) base ratios of the Mycoplasma span the range of 23-41% GC (10). Within this range the Acholeplasma species are 30-36% GC, the Mycoplasma species

(except for Mycoplasma pneumoniae) are 23-36% GC, and Mycoplasma pneumoniae is 39-41% GC.

Neimark (10) has tentatively divided the Mycoplasma into six subgroups taking into account their DNA composition together with metabolic activities:

1) Mycoplasma pneumoniae: GC content about 40% (separated from all other Mycoplasma)
2) Sterol-nonrequiring Mycoplasma: GC about 31-35%
3) High GC fermentative organisms: GC about 35%
4) Low GC fermentative organisms: GC about 23%
5) High GC nonfermentative organisms: GC about 34%
6) Low GC nonfermentative organisms: GC about 24%.

The Mycoplasmas as a group display a rather wide diversity of metabolic activities. Many degrade sugars with the production of lactic and acetic acids similar to the lactic acid bacteria (11). Some oxidize short chain fatty acids by the β -oxidative pathway and utilize the tricarboxylic acid cycle for acetate metabolism (12). Others convert arginine to ornithine by the arginine desimidase pathway (13). Glycerides are hydrolyzed by a variety of Mycoplasmas (14). Mycoplasmas also possess the ability to synthesize complex molecules but are limited in their ability to synthesize the basic chemical units making up these macromolecules (15).

Mycoplasma are relatively weak pathogens (16). A few exceptions are Mycoplasma mycoides, var. mycoides which

causes bovine pleuropneumonia, Mycoplasma mycoides, var. capri, the infectious agent of contagious caprine pneumonia, and Mycoplasma neurolyticum which by virtue of a toxin produces neurological disorders in mice (16). The usual Mycoplasma infections results in invasion of epithelial cells lining the respiratory or genitourinary tracts (16).

Respiratory infections are the most common and are seen in many species of animals. These include, in addition to the bovine, ovine, and caprine pleuropneumonias, primary atypical pneumonia of man caused by Mycoplasma pneumoniae (16), chronic respiratory disease of fowl caused by Mycoplasma gallisepticum and Mycoplasma meleagridis (16), chronic pneumonia of swine due to Mycoplasma hyopneumoniae (16), and infectious catarrh of rodents due primarily to Mycoplasma pulmonis (16). The typical gross pathology is consistent with an inflammatory response, i.e., congestion, edema, and the formation of a serous exudate which may become purulent in the event of secondary bacterial invasion.

The T strains found in the genital tracts of man and cattle and Mycoplasma bovigenitalium of cattle are found associated with inflammatory processes and in humans the T strains are considered responsible for some cases of nonspecific urethritis (17) and possibly responsible for reproductive failure (17).

Infections of joints and articular tissues are seen in many animals. Mycoplasma mycoides and several unclassified species produce arthritis in cattle (16). Mycoplasma granularum and Mycoplasma hyorhinis infected swine exhibit arthritis and polyserositis (16). Purulent arthritis of rats results from infection with Mycoplasma arthritidis (16).

Mycoplasma-like forms have been observed in electron micrographs of corn-stunt diseased plants (11,19,20). Until the presumed agents have been isolated and maintained on artificial culture medium there can be no proof of causal relationship for Mycoplasmas in these diseases.

LITERATURE REVIEW

As of now there has not been prepared a comprehensive review of the Mycoplasmas. However, three books have been written which give general information and references to the literature for these microorganisms (21-23).

A. Nutrition of the Mycoplasma

Although several attempts have been made by various investigators (24-28), a completely defined medium for any species of Mycoplasma has yet to be devised which will support growth to give cell yields equivalent to those obtained in complex media. An example of a defined medium is presented in Table 1 (p. 39-40).

1. Chemical Requirements for Growth

a) Proteins, Peptides, and Amino Acids

Smith and Morton (29) isolated and partially characterized a protein component from mammalian serum capable of supporting the growth of serum-requiring Mycoplasma. This protein retained its growth-promoting activity upon removal of lipids but was shown later to be inactive in a lipid-free basal medium. Refinement of fractionation procedures (30) resulted in the isolation of an electrophoretically and ultracentrifugally pure protein with properties similar to α_1-lipoprotein of serum. It

contained esterified cholesterol, phospholipid, a sedimentation coefficient of 3.76S, a molecular weight of about 200,000, and an electrophoretic mobility of 79.8 μ/sec/V/cm. Amino acid analysis demonstrated the presence of eight amino acids: L-tryptophan, L-leucine, L-valine, L-alanine, L-glutamic, L-arginine and L-lysine (31). This protein provides optimal growth when present at a level of 0.5 to 1.0 mg per ml of culture medium and can be replaced by 5 mg per ml quantities of bovine albumin (32-34) or β-lactoglobulin.

Rodwell (35) independently isolated a similar protein from horse serum which satisfies part of the protein requirement of Mycoplasma mycoides grown in defined media. This protein contains cholesterol and phospholipid,

Smith and Boughton (33) demonstrated that the ability of a protein to support growth correlated with its ability to regulate the incorporation of sterol into Mycoplasma arthritidis. All proteins capable of supporting growth were also capable of neutralizing the lytic effect of surface-active agents, such as detergents, bile salts, and salts of long-chain fatty acids. However, this property of detoxification was not the sole explanation of the protein requirement since proteins incapable of supporting growth, e.g., crystalline bovine albumin, also neutralized the lytic effects of surface active agents. These growth promoting proteins do not increase significantly the aqueous solubility of cholesterol. Nor do the organisms

incorporate the protein as demonstrated by the absence in the cells of the labeled protein growth factor (33).

Alteration of the terminal groups of the lipoprotein in an effort to determine the functional groups on the protein required for regulation of sterol incorporation showed that destruction of sulfhydryl and hydroxyl groups by iodination and periodate oxidation resulted in reduction or complete loss of growth-promoting activity and decrease of sterol uptake by Acholeplasma laidlawii B (33). The most probable conclusion as to the function of this protein alone or combined with fraction V bovine albumin is the regulation of sterol and undoubtedly also fatty acid incorporation into Acholeplasma laidlawii B (36).

Animal proteins have a stimulatory effect on the growth of sterol non-requiring Mycoplasmas, particularly those with fatty acid requirements such as Acholeplasma laidlawii B and Acholeplasma laidlawii A and when small inocula are used (36). Crystalline serum albumin promotes the growth of Acholeplasma laidlawii A, on a partially defined medium (37). This requirement is not absolute since this species can be grown in a peptone medium supplemented with glucose (38).

Peptides are required by Mycoplasma species strain Y, and Mycoplasma gallinarum strain J (24,39). Mycoplasma species strain Y can grow with added protease digest of purified proteins including insulin (26). The intact

B chain of insulin is fully active in growth-promoting ability after digestion with chymotrypsin, pepsin, and rennin but not with trypsin (24). Even in the presence of growth-promoting peptides, amino acids are required except aspartic and glutamic acids and cystine. However, these could be derived from asparagine, glutamine and cysteine, which are required.

Two functions have been postulated to explain the peptide requirements of Mycoplasma:

(1) An assimilable source of required amino acids (except Acholeplasma laidlawii B) (24).

(2) Neutralization of the toxicity of fatty acids (40).

To verify that peptides serve to detoxify free fatty acids, modifications of an early synthetic medium which supported growth of Acholeplasma laidlawii B were made (41). The basal medium contained glutamine (but no Tween 80) to which the following additions were made: (1) fatty acids only (oleic acid, palmitic acid, linoleic acid, linolenic acid, myristic acid), (2) fatty acids plus peptides (Thr-Thr-Glu-Ala-Asp-Lys and Lys-Glu-Thr-Ala-Ala-Ala-Lys), (3) peptides alone, (4) Tween 80 alone, and (5) Tween 80 plus fatty acids. Growth occurred only in modification 2, which contained both peptides and fatty acids, and 4 which contained Tween 80 alone (40). Since growth also did not occur in the presence of peptides alone, it was concluded

that certain fatty acids were required and the peptides were binding these essential fatty acids, rendering them nontoxic to the cells. The successful replacement of peptides and fatty acids with Tween 80 is presumably due to the presence of lipases in Acholeplasma laidlawii B (42) which hydrolyzed the Tween 80 (polyoxyethylene sorbitan monoester of oleic acid), slowly releasing free fatty acids required in the biosynthesis of cellular glycerolipids (43).

The amino acid requirements for growth have been defined for a few species: Mycoplasma arthritidis (25), Mycoplasma species strain Y (26), Acholeplasma laidlawii B (44), Acholeplasma laidlawii A (27), and Mycoplasma gallinarum J (24). Acholeplasma laidlawii B grows well in a medium containing 17 amino acids. Mycoplasma gallinarum J grows in a medium with 13 amino acids together with tryptic digest of casein. Acholeplasma laidlawii A requires at least 13 amino acids and Mycoplasma arthritidis nine amino acids, but in both instances dialyzed protein was added which conceivably could supply some of the amino acid requirements.

b) Nucleic Acids, Purines, and Pyrimidines

Early attempts to devise defined media showed the requirements for DNA and RNA by Mycoplasma arthritidis (25) and Mycoplasma mycoides (37). The nucleases of the organisms no doubt degrade these large molecules making

available the individual nucleosides. In Mycoplasma mycoides an excess of either nucleic acid inhibits growth by inhibiting the respective nucleases (37).

The minimal requirements for growth of Acholeplasma laidlawii A (28) and probably Mycoplasma gallinarum J (24) are adenosine, guanosine, and cytidine. In the absence of folinic acid, thymidine also is required. Acholeplasma laidlawii B has an absolute requirement for uridine and thymidine, a partial requirement for guanosine, deoxyguanosine, adenosine, deoxyadenosine, deoxyadenosine, cytosine, and deoxycytosine (5 mg/L.) (44). The latter two could partially satisfy the uridine requirement. Deprivation of thymidine but not the other growth requirements results in loss of viability, i.e., thymineless death.

Mycoplasma hominis produces an extracellular deoxyribonuclease which may degrade DNA outside the cell making thymidine nucleotides available for deoxyribonucleic acid synthesis (45).

c) Carbohydrates

Nonfermentative species such as Mycoplasma hominis type I and II and Mycoplasma salivarium are capable of formation of pentoses from hexoses via portions of the pentose phosphate and glycolytic pathways (43). No other carbohydrates appear essential for nonfermentative species.

All fermentative Mycoplasmas such as Acholeplasma laidlawii and Mycoplasma mycoides require hexoses as carbon and energy sources. Avian species (46), Mycoplasma mycoides (47), Acholeplasma laidlawii A and Acholeplasma laidlawii B (41,47) and Mycoplasma pneumoniae (48) require glucose, Glucose can be replaced by maltose, mannose or fructose, but not by galactose for Mycoplasma mycoides (35). Likewise maltose can replace glucose for growth of Acholeplasma laidlawii A, but mannose, fructose, galactose, sucrose and lactose are ineffective (27). Maltose can replace glucose for Mycoplasma gallinarum J (24).

d) Lipids

Early studies with Mycoplasma distinguished two nutritional types, those requiring mammalian serum and called "parasitic" and those having no such requirements and called "saprophytic." The necessary component of serum was shown to be the α_1-lipoprotein (30). Although all three components of this lipoprotein--protein, phospholipid, and sterol--are necessary for growth (30,49), only cholesterol is incorporated (50). Among all sterol-requiring Mycoplasmas there is an interrelationship between the levels of sterol and phospholipid or fatty acid (51,52,30). Usually twice as much sterol as phospholipid or fatty acid is required on a molar basis or growth inhibition occurs (53). It is probable that sterol in this proportion is necessary to prevent the lytic

action of the surface-active compounds since both protein and cholesterol counteract the lytic and growth inhibitory properties of this class of compounds (47,50).

Studies on the incorporation of sterols (cholesterol, cholestanol ergosterol) by resting and growing cells (50,54) and on the ability of these sterols to support growth (55) in relation to their molecular configuration and conformation led to the conclusion that only planar sterols possessing an equatorial 3-hydroxyl group and a nonpolar side chain were satisfactory. The nonpolar side chain is required for irreversible binding of the sterol in the membrane. Thus, steroids such as progesterone and testosterone which lack this side chain are not incorporated, while sterols, ketosteroids and hydrocarbon side chain steroids of both cholestane and coprostane series are bound whether or not they support growth. The requirements for planarity and an equatorial 3-hydroxyl group in order for growth stimulation to occur must be explained on some other terms than binding to the membrane (43).

Acholeplasma laidlawii species which do not require sterol, contain carotenoid pigments in their cell membrane. Incorporation of sterol or carotenoid into Mycoplasmas is dependent upon other components of the lipoprotein, i.e., protein and phospholipid. As already mentioned, the role of the protein moiety is regulation of sterol incorporation. The uptake of sterol is an

orderly process which occurs in both resting and growing cells (50).

The function of the phospholipid component of the lipoprotein is considered to be the aqueous solubilization of sterol or carotenoid making it available to the organisms in an assimilable form. This requirement is non-specific. Any surface-active agent (salts of long chain fatty acids, bile salts, and positively charged phospholipids) is functional as long as it is not so lytic as to be toxic to the organisms. Aqueous solubilization of sterol is the only property of these compounds which correlates with their ability to support growth (56). Neither uptake nor degradation of these compounds occur. The absence of incorporation of exogenously supplied phospholipids and the nonspecificity of their requirement is compatible with the conclusion that Mycoplasmas synthesize their phospholipids de novo (57).

Mycoplasmas can be divided into three classes with respect to fatty acid requirements for growth. One class, represented by Mycoplasma mycoides, requires both saturated and unsaturated fatty acids; another, represented by Acholeplasma laidlawii A, requires only unsaturated fatty acid; a third, represented by Acholeplasma laidlawii B, requires neither.

Mycoplasma mycoides requirements are met with equimolar amounts of saturated and an unsaturated fatty acid, e.g., palmitate or stearate and oleate (47). Growth in the

presence of a single fatty acid sometimes is initiated but always is followed by lysis. An apparent interrelationship exists between unsaturated and saturated fatty acid requirements which is thought to be governed by their fit into the membrane (31).

The fatty acid requirement of Acholeplasma laidlawii A can be met with oleic acid offered together with defatted bovine serum albumin or as Tween 80. Saturated acids have no stimulatory effect on growth (58).

Acholeplasma laidlawii B grows optimally in defined medium in the presence of five fatty acids, myristic, palmitic, oleic, linoleic, and linolenic (41), although these can be replaced with acetate together with Tween 80 (polyoxyethylene sorbitan monoester of oleic acid) (44).

Acetate is required by Acholeplasma laidlawii B for biosynthesis of carotenoids (59,60) and for saturated fatty acids (61,62). It can be supplied exogenously or be derived from hexose metabolism.

e) Vitamins and Coenzymes

Acholeplasma laidlawii A requires nicotinic acid, riboflavin, folinic acid, pyridoxine, and thiamine for growth in defined media (27,37). Acholeplasma laidlawii B requires thiamine, pyridoxine, nicotinic acid, biotin and coenzyme A and riboflavin (44). Mycoplasma arthritidis requires biotin, folic acid, pantothenate, pyridoxine and thiamine (25). Mycoplasma gallinarum J requires biotin,

B_{12}, pyridoxine, pantothenate and thioctic acid (24,63).

f) Inorganic Requirements

No well-controlled studies of inorganic requirements for Mycoplasma growth have been performed (43).

g) Aeration Requirements

Because aeration requirements ultimately reflect respiratory physiology, those species with a cytochrome-terminated respiratory pathway should grow better aerobically while those with a flavin-terminated pathway should prefer anaerobic or microaerophilic conditions. Since studies on aerobic requirements have been performed with complex culture media, accurate assessment is not possible. Most species grow equally well aerobically and under reduced oxygen tension on complex media (43).

2. Physical Requirements for Growth

a) Temperature

Edward (64) confirmed the generalizations of early workers that Mycoplasma species isolated from mammals grow best at 37°C while Acholeplasma laidlawii B grows at 22°C and 37°C but best at 30°C. The T strains from the genitourinary tract of man multiply satisfactorily between 30°C and 36°C but 36°C is optimal (65). Low temperatures, although not permitting growth, are not deleterious to viability.

Most species grow optimally at pH between 7 and 8. However, growth will occur over a broader range, i.e., pH 6.8 to 9.2 (64,66,67). An exception among the Mycoplasmas are the T strains which require a pH less than 7 for optimal growth (65,66).

b) Osmotic Requirement

Leach (68) reported that the optimal osmotic pressure requirements for growth of Mycoplasma gallisepticum lies between 6.8 and 14.0 atmospheres. Tonicity of the medium can be maintained with acetate, various inorganic salts, such as sodium chloride and sodium phosphate, and sucrose. There is little adaptation by Mycoplasma to hypotonic and hypertonic environments (68).

B. Metabolic Systems Found In Mycoplasma Species

1. Energy Production

a) Carbohydrate Metabolism

The dissimilation of glucose has been examined in some detail for three species, Mycoplasma mycoides (69-74), Mycoplasma gallisepticum (75) and Acholeplasma laidlawii (76) and the following conclusions have been drawn:

1) The Embden-Meyerhof-Parnas pathway is present in Mycoplasma mycoides (72,79). Glucose enters the pathway by phosphorylation with hexokinase, and mannose and fructose by their 6-phosphate intermediates. The sole products of hexose oxidation were acetate and CO_2. The oxidation of pyruvate to acetate and CO_2 is accomplished through the pyruvic oxidase system which requires inorganic phosphate, coenzyme A, α-lipoic acid, NAD and cocarboxylase. Glucose is not attacked anaerobically by intact cells of Mycoplasma mycoides even though an NAD-linked lactic dehydrogenase is present (74). Rodwell (75,74) postulated that pyruvate is removed preferentially by the pyruvate oxidase system and that the organism possesses no other anaerobic mechanism for the reoxidation of $NADH_2$ formed by the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Evidence favoring this hypothesis includes: (1) rapid fermentation of glucose in the presence of arsenite, an inhibitor of the pyruvate oxidase system, (2) production of a synergistic effect on glucose fermentation by exogenous pyruvate, and (3) ready attack of glucose by broken cells when coupled with a system for NAD regeneration, such as yeast alcohol dehydrogenase plus acetaldehyde.

2) Detailed studies have also confirmed the existence of the Embden-Meyerhof-Parnas pathway in cells of Mycoplasma gallisepticum (77). As in Mycoplasma mycoides, pyruvate is formed anaerobically and oxidized to

acetate and carbon dioxide aerobically with the requirements for coenzyme A, α-lipoic acid, and cocarboxylase.

3) The aerobic phosphogluconate pathway is absent as judged by the inability of Mycoplasma mycoides to oxidize 6-phosphogluconate and the absence of transaldolase and transketolase. Neither intact nor broken cells are capable of oxidation of di- or tricarboxylic acid cycles. Mycoplasma pneumoniae carries out glucose fermentations similar to Mycoplasma mycoides (78).

4) Acholeplasma laidlawii A and laidlawii B converted ^{14}C-glucose to CO_2, acetate, pyruvate, and lactate in both resting and growing states (76). Acholeplasma laidlawii B yielded CO_2 of higher specific activity from glucose-1-^{14}C than from glucose-6-^{14}C suggesting that the hexose monophosphate shunt mechanism was operative. This hypothesis was further substantiated by the demonstration of glucose-6-phosphate dehydrogenase, ribose-5-phosphate isomerase, and transketolase present in cell-free extracts of this organism. This pathway is absent in Acholeplasma laidlawii A.

Cursory examination of other fermentative species suggests the existence of the Embden-Meyerhof-Parnas pathway and the absence of the tricarboxylic acid cycle because resting cells oxidize glucose quantitatively to acetate and CO_2, while α-ketoglutarate, succinate, malate, and oxalacetate are oxidized slowly (79-81).

Nonfermentative species, in particular Mycoplasma arthritidis, are capable of oxidizing various intermediates of the Embden-Meyerhof-Parnas pathway and pentose phosphate pathway (82). Carbohydrate metabolism in these organisms probably reflects synthetic rather than catabolic functions. The existence of the tricarboxylic acid and the glyoxylate cycles have been shown in Mycoplasma arthritidis. With the exception of malate synthetase and citrate-condensing enzyme, cell extracts were shown to contain all the enzymes of the tricarboxylic acid and glyoxylate cycles, i.e., NADP-specific isocitric dehydrogenase, isocitratase, NAD-specific α-ketoglutaric dehydrogenase, succinic dehydrogenase, fumarase, NAD-specific malic dehydrogenase, acetyl coenzyme A synthetase, and citratase (83).

b) Nitrogen Metabolism

Proteolysis as measured by liquefaction of gelatin has been reported for Mycoplasma mycoides (69), Acholeplasma laidlawii (84), and certain strains from goats (85). Large amounts of ammonia are produced by the T strains as a result of the hydrolysis of urea (86). This urease has a pH optimum of 6.0, the optimal pH for growth of these organisms. Hydroxyurea, allylurea, thiourea, arginine, ornithine, citrulline, proline, and glutamine do not serve as substrates for this urease (86). The function of the urease is unknown but the absolute requirement of urea for growth of T strain Mycoplasma suggests that it may serve as a nitrogen source for growth.

Mycoplasma arthritidis grows well in media containing a variety of amino acids including arginine, glutamine, glutamic acid, aspartic acid, histidine, leucine, and threonine under aerobic conditions and tyrosine and tryptophan under anaerobic conditions (87,88). The metabolism of these amino acids was determined by measurement of disappearance of them and of ammonia production. Only two of these amino acids are related to the generation of energy: glutamine and arginine. Glutamine at alkaline pH undergoes hydrolytic deamination but at acid pH it undergoes phosphorolysis with the formation of ATP, glutamic acid, and ammonia. The phosphorolysis reaction is reversible with the equilibrium favoring ATP formation. Thus glutamine phosphorolysis could be a source of energy (89). Arginine undergoes quantitative hydrolytic desimidation to citrulline (87,88,90-92) mediated by arginine desimidase. Citrulline undergoes phosphorolysis by ornithine transcarbamylase to yield ornithine and carbamyl phosphate. Carbamyl phosphate is cleaved by carbamate kinase in the presence of ADP with the formation of ammonia, CO_2, and ATP. Both the ornithine transcarbamylase and the carbamate kinase reactions are reversible.

Of the species examined, only Mycoplasma hyorhinis and Mycoplasma canis of the nonfermentative group of organisms did not contain arginine desimidase activity while only Mycoplasma fermentans was positive among the fermentative species (92).

c) Lipid Metabolism

No phospholipase activity has been demonstrated in Mycoplasmas (93). A nonspecific lipase capable of hydrolysis of triglycerides, natural fats, and simple fatty acid esters is found in Mycoplasmas (94). It is distinct from the sterol esterase based upon its cellular locations, its heat lability and its lability to pretreatment at acid and alkaline pH. It is found in the soluble portion of the cell, has no requirement for inorganic ions, and has an alkaline pH optimum (95).

A membrane-associated sterol esterase has been studied in some detail in Mycoplasma arthritidis (94). It is capable of hydrolytic or thiolytic cleavage of steryl esters and carotenyl esters. The product of thiolytic cleavage appears to be an acyl coenzyme A based on the formation of hydroxylamine reactive products. The enzyme requires the micellar form of the ester formed in combination with some amphipathic lipid. It possesses little specificity toward different fatty acids but does have a specificity toward the sterol.

Nonfermentative species, in particular Mycoplasma arthritidis, are able to oxidize short-chain fatty acids, and derive a major portion of energy by this mechanism. Fatty acids of chain length 2 through 10 are oxidized, the ratio decreasing with increase in chain length (96). The mechanism of fatty acid oxidation follows the typical β-oxidative pathway. Mycoplasma arthritidis contains an

acetyl coenzyme A synthetase which also exhibits some activity on propionate but none with formate or other longer-chain fatty acids (83,97). Activation of fatty acids other than acetate or propionate occurs through the mediation of a propionate coenzyme A transferase. Although the specificity of this enzyme is not known, the inability of the organism to oxidize fatty acids of chain length greater than 10 suggests limited specificity.

2. Biosynthesis

a) Nucleic Acids and Their Precursors

All Mycoplasmas require for growth in laboratory media preformed nucleotides or purines and pyrimidines (98). As mentioned in the section on nutrition, these can be made available by the action of nucleases on RNA and DNA supplied in most of the culture media. A nucleoside phosphorylase catalyzes the phosphorolytic cleavage of thymidine, deoxyinosine, deoxyadenosine, and deoxyguanosine but not of deoxycytidine with the liberation of the free base and phosphorylated deoxypentose. This enzyme can carry out the transfer of the deoxypentose moiety from thymidine to hypoxanthine and xanthine but not to adenine to form the corresponding purine deoxyribosides. Thymine cannot act as a deoxypentose acceptor with purine deoxyribosides as deoxypentose donors (99). The biosynthetic pathway to pyrimidines is lacking in Mycoplasma arthritidis and Mycoplasma orale as judged by

the inability of these organisms to incorporate ureido-C^{14}-citrulline into orotic acid (100). These findings are consistent with the nutritional requirements. The pyrimidine nucleosides are required but purine nucleosides are not. Interconversions occur unidirectionally from pyrimidine to purine nucleosides.

Analysis of DNA fragment by cesium chloride equilibrium sedimentation following density labeling for varying time periods by substituting 5-bromodeoxyuridine for thymidine gives results consistent with semiconservative replication (101). Replication proceeds unidirectionally from a limited number of growing points.

While nothing is known about the biosynthesis of transfer RNA in Mycoplasma, transfer RNA has been isolated from three species: Acholeplasma laidlawii B, Mycoplasma gallisepticum A5969 and Mycoplasma species Kid (102), and all three species examined contain N-formyl-methionyl tRNA.

Formation of ribosomes also has not been studied. Their existence is known from electron micrographic examination and the appearance of large particulate RNA in sedimentation analysis. In Mycoplasma gallisepticum ribosomes constitute about 80% of the total RNA (103).

Messenger RNA synthesis inhibited by actinomycin D results in eventual cessation of protein synthesis consistent with turnover of mRNA in Mycoplasma gallisepticum A5969 (104). The half life of mRNA is approximately four

minutes. The delayed inhibition of protein synthesis and the immediate cessation of incorporation of C^{14}-uridine into RNA seen in several species is evidence for the existence of a DNA-dependent RNA polymerase responsible for transcription of the code from DNA (105).

b) Amino Acids and Proteins

Mycoplasmas as a group vary in their ability to synthesize amino acids. Mycoplasma arthritidis possesses weak transaminase activity principally forming alanine from pyruvate and glutamine (88). Mycoplasma gallisepticum contains a glutamic-aspartic transaminase system favoring the formation of aspartic acid (81). No transamination has been detected in Mycoplasma mycoides (69) or Acholeplasma laidlawii A (106). Aspartic acid is deaminated by Mycoplasma arthritidis and serves as a precursor of homoserine and threonine (88). Asparagine can be deaminated to give rise to aspartic acid and glutamine to glutamic acid. Lysine is formed by the decarboxylation of meso-$\alpha\varepsilon$-deaminopimelic acid. Synthesis of lysine in Mycoplasma does not involve the intermediate, α-aminoadipic acid. Glutamic acid is converted to proline via the intermediate formation of glutamic-γ-semialdehyde and Δ'-pyrroline-5-carboxylic acid. Ornithine and citrulline are derived from the degradation of arginine. Glutamic acid does not serve as a precursor for ornithine (88-90,107).

Protein synthesis in Mycoplasma occurs by the same mechanism as that found in other procaryotic cells. Amino acid activation is carried out by amino acid-specific tRNA synthetases in the presence of amino acid and ATP to form AMP-amino acid-tRNA synthetase complex and pyrophosphate. The amino acid is then transferred to amino acid-specific tRNA with the formation of aminoacyl tRNA and the liberation of AMP and free enzyme (108). Aminoacyl tRNA's have been demonstrated in Acholeplasma laidlawii B, Mycoplasma gallisepticum and Mycoplasma hominis (108,109); for valine, methionine, and N-formyl-methionine in Acholeplasma laidlawii B, Mycoplasma gallisepticum and Mycoplasma species Kid (102); for L-alanine in Mycoplasma laidlawii B (110).

c) Carbohydrates

Nutritional studies suggest that preformed ribose and deoxyribose is required by Mycoplasma arthritidis. However, this species possesses the hexose monophosphate pathway and probably is capable of deriving pentoses from glucose (111). Glucose carbon is incorporated into the galactan of Mycoplasma mycoides and into the glucan of the bovine arthritis strain as well as into the pentoses of the nucleic acids of both strains (112).

d) Lipids

(1) Fatty acids

There are two known types of Mycoplasma with respect to ability for fatty acid biosynthesis. One group is capable of the biosynthesis of only saturated fatty acids and is represented by Acholeplasma laidlawii. This species can be differentiated further into strains which require an unsaturated fatty acid (strain A) and strains whose requirements are satisfied with only saturated acids (strain B) (113). C^{14}-acetate is incorporated only into the saturated fatty acids of both strains A and B (114). A second group of Mycoplasmas is incapable of the biosynthesis of either saturated or unsaturated acids and includes Mycoplasma mycoides and probably Mycoplasma arthritidis, Mycoplasma gallinarum, and Mycoplasma gallisepticum (115).

A detailed study of saturated fatty acids has been performed by Panos and coworkers (116-118) using Acholeplasma laidlawii A and laidlawii B. The malonyl coenzyme-A pathway occurs in Acholeplasma laidlawii A. A soluble fatty acid synthetase system incorporates C^{14}-acetate into saturated fatty acids in the presence of malonyl coenzyme-A, $NADPH_2$ and Mg^{++}. The products are mainly stearic, palmitic, and myristic acids although a trace of C_{20} acid is formed.

(2) Glycolipids

The mechanism of biosynthesis of mono- and diglucosyl diglycerides has been established for Acholeplasma laidlawii B (119). The monoglucosyl diglyceride is synthesized by a membrane-associated enzyme from 1,2-diglyceride and uridine-5'-diphosphoglucose. A medium of high ionic strength and sodium dodecyl sulfate stimulates synthetase.

(3) Phospholipids

Mycoplasmas are able to synthesize all their phospholipids. Radiolabeling studies have shown the incorporation of glycerol and palmitate into the phosphatidyl glycerol and phosphatidyl monoglyceride of Mycoplasma pneumoniae (120), of glycerol, palmitate, and oleate into diphosphatidyl glycerol and phosphatidyl glycerol of Mycoplasma mycoides (121), of inorganic P^{32} and oleic acid into the phosphatidyl glycerol of Acholeplasma laidlawii (122-124).

(4) Polyterpenes

Nutritional studies have indicated that sterol nonrequiring Mycoplasmas are capable of de novo biosynthesis of polyterpenes, whereas sterol-requiring Mycoplasmas are either totally deficient or possess specific enzymic blocks. Three species have been examined for enzymes in the pathway to polyterpenes, Acholeplasma laidlawii B, Mycoplasma arthritidis and Mycoplasma gallinarum.

Acholeplasma laidlawii B, a sterol-nonrequiring species, can incorporate C^{14}-acetate and C^{14}-mevalonic acid into its unsaponifiable lipids while the other two, representative of sterol-requiring species, cannot (125).

C. Metabolites of Mycoplasma

Mycoplasma neurolyticum possesses a soluble exotoxin, while an endotoxin-like material has been isolated from Mycoplasma mycoides. Other metabolites produced by Mycoplasma have not been reported.

1) Galactan of Mycoplasma mycoides

Hot phenol extracts of Mycoplasma mycoides administered intravenously in rabbits elicits a febrile response accompanied by a leukopenia and followed by a leukocytosis (126). However, a considerable quantity of the purified galactan (1 mg) is necessary to show any febrile response although smaller does (1 mcg) induce a leukocytic response (126). Intravenous injection of the galactan in some cattle elicits an anaphylactoid reaction within a few minutes characterized by an increase in respiratory rate and salivation. Animals recover within an hour (127). The galactan itself is not a specific virulence factor of Mycoplasma mycoides as galactans from avirulent strains behave similarly (127).

2) Neurotoxin of *Mycoplasma neurolyticum*

The exotoxin of *Mycoplasma neurolyticum*, first noted in 1938 by Sabin (128), produces in mice the characteristic set of neurological manifestations known as "rolling disease." After a latent period, which varies in length depending on the dose of toxin, the animals suddenly begin to roll over and over on the long axis of the body until generalized convulsions and death (129).

The neurotoxin is produced only by logarithmically growing cultures and a loss in activity is noted by a drop in pH (less than 6.8) and in the stationary phase of growth (129). Filtrates of cultures free of organisms have a toxicity equivalent to the whole cultures. Nonproliferating cells and organisms suspended in nonnutritive media fail to elaborate additional toxin (130). Inhibition of its production by puromycin and chlortetracycline suggests that active protein synthesis is necessary for its formation (131,132). Freeze-thawing destroys the toxin and its preservation at -70°C is only partially successful (133). It is thermolabile at 45°C to 50°C (134). Its resistance to physical factors is greater in contact with the organisms than in cell-free culture filtrates. Sephadex filtration suggests that the toxic material in culture filtrates is a protein (absorption maximum 275 to 280 nm) of molecular weight in excess of 200,000 (129). However, similar material can be obtained from other nonneurotoxic Mycoplasmas (131). Its protein

nature is confirmed by its inactivation with trypsin (135).

Specific antibody is capable of neutralization of the toxin (131,135). Since antibody is ineffective 2 minutes after introduction of the toxin, it is presumed that toxin has already become bound to tissue receptors. Symptoms appear rapidly after the toxin reaches the central nervous system. The target site in brain is suspected to be a ganglioside (132).

D. Protein Synthesis In Mycoplasma

The mechanism of protein biosynthesis in procaryotic cells has been reviewed by Lengyel and Söll (136): the amino acid sequence of a particular protein is specified by the sequence of nucleotides in a segment of the deoxyribonucleic acid. The process of protein synthesis consists of two stages. First, the DNA is transcribed into a ribonucleic acid intermediate, messenger RNA, which has a ribonucleotide sequence complementary to that of the deoxyribonucleotide sequence of one of the strands of the DNA serving as template (137). The mRNA becomes attached to cytoplasmic ribonucleoprotein particles (ribosomes) which are the sites of protein synthesis, and there it determines the order of linkage of amino acids into a specific protein (138). The mRNA is translated in the 5' to 3' direction (139). The synthesis of a protein is initiated at the amino-terminal amino acid and proceeds towards the carboxyl-terminal amino acid (137,140,141).

During translation, a group of three adjacent nucleotides in the mRNA (codon) specifies which amino acid is to be linked to the growing peptide chain.

Protein synthesis in Mycoplasma occurs by the classical mechanism. Evidence for such an hypothesis is suggested by the following:

1) Mycoplasma ribosomes are 70S particles (142).
2) Mycoplasma tRNA has physical properties similar to E. coli tRNA (143).
3) N-formyl-methionine which is necessary for peptide initiation has been isolated from Mycoplasma (143).
4) Mycoplasma tRNA stimulates polypeptide formation in a tRNA-dependent E. coli cell-free amino acid incorporating system directed by synthetic and natural mRNAs (143).
5) tRNA synthetases have been shown to exist in Mycoplasma (143-146).

A cell-free protein synthesizing system in procaryotic cells can be prepared by disintegrating cells in aqueous media, removing unbroken cells and cell debris by low-speed centrifugation and small molecules by dialysis (147). To observe protein synthesis with such extracts, one requires the addition of adenosine triphosphate, guanosine triphosphate, and ATP-generating system, proper ions (Mg^{++} and either K^{+} or NH_4^{+}), sulfhydryl compounds, and amino acids (some of which are radioactively labeled) (136). In such a system, mRNA can be translated into protein. The translation is assayed by following the incorporation of labeled amino acids into protein (136). The messenger

may be present in the extracts (endogenous messenger), or it may be added (exogenous messenger). The exogenous messenger can be either a natural or a synthetic polyribonucleotide.

The cell extract can be further fractionated by centrifugation at high speed. The resulting supernatant fraction contains the following components of the protein synthesizing machinery: tRNA, AA-tRNA synthetases, and proteins involved in the elongation and termination of the peptide chain (136). The resulting pellet contains: ribosomes and proteins required for peptide chain initiation (136).

Cell-free protein synthesis in Mycoplasma has been reported using Acholeplasma laidlawii B as the test organism (144,145,148). For optimal protein synthesis in the cell-free system, ATP and an ATP-generating system, ribosomal (105,000 x g precipitate) and soluble (105,000 x g supernatant) fractions were essential. It was inhibited by ribonuclease, puromycin and chloramphenicol, while cycloheximide showed no effect. Omission of β-mercaptoethanol resulted in complete loss of activity. Addition of purified membrane preparation stimulated a 20-fold increase in incorporation.

The total amount of radioactive phenylalanine incorporated into protein as shown in Table 2 (p. 41) was small. The system did not show a requirement for GTP and attempts to stimulate incorporation of specific amino acids with

synthetic polyribonucleotides (Poly U and Poly A) were unsuccessful (145).

E. Mechanisms of Action of Tetracyclines In Bacterial Systems

The tetracyclines are included in a class of antibacterial agents known as broad spectrum antibiotics which inhibit the growth of Gram-negative as well as Gram-positive bacteria. In addition, the tetracyclines are effective against rickettsial organisms, Mycoplasma and some of the larger viruses.

For low concentrations of the drug the primary mechanism of action is an inhibition of protein synthesis (149). With high concentrations of drug, however, evidence suggests that inhibition of cell wall synthesis (150), of components of the respiratory chain (151), or of other biochemical systems (152,153), may contribute to the bactericidal effects of the tetracyclines.

Numerous studies (154-157) have demonstrated that tetracyclines inhibit bacterial protein synthesis by binding to the ribosome. The tetracyclines do not inhibit the binding to natural or synthetic mRNAs to the ribosomes and they are equally effective against protein synthesis directed by natural or synthetic messengers (158). Studies of the effects of the tetracyclines on the tRNA-ribosome interaction show that these drugs inhibit the binding of aminoacyl-tRNA, including f-Met $tRNA_f$, to the acceptor site

on the ribosome but have no effect on binding to the donor site (159-161). The tetracyclines apparently do not directly inhibit formation of the peptide bond or the translocation step (162). However, tetracycline binding is complicated. Large numbers of tetracycline molecules (about 300) can bind to the ribosome (163). Binding is to both 30S and 50S subunits (157,163).

The ability of the tetracyclines to form chelation complexes with polyvalent cations may have an important bearing on their inhibition of protein synthesis (164). It has been proposed that Mg^{++} attaches to the phosphate groups of the ribosomal RNA and may act as a link between the ribosome and tetracycline molecules. The 11, 12-beta diketone system, the 12α and 13 hydroxyl groups have all been implicated as possible complexing sites for polyvalent cations on the tetracycline molecule (164).

1. Mechanism of Resistance

The possible mechanisms by which cells might resist the toxic effects of a growth-inhibiting drug are: (165)

1) Conversion of an active drug to an inactive derivative by enzyme(s) produced by the resistant cells.
2) Modification of the drug-sensitive site.
3) Loss of cell permeability to a drug.
4) Increased levels of the enzyme inhibited by the drug.

5) Increased concentration of a metabolite that antagonizes the inhibitor.

6) Enhancement of an alternative metabolic pathway bypassing the inhibited pathway.

7) Decreased requirement for a product of the inhibited metabolic system.

Evidence of the first three mechanisms has been reported concerning the resistance of Gram-positive and Gram-negative bacteria to the tetracyclines: Okamoto and Suzuki (166) observed that tetracycline-resistant Escherichia coli strains selected by serial transfer on media containing the drug as well as a naturally occurring multiple drug resistant strain carrying episome 'R' were incapable of inactivating tetracycline. Sompolinsky, et al. (167) reported that a high-level tetracycline resistant strain of Staphylococcus aureus did not alter the spectrophotometric or chromatographic characteristic, of the drug. In addition, the tetracycline extracted from resistant organism after growth in tetracycline-containing medium was recognizable by its characteristic R_f on paper chromatography.

Craven, et al. (168) have isolated a tetracycline resistant Escherichia coli mutant containing ribosomes resistant to tetracycline in a cell-free system of protein synthesis. The location of the tetracycline resistance was examined by mixing supernatants with ribosomes from sensitive and resistant strains. This work is in direct contradiction to those who have demonstrated that

tetracycline and its derivatives inhibit protein synthesis in cell-free systems prepared from tetracycline-sensitive, in vitro-developed tetracycline resistant, and 'R' factor resistant strains of Escherichia coli to the same extent (169-172).

The uptake of oxytetracycline (and presumably all the related tetracyclines) by Escherichia coli was first shown by Arima and Izaki (173) to have some of the characteristics of an active transport process. At high oxytetracycline concentration the cells accumulated large quantities of the drug; accumulation was dependent on glucose added to the medium and was inhibited by dinitrophenol or azide. They (174) then found that a strain of Escherichia coli K-12 which exhibited multiple drug resistance accumulated much less oxytetracycline than a sensitive strain. Franklin (175) confirmed and extended the observation of Izaki, Kiuchi, and Arima (176) that uptake of tetracycline by an 'R' factor bearing tetracycline resistant Escherichia coli was decreased by incubation of the cells with tetracycline prior to the uptake measurement. Franklin used two resistant strains, one bearing an 'R' factor and the other obtained by serial passage through increasing concentration of chlortetracycline.

Recent studies have shown that resistance to the tetracyclines in Staphylococcus aureus (167,177) and Escherichia coli (178-180) mediated by 'R' factors, involves an inducible system which blocks tetracycline transport.

Thus, tetracycline-resistant cells exhibit two levels of resistance. 'R' factor bearing cells cultured in the absence of tetracycline are about 50% resistant (as compared to sensitive cells) when challenged with 50 mcg/ml of tetracycline (175). Cells cultured in the presence of a sub-inhibitory concentration of tetracycline, however, are not significantly inhibited by a subsequent challenge with 50 mcg/ml of tetracycline (175). This adaptation, which is apparently associated with a decrease in the absorption of tetracycline by the cells, can be suppressed with inhibitors of protein synthesis and RNA synthesis (175). The tetracycline resistance mechanism thus resembles an inducible system involving the usual components: repressor, operator and structural genes (180). A "constitutive" mutant in which tetracycline resistance is always expressed at a high level because of complete or partial loss of repressor control has also been isolated (180).

Independent lines of evidence strongly suggest that resistance to the tetracycline antibiotics is due to a failure of the antibiotics to reach their site of action rather than to drug inactivation or to a modification of the drug-sensitive site.

TABLE 1

Defined Medium for Growth of *Acholeplasma laidlawii* B*

Amino Acids Content	mg/L.
L-Alanine	725
L-Arginine	440
L-Aspartic acid	90
L-Cystine	30
L-Glutamic acid	75
L-Glutamine	1110
Glycine	240
L-Histidine	310
L-Isoleucine	480
L-Leucine	1010
L-Lysine	840
L-Methionine	325
L-Proline	1310
L-Serine	725
L-Threonine	460
L-Tryptophan	20
L-Tyrosine	725
Nucleic Acid Derivatives	
Adenosine	5
Cytidine	5
Guanosine	5
Uradine	5
Thymidine	5
Deoxyadenosine	5
Deoxyguanosine	5
Deoxycytidine	5
Vitamins and Coenzymes	
D-Biotin	0.01
Coenzyme A	1.0
Nicotinic acid	0.025
Pyridoxine hydrochloride	0.025
Riboflavin	0.01
Thiamine hydrochloride	0.01
Lipid Precursor	
Tween 80 (polyoxyethylene sorbitan monoester of oleic acid)	5
Sodium acetate	50

TABLE 1 - Cont.

Carbohydrates	mg/L.
Glucose	10,000
Inorganic Salts	
NaCl	8,000
KCl	400
Na_2HPO_4	120
$MgCl_2 \cdot 7H_2O$	200
$(NH_4)_2SO_4$	120
Tris (Hydroxymethyl aminomethane buffer)	1,000

The pH of this medium is 8.0.

*Taken from Smith (44).

TABLE 2

Cell-Free Protein Synthesis In *Acholeplasma laidlawii* B*

Additions or Deletions	Counts/min/mg of Protein
Complete System	1,104
-105,000 x g supernatant	120
-ribosomes (105,000 x g ppt.)	285
-ATP, PEP, and PEP Kinase	250
+ribonuclease (10 mcg/ml)	262
+puromycin (10 mcg/ml)	155
+chloramphenicol (50 mcg/ml)	186
+cycloheximide (10 mcg/ml)	1,010
+membrane fraction	19,120

Complete system contains ribosomes; 105,000 x g supernatant; 10 μC ^{14}C-phenylalanine; plus the following (μmoles/ml): 19 L-amino acids, 0.05; ATP, K^+ salt, 1.0; phosphoenolpyruvate, K^+ salt (PEP), 5.0; and PEP Kinase, 20 mcg in a total volume of 1 ml. Incubated 30 minutes at 37°C. Deproteinized with equal volumes of 10% TCA.

*Tourtellotte, *et al.* (144).

PART I

MECHANISM OF TETRACYCLINE RESISTANCE IN ACHOLEPLASMA LAIDLAWII B

A. Materials and Methods

1. Materials

The following chemicals, biochemicals and their sources were used in these studies:

L-Phenylalanine-U-^{14}C (sp. act. 374 mc/mM) was obtained from New England Nuclear Corp. Adenosine triphosphate, guanosine triphosphate, phosphoenolpyruvate, phosphoenolpyruvate kinase, Tris buffer, and polyuridylic acid were all purchased from Sigma Chemical Company. t-Ribonucleic acid (as sodium salt) was obtained from General Biochemicals Inc. Dithiothreitol and β-mercaptoethanol were obtained from Calbiochem. L-amino acids were obtained from Mann Research Laboratories. Tetracycline hydrochloride was obtained from Lederle Laboratories.

Acholeplasma cultures. A culture of Acholeplasma laidlawii B was obtained from Prof. H. E. Morton (University of Pennsylvania) and was maintained on PPLO agar supplemented with PPLO serum fraction which were obtained from the Difco Company. A mutant of this culture resistant to 50 mcg/ml tetracycline was obtained by repeated exposure of the parent (which is sensitive to 2 mcg/ml tetracycline).

This mutant has maintained its resistance when transferred on media containing 5 mcg/ml tetracycline. Both cultures were transferred weekly by the agar block method (181).

2. Preparation of Acholeplasma laidlawii B Cells

Four 2-liter cotton-plugged flasks each containing 1-liter quantities of sterile tryptose broth (Difco Tryptose, 20 g; NaCl, 5 g; Tris buffer, 3.7 g; glucose, 10 g; distilled water, q.s. 1 liter; pH adjusted with NaOH to pH 8.0-8.2 before autoclaving for 30 minutes at 121°C) were supplemented with 5 ml of Difco PPLO serum fraction and inoculated with 50 ml of a 24-hour old culture of Acholeplasma laidlawii B grown in the same medium. The flasks were incubated for 12 hours at 37°C (without shaking) and the cells collected by centrifugation at 3,000 x g for 15 minutes at 4°C. The 1 g of collected cells from the four liters medium was washed with 10 ml of chilled medium and centrifuged at 5,000 x g for 20 minutes at 4°C.

3. Preparation of French Press Broken Cells

One gram of wet cells was suspended in 7 ml of 0.01 N pH 7.8 Tris buffer containing $Mg(C_2H_3O_2)_2$ (final concentration 0.01 M), KCl (final concentration 0.06 M), β-mercaptoethanol (final concentration 0.0006 M) and crushed twice in a chilled Aminco French press (4,000 lbs/sq.

in.). The resulting paste was then homogenized in a glass Potter-Elvehjem homogenizer. The resulting slurry was utilized immediately without storage.

4. Preparation of S-30 Fraction from Vibrogen Mill Broken Cells

One gram of cells was suspended in 4 ml of 0.01 M pH 7.6 Tris buffer containing $Mg(C_2H_3O_2)_2$ (final concentration 0.016 M), KCl (final concentration 0.06 M), and dithiothreitol (final concentration 0.1 mM). This suspension was placed in a 5 ml cup containing 3 ml of 120 micron glass beads attached to a Vibrogen cell mill (Model 600-00, manufactured by the Edmund Buhler Co.). The cell was operated at maximum speed for 30 minutes and the cup was maintained at 0°C by continuously passing chilled ethylene glycol through the jacket. The broken cell-bead mixture was then centrifuged at 30,000 x g for 30 minutes at 4°C and the supernatant solution (designated the S-30 fraction) was dialyzed at 4°C overnight against the Tris buffer-$Mg(C_2H_3O_2)_2$-KCl-dithiothreitol solution. This dialyzed S-30 fraction was stored in small quantities at -20°C and cell free protein synthesis activity was maintained for at least 10 days under these storage conditions.

5. Preparation of 105,000 x g Supernatant and Ribosomes

The S-30 fraction obtained from 1 gm of cells was diluted to 11 ml with Tris buffer-$Mg(C_2H_3O_2)_2$-KCl-dithiothreitol solution. This preparation was then centrifuged at 105,000 x g for 90 minutes in a Beckman model L ultracentrifuge at 4°C. The supernatant was carefully removed and the precipitated ribosomes washed with buffer and again centrifuged. The ribosomes were resuspended in 1 ml of buffer and dialyzed together with the 105,000 x g supernatant overnight at 4°C against the Tris buffer-$Mg(C_2H_3O_2)_2$-KCl-dithiothreitol solution. These dialyzed fractions were then stored in small quantities at -20°C.

6. Protein Synthesizing Reaction Mixture for French Press Broken Cells

Tris buffer, pH 7.8, 100 μM; KCl, 50 μM; $Mg(C_2H_3O_2)_2$, 10 μM; β-mercaptoethanol, 6 μM; adenosine triphosphate, 1 μM; phosphoenolpyruvate, 5 μM; phosphoenolpyruvate kinase, 2 μg; the following amino acids: glycine, L-alanine, L-serine, L-threonine, L-asparagine, L-glutamine, L-methionine, L-isoleucine, L-leucine, L-cysteine, L-proline, L-valine, L-lysine, L-tyrosine, L-histadine, L-arginine, L-cystine, L-aspartic acid, and L-glutamic acid, 0.05 μM; L-phenylalanine-U-^{14}C (sp. act. 187 μC/μM), 0.1 μC; L-phenylalanine, 26 μM; crushed cells, 350 mcg of protein; total volume, 0.10 ml.

7. Protein Synthesizing Reaction Mixture for the S-30 Fraction

Tris buffer, pH 7.6, 50 μM; $Mg(C_2H_3O_2)_2$ 18 μM; NH_4Cl, 50 μM; dithiothreitol, 0.1 μM; adenosine triphosphate, 1 μM; guanosine triphosphate, 0.5 μM; phosphoenolpyruvate, 5 μM; phosphoenolpyruvate kinase, 2 μg; polyuridylic acid, 10 μg; t-ribonucleic acid (from Escherichia coli B), 0.1 mg; L-phenylalanine-U-^{14}C (sp. act. 187 μC/μM) 0.1 μC; L-phenylalanine, 26 μM; total volume, 0.10 ml. In those reactions in which the S-30 fraction was used, there was approximately 0.1 mg protein added to the reaction mixture. In the other experiments where the ribosomes were separated from the supernatant solution, 0.09 mg of ribosomal protein and 0.09 mg of supernatant protein were added to the reaction mixture.

8. Determination of Protein Synthesizing Ability

The reaction mixtures were incubated for up to 60 minutes at 37°C and then chilled in ice water. One ml of distilled water and 0.05 ml of 10% bovine serum albumin were added followed by 1.0 ml of 10% trichloroacetic acid. The resulting suspension was heated at 100°C for 15 minutes, chilled, and the precipitate collected on a Millipore® filter. The collected precipitate was washed with 5% trichloroacetic acid, the filters dried and the

absorbed radioactivity determined in a Packard TriCarb Liquid Scintillation Spectrometer (model 2002) using Bray's solution (182).

9. Protein Determination

The biuret method was used with crystalline bovine serum albumin as standard (183).

B. Results and Discussion

1. Cell Free Protein Synthesis by French Press Broken Cells of Acholeplasma laidlawii B

Optimum conditions for ^{14}C-phenylalanine incorporation into protein appeared to be those as stated by Tourtellotte, et al. (144):

a) 30 minutes incubation time
b) 37°C incubation temperature
c) 10 uM of magnesium acetate
d) pH 7.8 incubation buffer
e) cell membranes.

Incorporation of ^{14}C-phenylalanine into protein was directed by endogenous mRNA; no requirement for polyuridylic acid could be shown in these preparations. Data collected in two studies of the time course of incorporation of ^{14}C-phenylalanine into protein directed by endogenous mRNA in both tetracycline sensitive and tetracycline resistant

Acholeplasma laidlawii B are summarized in Figure 1 (p. 56).

2. Inhibition of Cell Free Protein Synthesis by French Press Broken Cells by Tetracycline

The inhibition of protein synthesis resulting when tetracycline was added to the reaction mixture of a tetracycline-sensitive Acholeplasma laidlawii B and a tetracycline-resistant mutant is shown in Figure 2 (p. 58). 6.2×10^{-6} M (3 mcg/ml) concentration of tetracycline has a significant effect on phenylalanine incorporation regardless of whether the cell-free system is prepared from tetracycline-resistant or tetracycline-sensitive cells.

3. Cell Free Protein Synthesis by S-30 Fraction from Vibrogen Mill Broken Cells of Acholeplasma laidlawii B

a) Requirements for Incorporation of Phenylalanine into Protein

An incubation mixture consisting of dialyzed 105,000 x g supernatant fluid and ribosomes required ATP and an ATP generating system for incorporation of ^{14}C-phenylalanine into protein (Table 3, p. 54). Omission of GTP resulted in about 65% reduction in ^{14}C-phenylalanine incorporation into protein. The 105,000 x g supernatant fluid and ribosomes were necessary for complete activity of the system.

b) Response To Polyuridylic Acid

Increasing concentrations of polyuridylic acid effect an increase in ^{14}C-phenylalanine incorporation into protein (Figure 3, p. 60). This phenomenon could not be shown for French press broken cells of Acholeplasma laidlawii B.

c) Time Course of Phenylalanine Incorporation Into Protein

Incorporation of ^{14}C-phenylalanine increases for 30 minutes and then stops (Figure 4, p. 62). Increase in ^{14}C-phenylalanine incorporation into protein was much more rapid in the S-30 fraction from Vibrogen mill broken cells than that found using the French press broken cells.

d) Effect of pH on the Incorporation of Phenylalanine Into Protein

Polyuridylic acid directed incorporation of ^{14}C-phenylalanine into protein was sensitive to changes of pH in the incubation buffer (Figure 5, p. 64). An incubation buffer of pH 7.4 gave the maximum incorporation while buffers of higher pH caused a sharp decrease in phenylalanine incorporation into protein.

e) Effect of Magnesium Ion Concentration on the Incorporation of Phenylalanine Into Protein

Both high and low concentrations of Mg^{++} ion cause a decrease in C^{14}-phenylalanine incorporation into protein. Maximum stimulation of phenylalanine into protein as shown in Figure 6 (p. 66) requires a magnesium ion concentration of at least 18 μM.

4. Inhibition of Cell Free Protein Synthesis by S-30 Fraction from Vibrogen Mill Broken Cells by Tetracycline

Tetracycline inhibits the incorporation of C^{14}-phenylalanine in cell free systems from both tetracycline sensitive and resistant Acholeplasma laidlawii B (Figure 7, p. 68). 6.2×10^{-6} M (3 mcg/ml) concentration of tetracycline inhibits incorporation of phenylalanine more than 20% in each preparation.

As previously stated in the section dealing with protein synthesis in Mycoplasma (p. 33), the only previous report of cell-free protein synthesis in the Mycoplasmas is that by Tourtellotte, et al. (144) who used Acholeplasma laidlawii B. They noted that for incorporation of C^{14}-phenylalanine into protein, the Acholeplasma laidlawii B system required ATP and an ATP-generating system, ribosomes, and the 105,000 x g supernatant fraction. Since the cell-free system was undialyzed (144), a requirement for

guanosine triphosphate, essential in other systems (184, 185), could not be demonstrated. In addition, the total amount of radioactive phenylalanine incorporated into protein was small (144). Furthermore, attempts to stimulate incorporation of specific amino acids in the cell-free Acholeplasma laidlawii B system with synthetic polyribonucleotides were unsuccessful (145). Although the observation that the addition of a purified cell membrane fraction to the complete Acholeplasma laidlawii B cell-free system stimulated a 20-fold increase in C^{14}-phenylalanine incorporation into protein (141), a better-defined cell-free system was needed.

The S-30 fraction of Acholeplasma laidlawii B prepared by the Vibrogen cell mill actively incorporated radioactive phenylalanine into protein. The requirements for phenylalanine incorporation of this preparation were similar to those reported for Escherichia coli (183) and Staphylococcus aureus (185):

1) ribosomes
2) 105,000 x g supernatant
3) ATP and an ATP generating system
4) magnesium ion
5) polyuridylic acid
6) guanosine triphosphate.

Two independent lines of evidence strongly suggest that resistance to the tetracycline antibiotics is due to a failure of the antibiotics to reach their site of action rather than to drug inactivation or to the biosynthesis of ribosomes resistant to inhibition by the drug (186).

1) Polypeptide synthesis of cell-free extracts prepared from either sensitive or resistant strains of bacteria was inhibited to the same extent by the tetracycline antibiotic (186).

2) Resistant strains of bacteria, when compared with sensitive strains of the same organisms, accumulated much less antibiotic from the culture medium (187).

The results of the addition of tetracycline to cell-free systems of tetracycline-resistant and tetracycline-sensitive Acholeplasma laidlawii B are summarized in Figures 2 and 7 (p. 58 and p. 68). If the ribosomes of the tetracycline-resistant strain were in some way resistant to the action of tetracycline it would be expected that phenylalanine incorporation into protein would not be inhibited. It is apparent from the data that the ribosomes regardless of origin or preparation are significantly sensitive to the action of the antibiotic in cell-free systems. Schwartz (188) concluded from his adsorption studies that this tetracycline-resistant mutant of Acholeplasma laidlawii B accumulated less tetracycline than its sensitive parent at low concentrations of the antibiotic. Although these results in themselves do not preclude the

possibility of another mechanism of resistance in other strains of Mycoplasma, these data together with previous experiments on other procaryotic cells suggest that resistance to tetracycline by this Acholeplasma laidlawii B mutant is one of impermeability of the antibiotic.

TABLE 3

Requirements for Incorporation of L-^{14}C-Phenylalanine into Protein by the S-30 Fraction from Vibrogen Mill Broken Cells of *Acholeplasma laidlawii* B

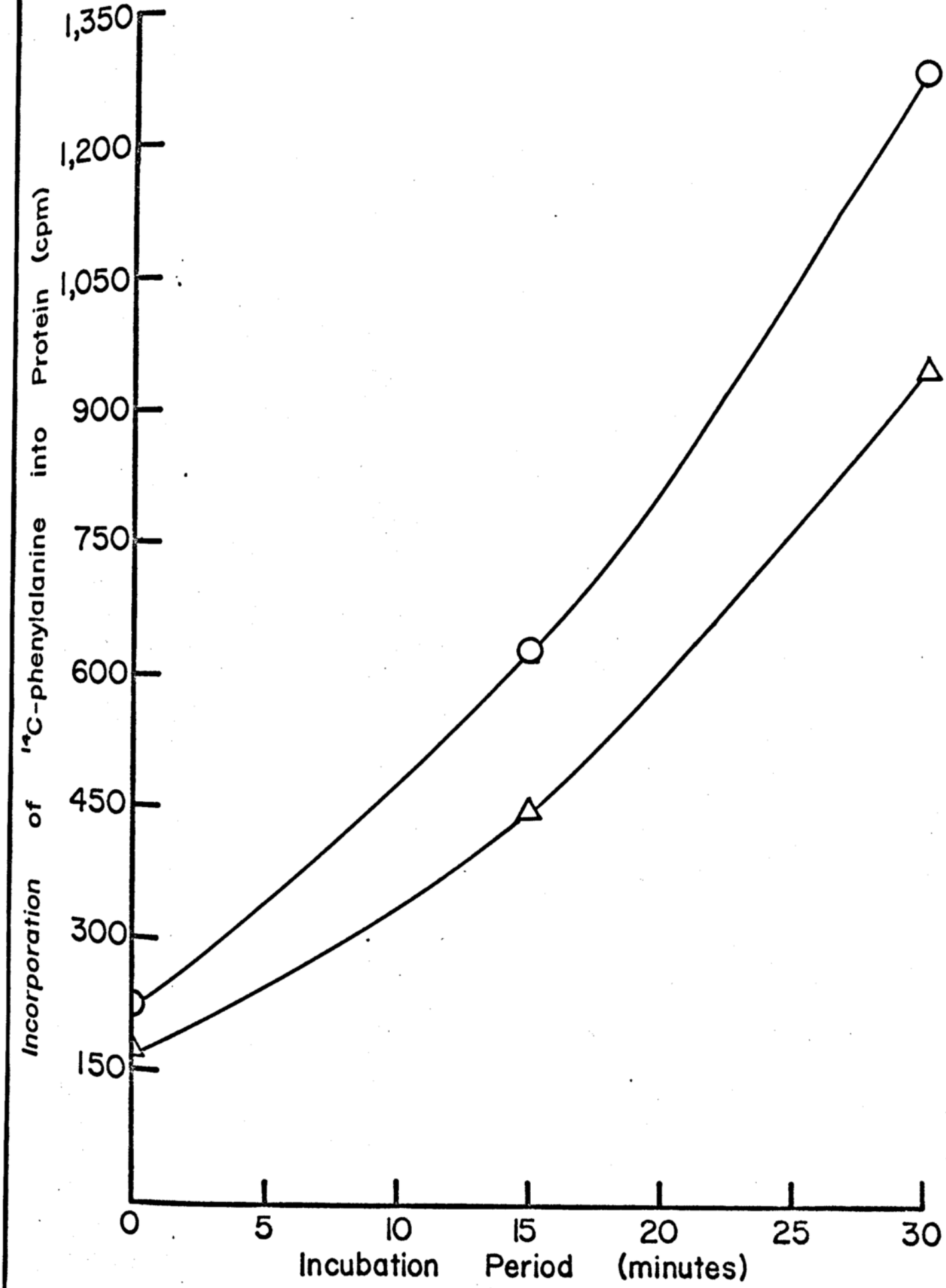
Experimental Conditions	^{14}C-Phenylalanine Incorporation cpm/mg of protein
Complete system	47,000
-105,000 x g supernatant	16,200
-ribosomes (105,000 x g ppt.)	3,150
-ATP, PEP, PEP Kinase	8,400
-GTP	17,300

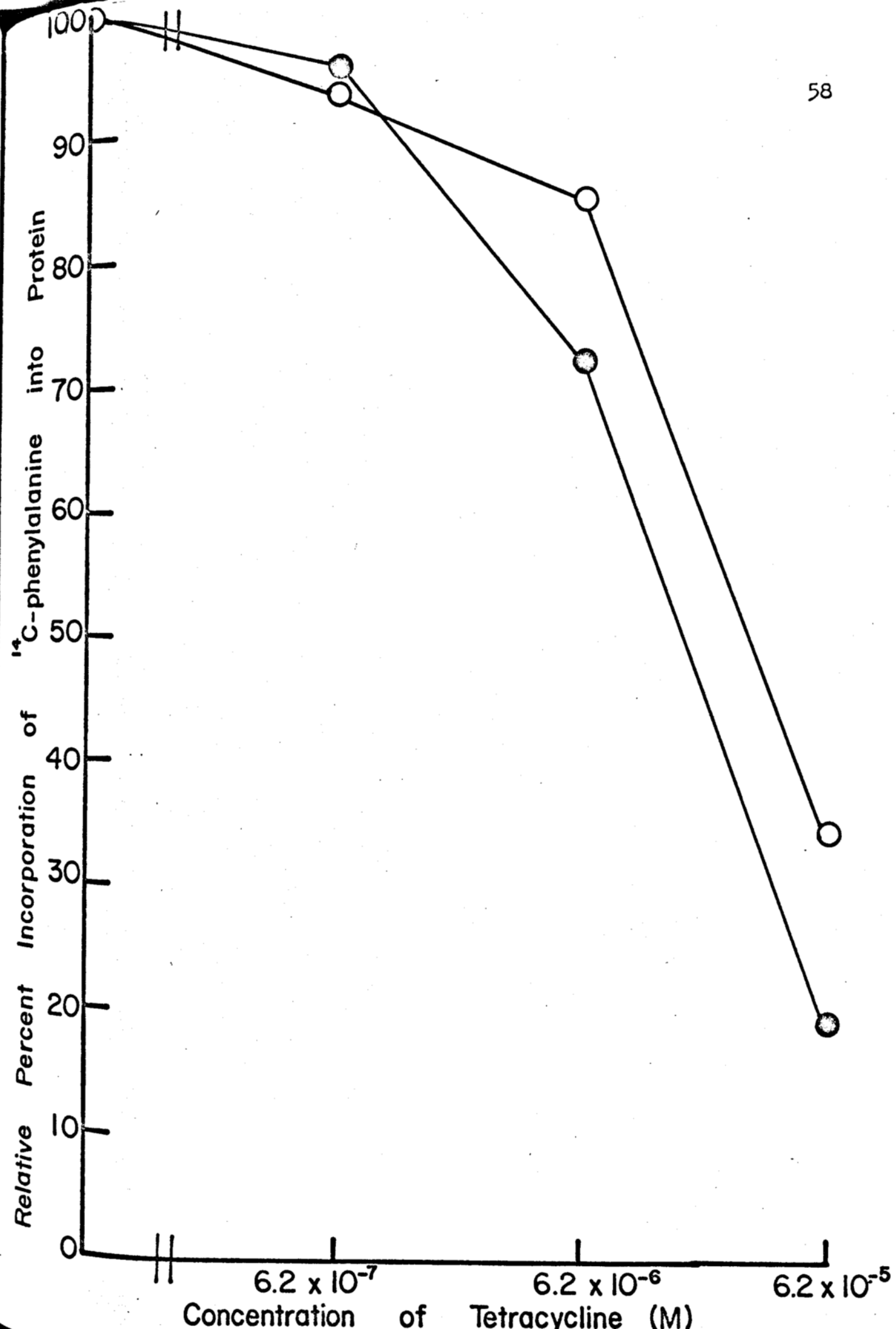
Incubation time: 30 minutes

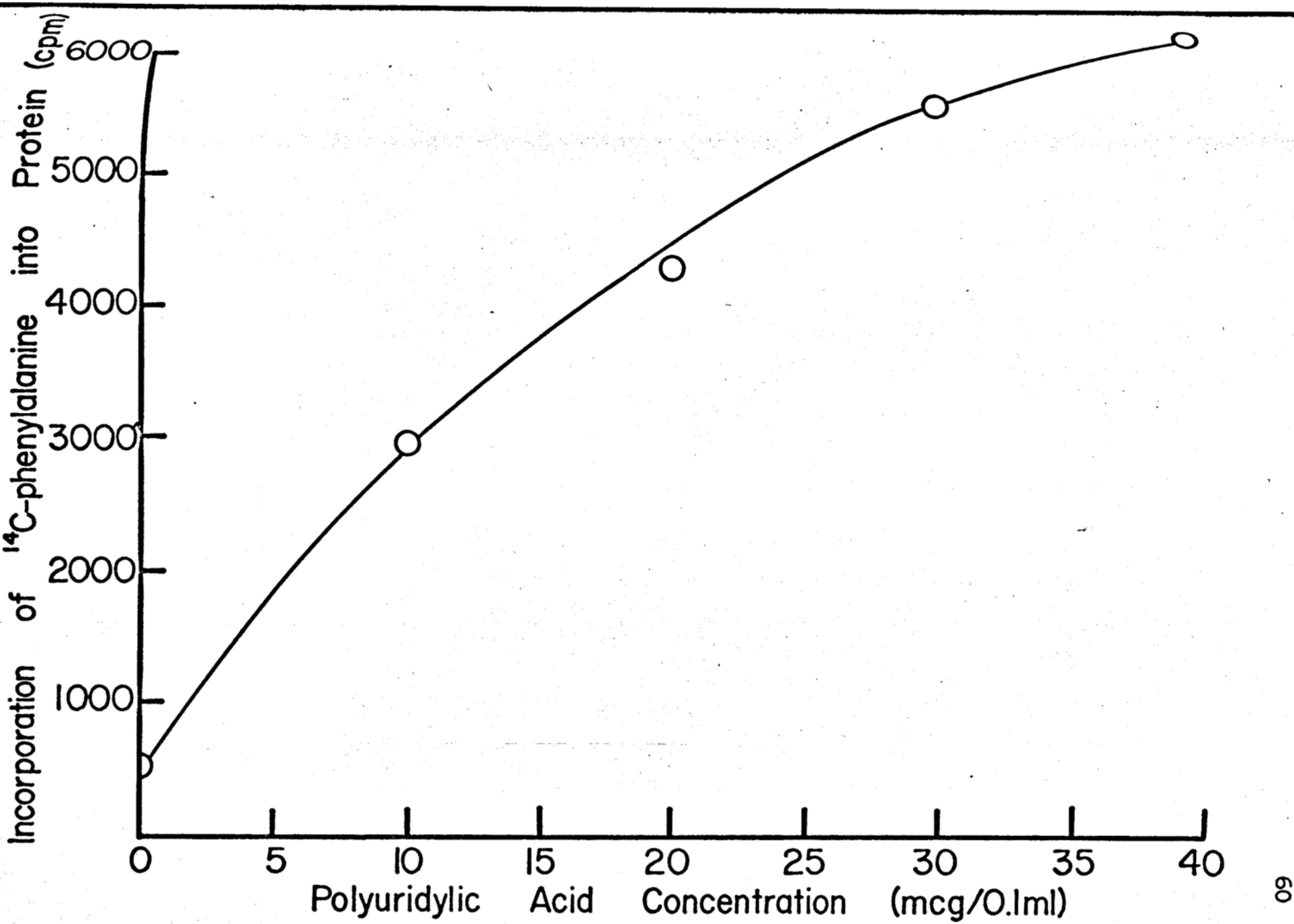
Figure 1. Incorporation of ^{14}C-phenylalanine into protein by French press broken cells of Acholeplasma laidlawii B as a function of time.

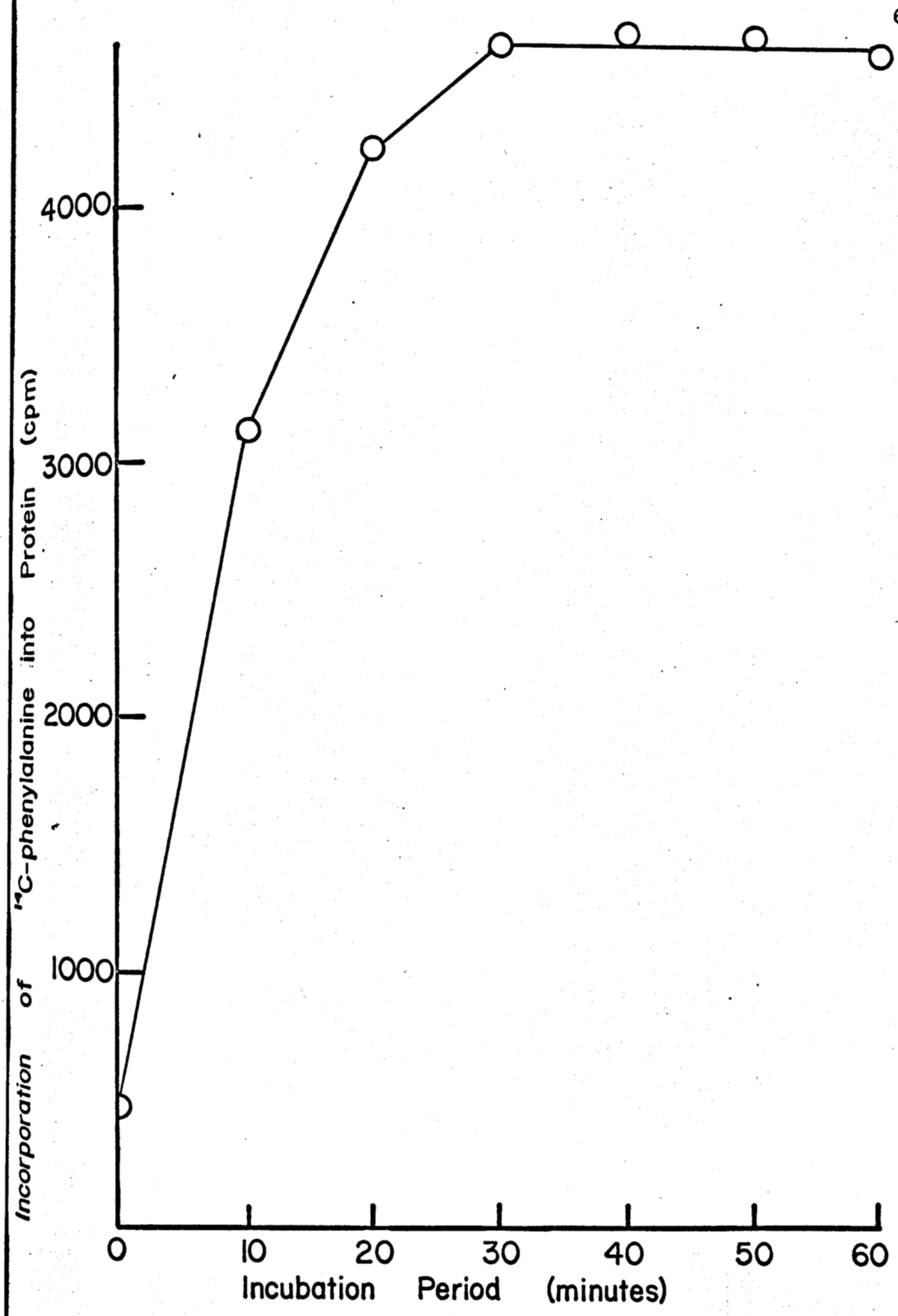
Tetracycline-sensitive cells: O

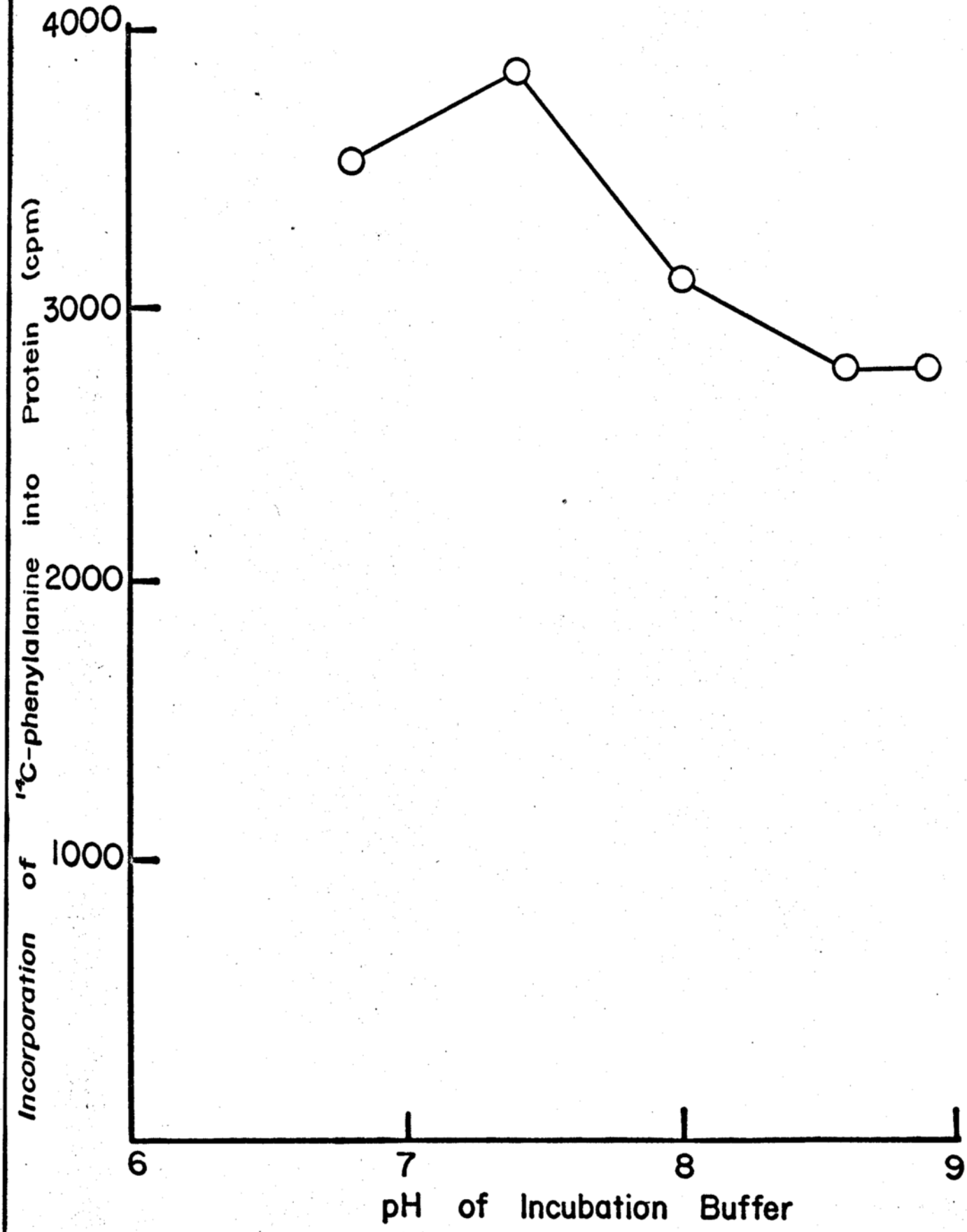
Tetracycline-resistant cells: Δ

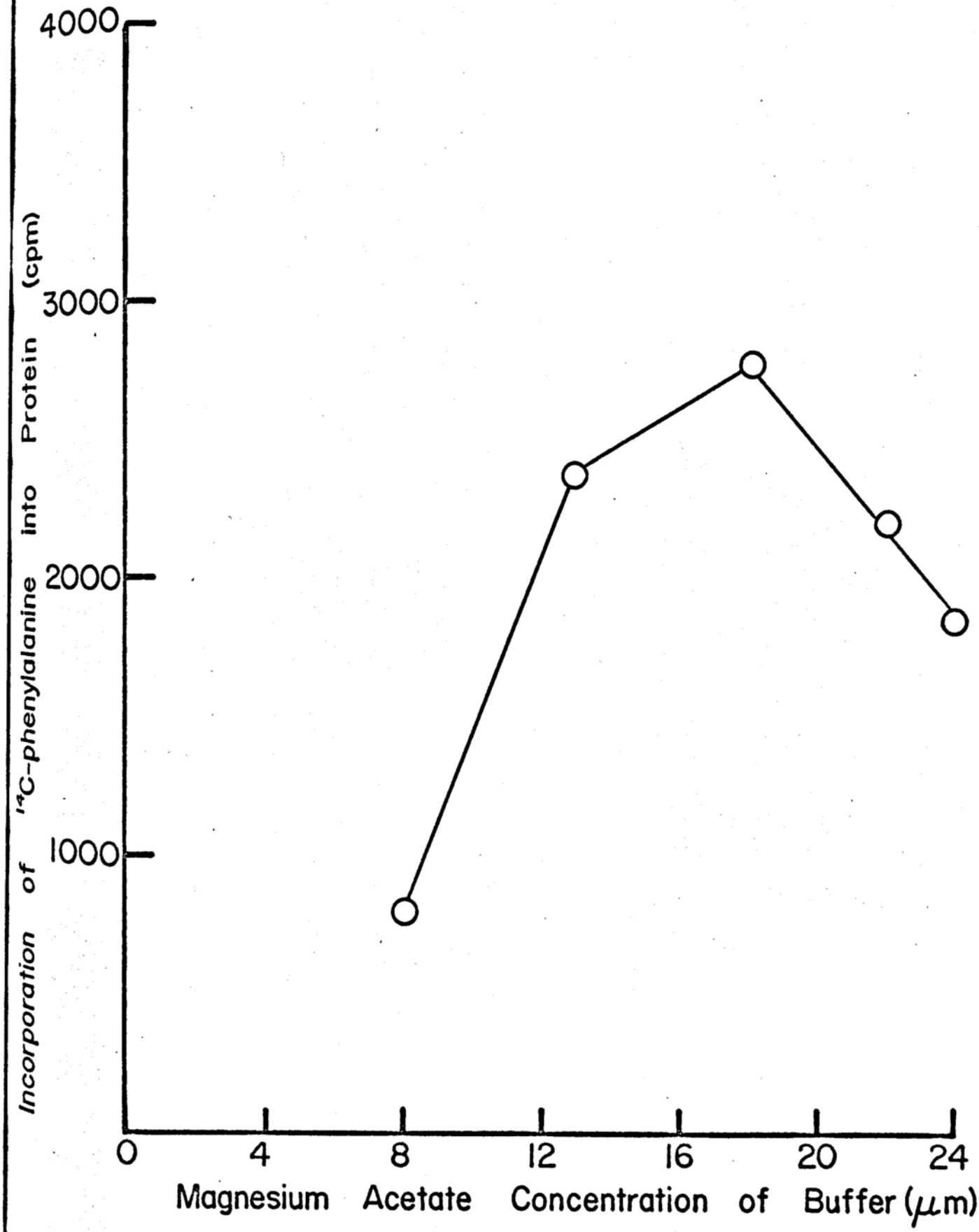

Incorporation of ^{14}C-phenylalanine into Protein (cpm)
1,350
1,200
1,050
900
750
600
450
300
150
0
5
10
15
20
25
30
Incubation Period (minutes)

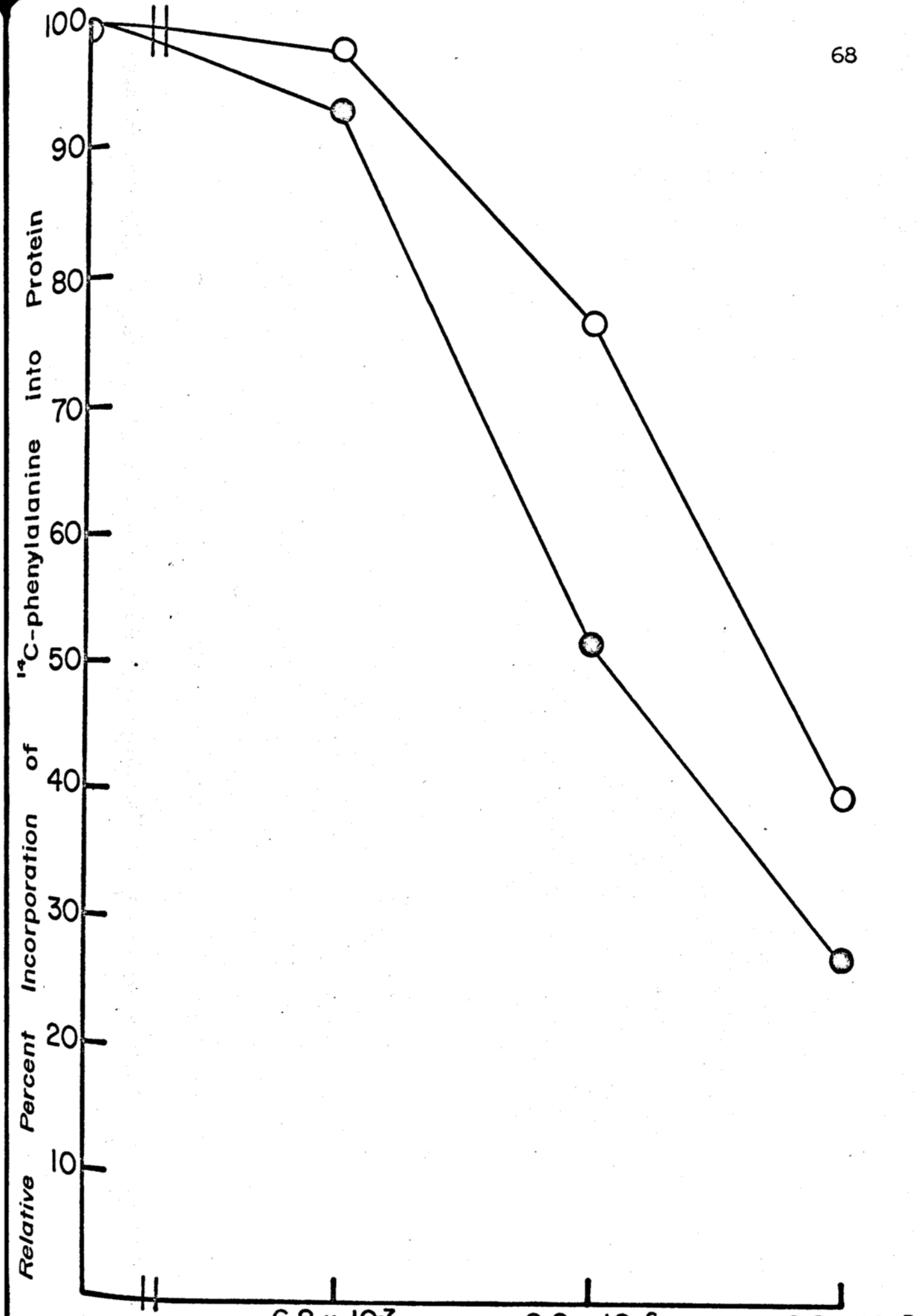

Relative Percent Incorporation of ^{14}C-phenylalanine into Protein
100
90
80
70
60
50
40
30
20
10
0
6.2 x 10^{-7}
6.2 x 10^{-6}
6.2 x 10^{-5}
Concentration of Tetracycline (M)


Incorporation of ^{14}C-phenylalanine into Protein (cpm)
6000
5000
4000
3000
2000
1000
0
5
10
15
20
25
30
35
40
Polyuridylic Acid Concentration (mcg/0.1ml)


Incorporation of ^{14}C-phenylalanine into Protein (cpm)
4000
3000
2000
1000
0
10
20
30
40
50
60
Incubation Period (minutes)


4000
3000
2000
1000
Incorporation of ^{14}C-phenylalanine into Protein (cpm)
6
7
8
9
pH of Incubation Buffer


4000
3000
2000
1000
Incorporation of ^{14}C-phenylalanine into Protein (cpm)
0
4
8
12
16
20
24
Magnesium Acetate Concentration of Buffer (μm)


Relative Percent Incorporation of ^{14}C-phenylalanine into Protein
100
90
80
70
60
50
40
30
20
10
6.2 x 10^{-7}
6.2 x 10^{-6}
6.2 x 10^{-5}

PART II

CHARACTERISTICS OF ANTIMICROBIAL AGENTS PRODUCED BY ACHOLEPLASMA LAIDLAWII B (TETRACYCLINE RESISTANT)

A. Materials and Methods

1. Materials

The following chemicals, biochemicals and their sources were used in these studies:

Uridine-5-^{3}H, 25 C/mM and thymidine-methyl-^{3}H, 10 C/mM (New England Nuclear Corp.) were gifts from Prof. B. Weisblum (University of Wisconsin). L-Phenylalanine-U-^{14}C, 374 mC/mM was obtained from New England Nuclear Corp. L-Leucine-U-^{14}C, 216 mC/mM was obtained from CalAtomic. Adenosine triphosphate, guanosine triphosphate, phosphoenolpyruvate, phosphoenolpyruvate kinase, Tris buffer, and polyuridylic acid were all purchased from Sigma Chemical Company. t-Ribonucleic acid (as sodium salt) was obtained from General Biochemical Inc. Dithiothreitol and β-mercaptoethanol were obtained from Calbiochem. L-Phenylalanine was purchased from Mann Research Laboratories. Rifampin (Lepetit, Milano, Italy) and Novobiocin (Upjohn Co., Kalamazoo, Mich.) were both gifts from Prof. B. Weisblum.

Acholeplasma Cultures. A culture of Acholeplasma laidlawii B was obtained from Prof. H. E. Morton (University of Pennsylvania) and was maintained on PPLO agar supplemented

with PPLO serum fraction which were obtained from the Difco Company. A mutant of this culture resistant to 50 mcg/ml tetracycline was obtained by repeated exposure of the parent (which is sensitive to 2 mcg/ml tetracycline). This mutant has maintained its resistance when transferred on media containing 5 mcg/ml tetracycline. Both cultures were transferred weekly by the agar block method (181).

Test Organisms. Organisms used to assay for the production of the antimicrobial agents produced by Acholeplasma laidlawii B (tetracycline resistant) were Sarcina lutea, Bacillus subtilis Marburg, Bacillus megaterium, Staphylococcus aureus 209, Saccharomyces cerevisiae L-24 (all obtained from the Department of Bacteriology, University of Wisconsin), Staphylococcus aureus 1206 (antibiotic resistant) was obtained from Prof. B. Weisblum (University of Wisconsin), and Escherichia coli B (obtained from Squibb Institute for Medical Research).

Media. Bacto® PPLO broth w/o crystal violet, supplemented with 0.5% Bacto® yeast extract, 0.1% sodium acetate, and 0.1%, 2.5% calf serum or 2.5% swine serum (Flow Laboratories), was used for broth culture of Acholeplasma laidlawii B (tetracycline resistant). Bacterial cultures were maintained on Bacto® nutrient agar slants, and Bacto® nutrient broth was used for preparation of liquid cultures. Sensitivity of the

bacterial cultures to the antimicrobial agents were assayed using Bacto® Penassay seed agar except for Saccharomyces cerevisiae which was grown on Bacto® Sabouraud Dextrose Agar. Antibiotic Medium 3 (Difco Company) was used in experiments studying the effect of antibiotics on the growth of Bacillus subtilis Marburg or Bacillus megaterium in liquid medium.

2. Production of Antimicrobial Substances by Acholeplasma laidlawii B (Tetracycline Resistant)

Inoculum for fermentations were prepared by transferring a 1 x 1 cm agar block containing colonies of the tetracycline-resistant mutant of Acholeplasma laidlawii B to a sterile cotton stoppered 0.5 x 6 inch test tube containing 10 ml of sterile PPLO broth. The tube was incubated for 3 days at 37°C. 0.5 ml of this broth was used to inoculate 500 ml of media in 1 L. cotton stoppered flasks or 0.1 ml of the broth was used to inoculate 100 ml of media in 250 ml cotton stoppered flasks. The flasks were incubated at 37°C without aeration. At approximately 24-hour intervals one of a series of replicate flasks was removed and a 1 ml sample was used for colony counts. Acholeplasma laidlawii B colony counts were performed according to Smith (181). The procedure consists of diluting samples in PPLO broth and spotting 0.01 ml of the dilution on PPLO agar supplemented with serum fraction.

After 3 days the colonies are counted with the aid of a microscope (15X magnification). The remainder of the fermentation was lyophilized and stored at -20°C in wide mouth 2 oz. jars.

3. Assay for Antimicrobial Agent Production from Acholeplasma laidlawii B (Tetracycline Resistant)

Bacillus subtilis Marburg spores were used for preliminary assay of antimicrobial activity in fermentations from Acholeplasma laidlawii B (tetracycline resistant). The spore suspension was prepared by incubating 100 ml of nutrient broth inoculated from a slant of Bacillus subtilis at 35°C on a shaker for 7 days. The cells and spores were then centrifuged and resuspended in 30 ml of 0.9% sodium chloride. The cells and spores were heated for 30 minutes at 70°C in a water bath. The suspension contained 1.0×10^8 spores/ml. The spore suspension was stored at 4°C when not in use for antibiotic assays.

Bacto® Penassay seed agar was inoculated with 1×10^6 spores/ml of Bacillus subtilis Marburg and 10 ml of this agar was pipetted into 100 x 15 mm sterile plastic Petri dishes. When the agar solidified, 13 mm wells were formed in the agar using a cork borer. The lyophilized powder from the fermentations (mentioned above) was reconstituted with deionized water to give a 20 x concentrated solution.

Duplicate agar wells were then filled with liquid sample (approximately 0.2 ml) and incubated at room temperature, e.g., 25°C for 16 hours. Active samples gave clear zones around the circular wells.

4. Preparation of Bioautographs for Paper Ionophoresis and Paper Chromatographic Studies

150 ml of Penassay® agar at 55°C (pH 7.0) was inoculated with Bacillus subtilis Marburg spores (1 x 10^6/ml). The liquid agar was poured into a 3 quart Pyrex® baking dish and cooled. The paper sheets from ionophoresis or paper chromatography were then placed on the agar surface for 15 minutes. After removing the sheets, the plates were incubated at room temperature, e.g., 25°C, overnight.

5. Determination of Antibiotic Spectrum

Test organisms were grown overnight in nutrient broth on a shaker (150 rpm) at 35°C. 0.1 ml of each culture was added to 50 ml of Penassay® agar (pH 7.0) except for Saccharomyces cerevisiae L-24 which was added to Bacto® Sabouraud dextrose agar. 10 ml of the inoculated agar was then pipetted into 100 x 15 mm sterile plastic Petri dishes. When the agar had solidified, 13 mm wells were formed with a cork borer. The wells were then filled with liquid sample and the plates incubated overnight at room temperature, e.g., 25°C, for 16 hours. Cytotoxicity-

screening of mammalian cells was also performed using the differential agar-diffusion bioassay of Perlman, et al. (189).

6. Bioassay Dose-Response Curve of Antibiotics from Acholeplasma laidlawii B (Tetracycline Resistant)

Agar plates were prepared as stated earlier in the method for assay of antibiotic samples. The 20 x concentrated sample was diluted 1:1, 1:4, and 1:8 with deionized water. The samples were then added to the agar wells and the Petri dishes incubated at room temperature overnight.

7. Effect of pH on Sensitivity of Bacillus subtilis to Antibiotics Produced from Acholeplasma laidlawii B (Tetracycline Resistant)

Penassay® agar was adjusted to pH 6, 7 and 8 with 2 N HCl or 2 N NaOH after autoclaving. The agar was inoculated with spores of Bacillus subtilis Marburg (1×10^6/ml) and 10 ml pipetted into sterile plastic Petri dishes. 13 mm wells were formed in the agar when cool. 0.5 ml of samples of the antibiotic were adjusted to pH 6, 7 and 8 using 1 N HCl or 1 N NaOH. 0.2 ml of each sample was added to the corresponding agar at that pH value and the plates were incubated overnight at room temperature.

8. Determination of Heat Stability

0.5 ml of antibiotic samples were pipetted into 10 x 75 mm test tubes. 1 N NaOH or 1 N HCl was added to adjust the pH to 2, 5, 7 or 9. The test tubes were then covered with Parafilm® and placed in a boiling water bath for 15 minutes. The tubes were cooled and adjusted to pH 7 with 1 N NAOH or HCl. The samples were then added to agar wells as previously described in the methods for assay of antimicrobial agent production.

9. Determination of MIC Using Bacillus subtilis Marburg

A 24-hour culture of Bacillus subtilis Marburg grown in Antibiotic Medium 3 (Difco) at 30°C was diluted 1000 x in fresh Antibiotic Medium 3 (Difco). 0.5 ml of this inoculated medium was pipetted into sterile cotton stoppered 10 x 100 mm test tubes. 0.5 ml of antibiotic solution was then added into the first tube. The contents of this tube were mixed and 0.5 ml was transferred to the next tube. This procedure was repeated for the next 8 tubes. The tubes were incubated at 30°C in a water bath without shaking and were visually checked for growth at 16, 24, and 42 hours by stirring the contents of each tube and noting turbidity.

10. Study of Mechanism of Inhibition of Bacillus subtilis Marburg Growth by Acholeplasma laidlawii B Antibiotics

Inoculated media was prepared as in the determination of MIC. 3 ml of the media (6.5×10^7 cfu/ml) was pipetted into sterile cotton stoppered 10 x 100 mm test tubes. Antibiotic was added and the tubes were incubated in a 30°C water bath without shaking. Samples were taken periodically and diluted in Antibiotic Medium 3 (Difco). 0.1 ml samples from the dilution tubes were spotted on 100 x 15 mm sterile plastic Petri dishes containing 10 ml of Penassay® agar and spread out with a glass rod. The plates were incubated at 37°C overnight and colonies counted the next day.

11. Study of Protein Synthesis Inhibition by Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

a) Inhibition of L-Leucine-U-^{14}C, Uridine-5-^{3}H and Thymidine-methyl-^{3}H Incorporation by Whole Cells of Bacillus megaterium

4.5 ml of Antibiotic Medium 3 (Difco) in each of two cotton stoppered 25 ml Erlenmeyer flasks was inoculated with 0.5 ml of a 12-hour culture of Bacillus megaterium. The flasks were shaken at 37°C in a water bath for 1.5 hours before the addition of 1 µC of ^{14}C-L-leucine,

5 μC of uridine-5-^{3}H or 5 μC of thymidine-methyl-^{3}H. At periodic intervals before and after the addition of various antibiotics, 0.5 ml samples were withdrawn, filtered through Millipore® filters and washed with 5 ml of fresh Antibiotic Medium 3 (Difco). The filters were dried and counted in 10 ml of Bray's solution (182) using a Packard Tricarb Liquid Scintillation Counter (model 2002).

b) Preparation of S-30 Fraction from Escherichia coli B

4 gm (wet weight) of Escherichia coli B cells in log phase were washed with 0.01 M pH 7.6 Tris buffer containing 0.01 M $Mg(Cl)_2$ and 0.9% NaCl. The subsequent steps were all performed at 4°C. 8 gm of levigated alumina (Alundum®) was slowly added to the cells in a porcelain mortar and ground for 15 minutes. 5 ml of 0.01 M pH 7.8 Tris buffer containing 0.01 M, $Mg(C_2H_3O_2)_2$, 0.06 M, KCl and 0.006 M, β-mercaptoethanol was added to the broken cells and alumina to form a suspension. The alumina, cell walls, membranes and unbroken cells were then removed by centrifugation. The supernatant (S-30 fraction) was stored in small quantities at -20°C.

c) Inhibition of ^{14}C-Phenylalanine Incorporation into Protein by the S-30 Fraction from Escherichia coli B by Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

The incubation mixture for L-phenylalanine-U-^{14}C incorporation contained: Tris buffer, pH 7.6, 50 μM; $Mg(C_2H_3O_2)_2$, 13 μM; NH_4Cl, 50 μM; dithiothreitol, 0.1 μM; adenosine triphosphate, 1 μM; guanosine triphosphate, 0.5 μM; phosphoenolpyruvate, 5 μM; phosphoenolpyruvate kinase, 2 μg; polyuridylic acid, 10 μg; tRNA (from Escherichia coli B), 0.1 mg; L-phenylalanine-U-^{14}C (sp. act. 187 μC/uM), 0.1 μC; L-phenylalanine, 26 μM; S-30 fraction, 1.5 mg; antibiotic produced by Acholeplasma laidlawii B, equivalent to 0.01, 0.1 or 1.0 units/ml; total volume, 0.1 ml. The reaction mixtures were incubated for 30 minutes at 37°C and then chilled in ice water. The samples were then treated as previously described in section 8 of the materials and methods of Part I (p. 46).

12. Isolation of a Bacillus subtilis Marburg Mutant Resistant to Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

Ten colonies of Bacillus subtilis Marburg which were growing inside a zone of inhibition produced by the antimicrobial substances from Acholeplasma laidlawii B on an agar plate, were selected and inoculated into sterile

cotton stoppered 10 x 100 mm test tubes containing 1 ml of Antibiotic Medium 3 (Difco) and 0.1 units of the antibiotic. The tubes were incubated for 16 hours at 30°C. Those tubes showing growth were transferred to fresh media containing twice the concentration of antibiotic than the previous tubes. This procedure was repeated until a Bacillus subtilis Marburg mutant resistant to 25 x the MIC was isolated.

Cross resistance of this organism to other known antibiotics was studied by following the same procedure as that in determination of antibiotic spectrum (p. 73), except antibiotic solutions to be tested were applied to the agar surface by means of 6.35 mm filter paper discs.

13. Recovery and Purification of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

a) Adsorption of Antibiotics on Activated Charcoal

One ml of 20 x concentrated sample was mixed with equal volumes of 95% ethanol. The solution was cooled in an ice bath for 30 minutes and then centrifuged to remove the whole cells and precipitated material. The supernatant solution was evaporated to dryness in vacuo (30°C) and dissolved in 1 ml of deionized water. The 1 ml of solution was adjusted to pH 5 or 9 using 1 N HCl or 1 N

NaOH. 50 mg of activated charcoal (Norite A) was then added, and the mixture agitated on a Vortex-Genie for 10 minutes. The charcoal was removed by filtration using a Millipore® filter and the filtrate collected together with 3 ml of 50% ethanol containing 0.3 N NH_4OH, or 3 ml of 50% ethanol adjusted to pH 2 with 1 N HCl, respectively. The fractions were evaporated to dryness in vacuo (30°C) and dissolved in 1 ml of deionized water. The pH of all samples was adjusted to pH 7 and the solutions tested as described above in the method for assay of antimicrobial substances.

b) Adsorption on Ion Exchange Resins

The strong cationic exchange resin (Dowex 50 X2) was regenerated by first washing with 1 N NaOH, followed by water. When the pH of the filtrate was neutral, 1 N HCl was passed through the column and followed by water until the filtrate was neutral. Strong anionic exchange resins (Dowex 1 X2, Amberlite CG-400) were regenerated by washing with 1 N HCl, followed by water. When the pH of the filtrate was neutral 1 N NaOH was passed through the column followed by water until the filtrate was neutral pH.

One ml of filtrate from the pH 5 charcoal treatment was placed on an Amberlite CG-400 column (OH^- form) of 2 ml retention volume. The column was washed with 10 ml of water, 10 ml of 0.8 N acetic acid and 10 ml of 1 N HCl

sequentially. Each fraction was evaporated to dryness *in vacuo* (30°C) and the solids dissolved in 1 ml of deionized water and adjusted to pH 7. The bioassay of each sample was performed as described in the methods for assay of antimicrobial substances.

One ml of the acidic eluate from the pH 9 charcoal treatment was placed on a Dowex 50 X2 column (H^+ form) of 2 ml retention volume. The column was washed with 10 ml of water, 10 ml of 0.5 N NH_4OH and 10 ml of 1 N NH_4OH. sequentially. Each fraction was evaporated to dryness *in vacuo* (30°C) and the solids dissolved in 1 ml of deionized water and adjusted to pH 7. The bioassay of each sample was performed as described in the methods for assay of antimicrobial substances.

c) Large-Scale Purification Method for Antibiotics from *Acholeplasma laidlawii* B (Tetracycline Resistant)

15 ml of 20 x concentrated sample were mixed with 15 ml of 95% ethanol. The suspension was cooled in an ice bath for 30 minutes and then centrifuged to remove whole cells and precipitated material. The supernatant solution was evaporated *in vacuo* and dissolved in 15 ml of deionized water. This solution (pH 7.0) was placed on a Dowex 50 X2 column (H^+ form) of 30 ml retention volume. The resin was washed with 150 ml of deionized water and then eluted with 150 ml 1 N NH_4OH. Each sample was

lyophilized and the solid material dissolved in 15 ml of deionized water. After the samples were adjusted to pH 7, bioactivity was tested as described above in the method for assay of antimicrobial substances.

The active fraction was then adjusted to pH 9 with 6 N NaOH and placed on a Dowex 1 X2 column (OH^- form) of 30 ml retention volume. The resin was washed with 150 ml of deionized water and eluted with 150 ml of 0.8 N acetic acid. Each sample was lyophilized and dissolved in 5 ml of deionized water. After the fractions were adjusted to pH 7, bioactivity was tested as described above in the method for assay of antimicrobial substances. Yield of antibiotic from each column in units/ml was calculated by referring back to a standard curve of the 20 x concentrated sample.

14. Ionophoresis of Antibiotics of Acholeplasma laidlawii B (Tetracycline Resistant)

Whatman #1 paper sheets (56.5 cm x 11 cm) were spotted with active compound. The paper was then moistened with either pyridine-acetic acid-water buffer (pH 6.4) or acetic acid-formic acid-water buffer (pH 1.9). The paper sheets were then placed in Savant electrophoresis cells and subjected to 19 V/cm for 1 hour. After the sheets were air dried, bioautographs were prepared as described above.

15. Paper Chromatographic Properties of Antibiotics from Acholeplasma laidlawii B (Tetracycline Resistant)

Whatman #1 paper strips (16 cm x 2.5 cm) were spotted with active compound and suspended from rubber stoppers fitted into 20 x 40 cm test tubes. The paper was allowed to equilibrate for one hour over the 20 ml of solvent before ascending development. The strips were air dried and bioautographed as described above.

Descending paper chromatography was also performed using Whatman #1 paper. A 50 cm x 9 cm strip was spotted with active compound and developed by descending chromatography with n-butanol:ethanol:water (2:1:1). The paper was air dried and bioautographed as described above.

B. Results and Discussion

1. Relationship of Growth of Acholeplasma laidlawii B (Tetracycline Resistant) to Antibiotic Production

Data collected from four active fermentations of Acholeplasma laidlawii B (tetracycline resistant) are summarized in Table 4 (p. 94-95). The results of these four fermentations can be interpreted as showing that

there is no correlation between production of the antimicrobial substance, pH of the fermentation culture media, formation of colony forming units, length of incubation period, or quantity or type of serum added. Production of the antibiotic takes place in a period of 24 hours. Antimicrobial activity has not been found in replicate flasks which have been incubated 24 hours before or 24 hours after that period in which antimicrobial activity has been found. Although short-term occurrence of antibiotic production is usually observed it is not possible at this time to exclude the possibility for the presence of inhibitors which are also formed and which reverse the activity of the antimicrobial substance in the assay system.

2. Antibiotic Spectrum

The spectrum of activity of the antibiotics produced by Acholeplasma laidlawii B is summarized in Table 5 (p. 96). The antibiotics have a Gram-positive spectrum, although they show no activity against Sarcina lutea. They are effective against both spores and vegetative cells of Bacillus subtilis Marburg, and against Staphylococcus aureus 1206 (a clinical isolate naturally resistant to tetracycline, erythromycin and penicillin G) and Staphylococcus aureus 209 (a standard strain for antibiotic assays). The antibiotics are inactive against

yeast, Gram-negative organisms, tissue culture cells and Acholeplasma laidlawii B (tetracycline sensitive).

3. Dose-Response of Bacillus subtilis Marburg to Antibiotics

A typical dose-response curve obtained in the agar diffusion bioassay is shown in Figure 8 (p. 98). The test solution was the reconstituted broth prior to ion exchange resin treatment. A twofold concentration results in a 2 mm increase in zone of inhibition diameter, e.g., a slope of 2.

4. Definition of Unit of Antimicrobial Activity

That quantity of antibiotic giving a 25 mm zone diameter in the Bacillus subtilis Marburg agar diffusion assay as described on p. 72. The 20 x concentrated broth was found to contain 1 unit/ml.

5. Determination of Mechanism of Inhibition for Bacillus subtilis Marburg

The MIC of Bacillus subtilis Marburg was found to be 0.066 units/ml under the assay conditions used. Bacteriostatic or bacteriocidal action of this antibiotic on Bacillus subtilis Marburg at its MIC was determined by adding 0.066 units/ml of the antibiotic to static cultures of the organism and carrying out viable counts over a

12-hour incubation period. Data collected in this study are summarized in Figure 9 (p. 100). At its MIC this antibiotic acts bacteriostatically on Bacillus subtilis Marburg since the organism is still viable after 3 hours incubation with the antibiotic and continues to reproduce at increasing rates with time.

6. Effect of pH on Sensitivity of Bacillus subtilis Marburg to the Antibiotics

A significant increase in zone of inhibition size is noted when the antibiotic solution is adjusted to pH 8. This effect is also seen for other basic antibiotics such as the aminoglycosides (190).

7. Effect of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant) on Incorporation of L-Leucine-U-^{14}C, Uridine-5-^{3}H or Thymidine-Methyl-^{3}H by Whole Cells of Bacillus megaterium

After the addition of antibiotic to actively growing cells of Bacillus megaterium a significant inhibition in the incorporation of ^{14}C-leucine was noted compared to the control (Table 6, p.101). ^{14}C-leucine incorporation was inhibited within 5 minutes after addition of the antibiotic (Figure 10, p.103). Although the antibiotic also affects the incorporation of uridine-5-^{3}H and thymidine-methyl-^{3}H, the overall inhibition of these

nucleotides proceeds at a slower rate (Tables 7 and 8, p. 104 and 105). The delayed effect of the antibiotic on uracil and thymidine metabolism (Figures 11 and 12, p. 107 and 109) is due to a direct inhibition of protein synthesis and therefore a decreased need for biosynthesis of new DNA and RNA.

8. Effects of Known Inhibitors on the Incorporation of Uridine-5-^{3}H and Thymidine-Methyl-^{3}H by Whole Cells of Bacillus megaterium

Novobiocin inhibits DNA synthesis (191) while rifampin inhibits RNA synthesis (192). Upon the addition of each, there is a significant decrease in the incorporation of thymidine-methyl-^{3}H and uridine-5-^{3}H (Figures 13 and 14, p. 111 and 113) by whole cells of Bacillus megaterium. Each of these antibiotics selectively inhibits the incorporation of that nucleotide which is involved with its mechanism of action.

These data (Figures 13 and 14) together with Figures 10, 11, and 12 represent collectively a series of experiments which show three possible mechanisms of action for three different antibiotics. Although each experimental result does not in itself conclusively define the antibiotic's mechanism of action, they do reveal certain differences with respect to the nature of the inhibition.

In each case further experimental evidence would be required to establish the exact nature of the inhibition.

9. Effect of Antibiotics from Acholeplasma laidlawii B (Tetracycline Resistant) on the Incorporation of L-Phenylalanine-U-^{14}C into Protein by a Cell-free System from Escherichia coli B Using Polyuridylic Acid as Messenger

Antibiotics produced by Acholeplasma laidlawii B (tetracycline resistant) inhibit the incorporation of L-phenylalanine-U-^{14}C into protein by a cell-free system from Escherichia coli B. Data from Figure 15 (p. 115) concurs with the results of the L-leucine-U-^{14}C incorporation study (Figure 10, p. 103). Although the exact site of protein inhibition is not known, the primary mechanism of action of these antibiotics produced by Acholeplasma laidlawii B is inhibition of protein synthesis.

10. Cross Resistance Studies to Known Antibiotics Using a Mutant of Bacillus subtilis Marburg Resistant to Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

A mutant of Bacillus subtilis Marburg resistant to antibiotics produced by Acholeplasma laidlawii B (tetracycline resistant) was studied for resistance to a number of known antibiotics representing certain structural families. The mutant was not resistant to: ampicillin; tetracycline; streptomycin; chloramphenicol; erythromycin;

neomycin; novobiocin; capreomycin; mikamycin; lincomycin; and staphylomycin (Table 9, p. 116).

11. Chemical Properties of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant)

a) Stability of Antibiotics to Heat

The antibiotics produced by Acholeplasma laidlawii B (tetracycline resistant) were stable in both acid and base when heated in boiling water (Table 10, p. 117). The active substances were also stable in freezing and thawing.

b) Adsorption of Antibiotics on Activated Charcoal

The 20 x concentrated solution of antibiotic was adsorbed by activated charcoal at pH 9. It was eluted using 50% ethanol adjusted to pH 2. Antibiotics were not adsorbed onto the charcoal at pH 5.

c) Adsorption on Ion Exchange Resins

The 20 x concentrated solution of antibiotics, adjusted to pH 7, was retained on Dowex 50 X2 (H^+ form), but not on Amberlite CG-400 (OH^- form).

d) Attempts at Purification of Antibiotics from Acholeplasma laidlawii B (Tetracycline Resistant)

15 ml of the 20 x concentrated solution was passed through two ion exchange resin columns. The purification information is summarized in Table 11, p. 118, and presented in flow sheet form in Figure 16, p. 119. Application of the supernatant after alcohol precipitation to a Dowex 50 X2 resin and elution of the active material with NH_4OH represents an approximate 3 x purification. At this stage of purification the solution still had a deep yellow color. When the antibiotic solution was adjusted to pH 9 and applied to a Dowex 1 X2 resin column, bioactivity was found in the water eluate which was clear and colorless. Although the purification steps only represented an approximate 8 x purification, the absence of a number of ninhydrin positive spots as detected with ionophoresis on the 8 x purified sample appears to warrant a greater consideration of this method for purification than 8 x.

There was an approximate 41% recovery of the antibiotic after treatment on two ion exchange columns. A 59% loss of activity occurred upon treatment of the sample with Dowex 1 X2 resin. This loss was unexplainable but may be due to decomposition or a mixture of compounds in the original solution which adhered to the Dowex 1 X2 resin and were not eluted.

12. Ionophoresis of Antibiotics

Ionophoresis of the 8 x purified sample at pH 6.4 showed the presence of two active compounds using bioautography. The faster moving compound had a positive charge and moved 3.5 cm from the point of application, while the second compound also had a positive charge and moved 1 cm. At pH 1.9 only one antibiotic compound could be detected with bioautography. It had a positive charge and moved 7.5 cm from the point of application. Alanine was used as a reference standard and at pH 1.9 it moved 7.5 cm toward the cathode, at pH 6.4 it was neutral.

13. Paper Chromatography of Antibiotics

Several different solvent systems for ascending paper chromatography were tested (Table 12, p. 120). n-Butanol: ethanol:water (2:1:1) was chosen for descending paper chromatography. Three separate compounds could be distinguished. The active compounds as detected by bioautography had R_f values of 0.01, 0.15 and 0.33.

14. Conclusions

A tetracycline resistant Acholeplasma laidlawii B mutant produces a mixture of antimicrobial agents when grown in PPLO Broth (Difco) supplemented with yeast extract, sodium acetate and calf or swine serum. There is apparently no relationship of growth of the organism to antibiotic production.

The antibiotics have a Gram-positive spectrum, although they are not effective against Sarcina lutea.

The mechanism of action of these antibiotics involves inhibition of protein synthesis; the antibiotics inhibit the incorporation of ^{14}C-leucine by actively growing whole cells of Bacillus megaterium and the incorporation of ^{14}C-phenylalanine into protein by a cell-free system from Escherichia coli B. The specific site of protein inhibition is unknown. Preliminary experiments suggest that they are bacteriostatic against Bacillus subtilis Marburg at MIC. Cross resistance to a number of antibiotics known to inhibit Gram-positive bacteria could not be demonstrated.

The mixture of antibiotic compounds is water soluble. A number of criteria can be used to prove their basic character:

1) adsorption onto charcoal at pH 9
2) retained on Dowex 50 X2 at pH 7

3) not retained on Amberlite CG-400 at pH 7

4) movement toward cathode at pH 6.4 and 1.9 when subjected to ionophoresis

5) increased bioactivity at alkaline pH.

Taking advantage of the basic character of these compounds, an 8 x purification was accomplished by using two ion exchange resins. Although 59% of the bioactivity was lost during these procedures, the resulting solution was clear and colorless.

The antibiotic compounds are heat stable in acidic and alkaline solutions.

In order to eliminate the possibility that the A. laidlawii B (tetracycline resistant) culture inoculum contained sufficient residual tetracycline from the agar block transfer (page 71) to account for the antibacterial activity found in the A. laidlawii B fermentations, we compared the A. laidlawii antibiotics with tetracycline in the following systems: paper chromatography; paper ionophoresis; and antimicrobial spectrum. No similarity between the A. laidlawii B antibiotics and tetracycline was noted in any of these test systems.

TABLE 4

Relationship of Growth of Acholeplasma laidlawii B (Tetracycline Resistant) to Production of Antimicrobial Substances

A. 100 ml of fermentation volume with 2.5% calf serum containing media

Incubation Period Hours	Colony Forming Units/ml	pH	Inhibition Zone Diameter, mm
0	2.6×10^3	7.6	--
24	1.0×10^5	7.6	18
42	1.0×10^6	7.5	0
74	1.7×10^7	7.5	0
92	2.7×10^8	7.45	0
120	7.0×10^8	7.4	0
Uninoculated control	---	7.6	0

B. 100 ml fermentation volume with 2.5% swine serum containing media

Incubation Period Hours	Colony Forming Units/ml	pH	Inhibition Zone Diameter, mm
0	3.0×10^3	7.6	--
20	1.0×10^4	7.6	0
54	1.0×10^6	7.5	0
78	4.5×10^6	7.5	0
96	8.0×10^7	7.5	0
120	5.2×10^8	7.4	18
144	6.0×10^8	7.4	0
Uninoculated control	---	7.6	0

TABLE 4 - Cont.

C. 500 ml fermentation volume with 0.1% calf serum containing media

Incubation Period Hours	Colony Forming Units/ml	pH	Inhibition Zone Diameter, mm
0	1.0×10^3	7.6	--
18	4.0×10^4	7.6	0
42	1.5×10^7	7.55	0
66	4.0×10^8	7.45	18
Uninoculated control	---	7.60	0

D. 500 ml fermentation volume with 0.1% calf serum containing media

Incubation Period Hours	Colony Forming Units/ml	pH	Inhibition Zone Diameter, mm
0	9.0×10^6	7.6	--
24	1.1×10^5	7.6	0
48	8.0×10^5	7.5	17
72	5.4×10^7	7.5	0
96	1.4×10^8	7.45	0
120	1.6×10^9	7.40	0
Uninoculated control	---	7.6	0

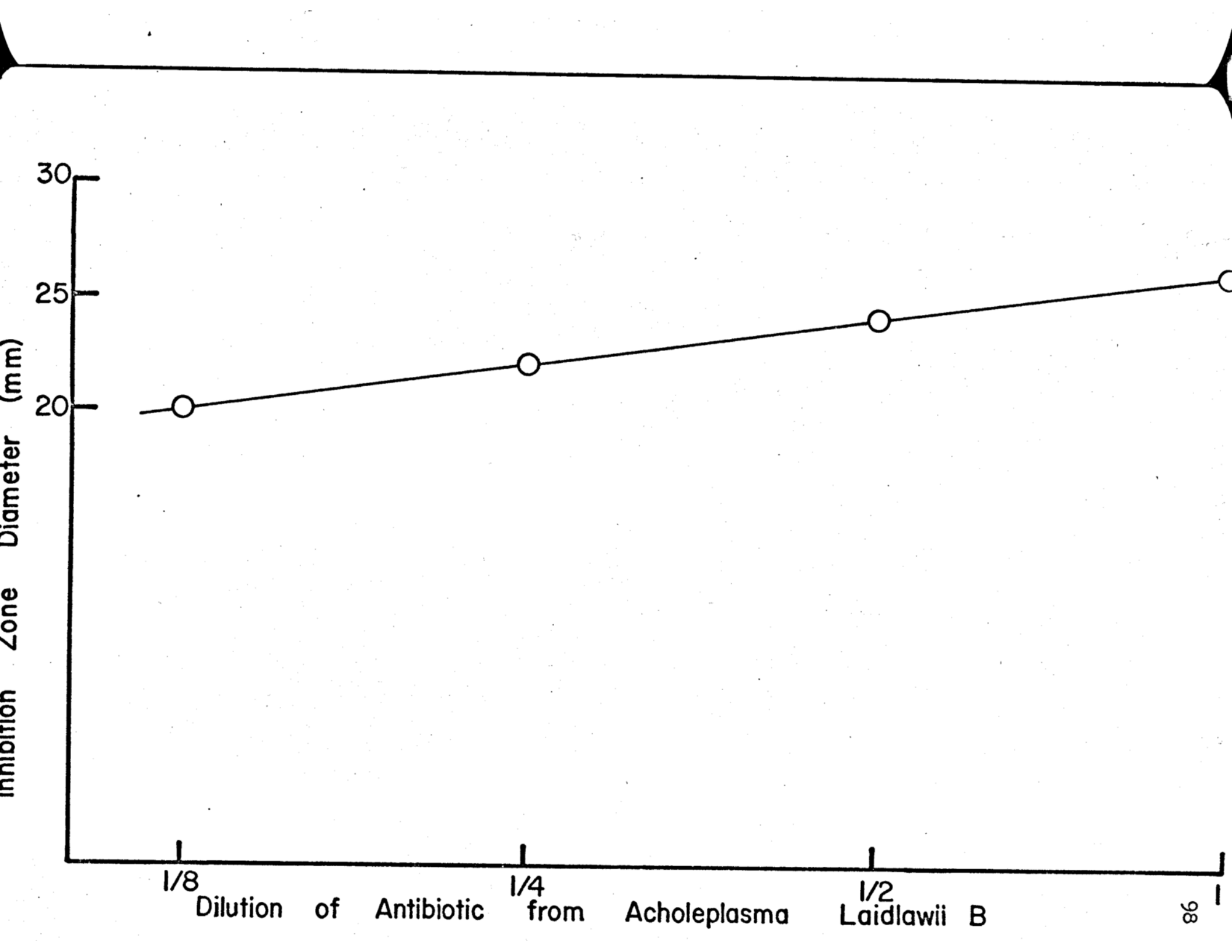
TABLE 5

Antimicrobial Spectrum* and Cytotoxicity-Screening** of *Acholeplasma laidlawii* B (Tetracycline Resistant) Antibiotics

Test Organisms and Mammalian Cells	Inhibition Zone Diameter, mm
Bacillus subtilis Marburg (spores)	25
Bacillus subtilis Marburg (vegetative cells)	22
Bacillus megaterium (vegetative cells)	25
Staphylococcus aureus 209	20
Staphylococcus aureus 1206	18
Sarcina lutea	0
Saccharomyces cerevisiae L-24	0
Escherichia coli B	0
Acholeplasma laidlawii B (tetracycline sensitive)	0
Eagle's KB Cells	0
WI-38 Cells	0
Hela Cells	0

*as determined in agar diffusion assay

**sensitivity test performed by Mr. B. Haase (189)


30
25
20
Inhibition Zone Diameter (mm)
1/8
1/4
1/2
1
Dilution of Antibiotic from Acholeplasma Laidlawii B

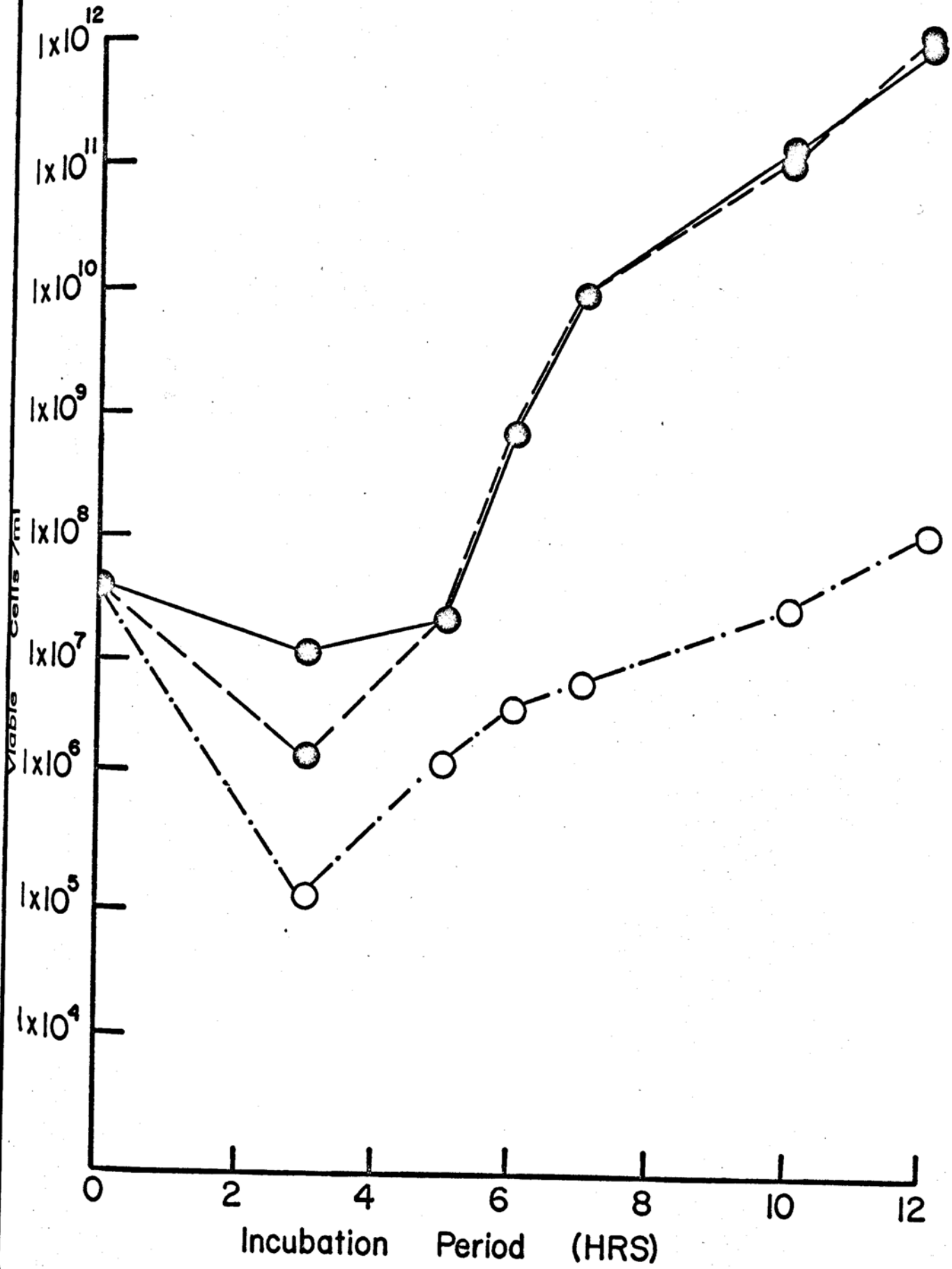

1x10^12
1x10^11
1x10^10
1x10^9
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
Viable Cells /ml
0
2
4
6
8
10
12
Incubation Period (HRS)

TABLE 6

Effect of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant) on Incorporation of L-Leucine-U-^{14}C by Whole Cells of Bacillus megaterium

Incorporation of L-Leucine-U-^{14}C by Whole Cells of Bacillus megaterium		Antibiotic* Inhibition of Incorporation of L-Leucine-U-^{14}C by Whole Cells of Bacillus megaterium	
Time (minutes)	cpm	Time (minutes)	cpm
0	449	0	449
10	623	10	623
20	1012	20	1012
30	1633	30	1633
35	1995	35	1767
40	2400	40	1869
45	3013	45	2000
50	3600	50	2050
60	4981	60	2290
70	5810	70	2600

Incubation flasks contained 1 μC of L-leucine-U-^{14}C in 5 ml of media.

*0.066 Units/ml of antibiotic from Acholeplasma laidlawii B added after 30 minutes.

Figure 10. Effect of antibiotics produced by *Acholeplasma laidlawii* B (tetracycline resistant) on incorporation of L-leucine-U-^{14}C by whole cells of *Bacillus megaterium*.

Control ○

0.066 Units/ml of antibiotic from *Acholeplasma laidlawii* B (tetracycline resistant) added ●

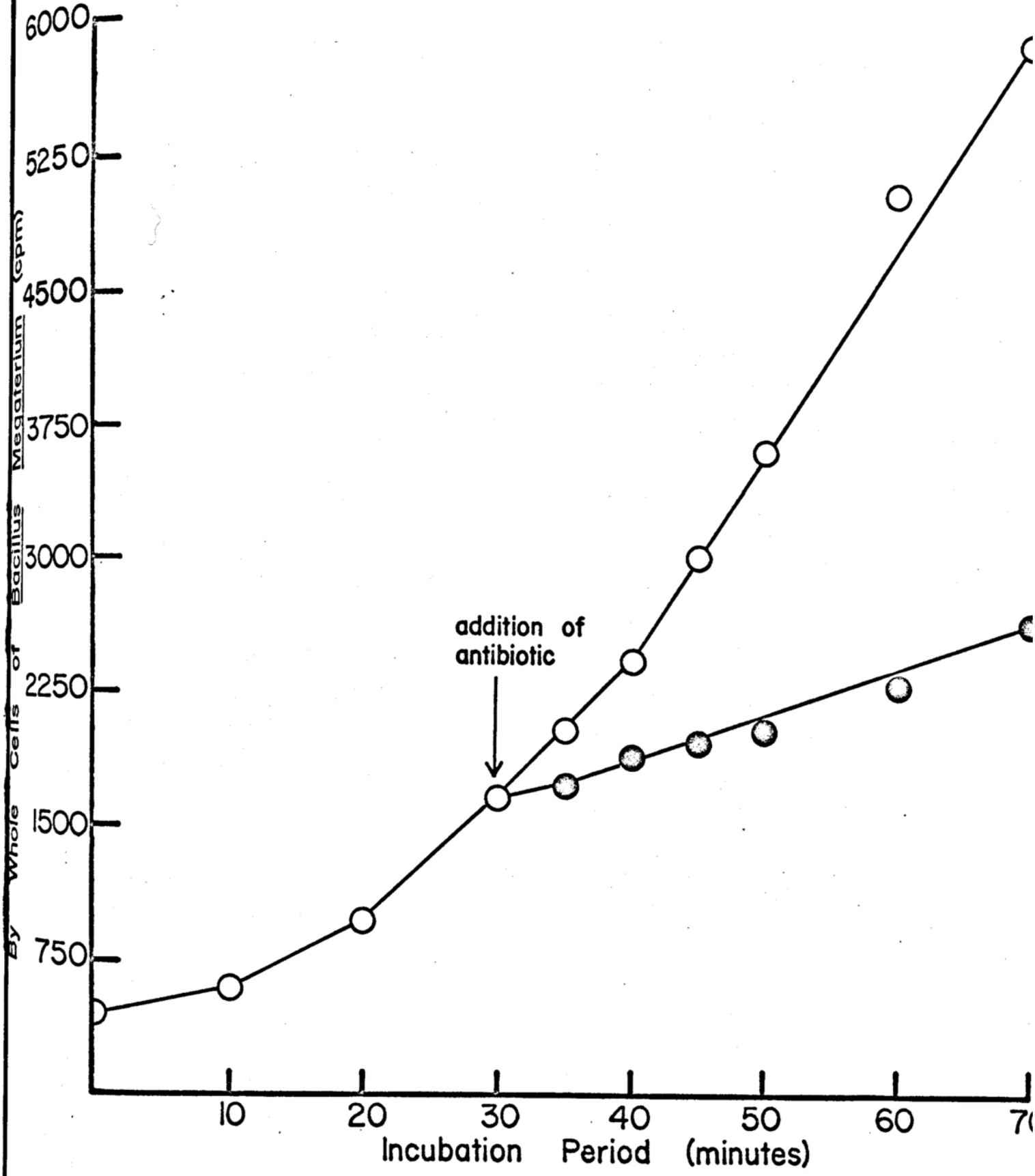

6000
5250
4500
3750
3000
2250
1500
750
By Whole Cells of Bacillus Megaterium (cpm)
addition of antibiotic
10
20
30
40
50
60
Incubation Period (minutes)

TABLE 7

Effect of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant) on Incorporation of Uridine-5-^{3}H by Whole Cells of Bacillus megaterium

Incorporation of Uridine-5-^{3}H by Whole Cells of Bacillus megaterium		Antibiotic* Inhibition of Incorporation of Uridine-5-^{3}H by Whole Cells of Bacillus megaterium	
Time (minutes)	cpm	Time (minutes)	cpm
0	498	0	498
5	1210	5	1210
10	1977	10	1873
15	2890	15	2690
20	3979	20	3476
25	4875	25	4038
30	5800	30	4623
40	7790	40	5399
50	8673	50	6113
60	8964	60	6513

Incubation flasks contained 5 μC of uridine-5-^{3}H in 5 ml of media.

*0.066 Units/ml of antibiotic from Acholeplasma ladilawii B added after 5 minutes.

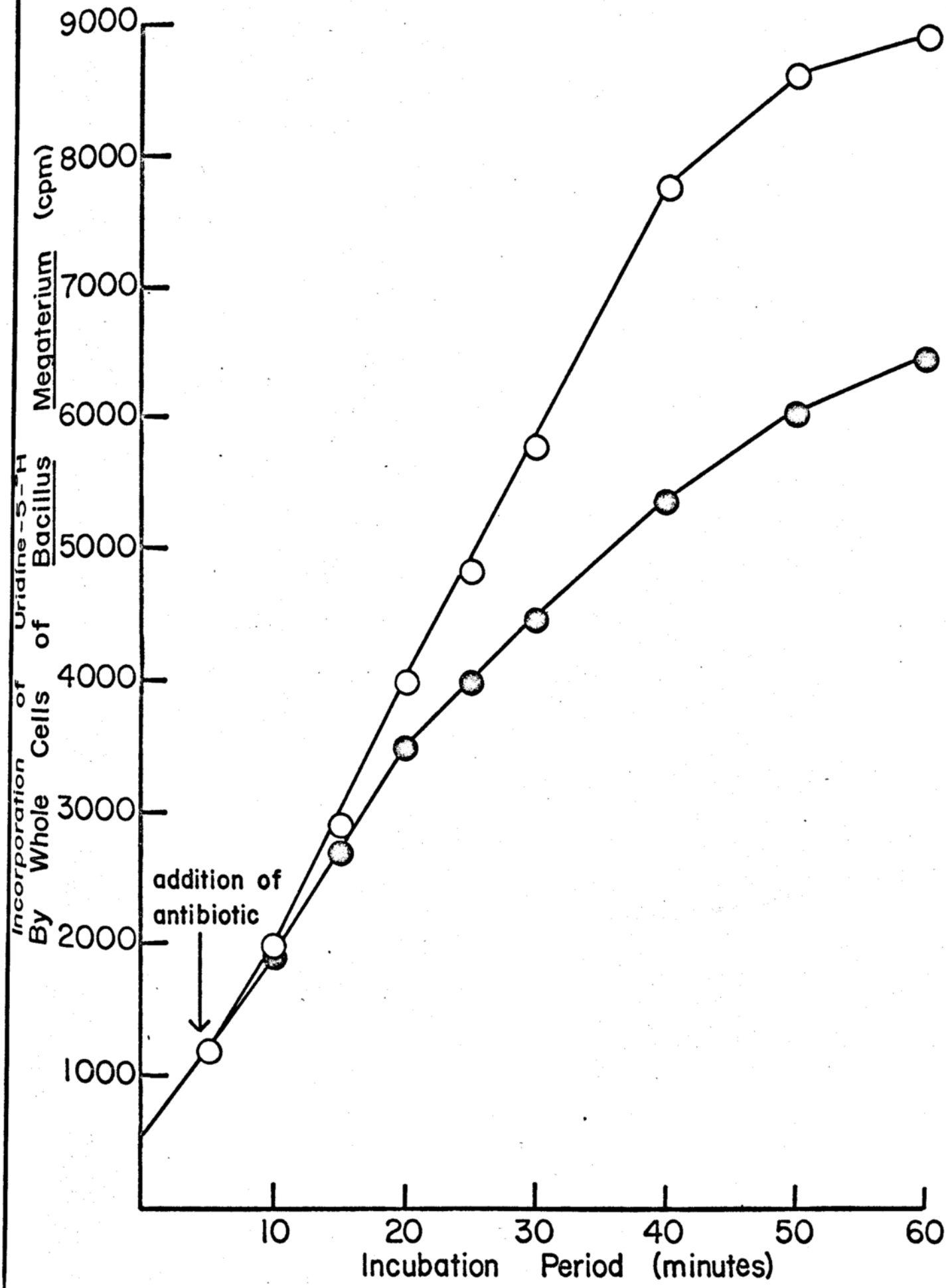
TABLE 8

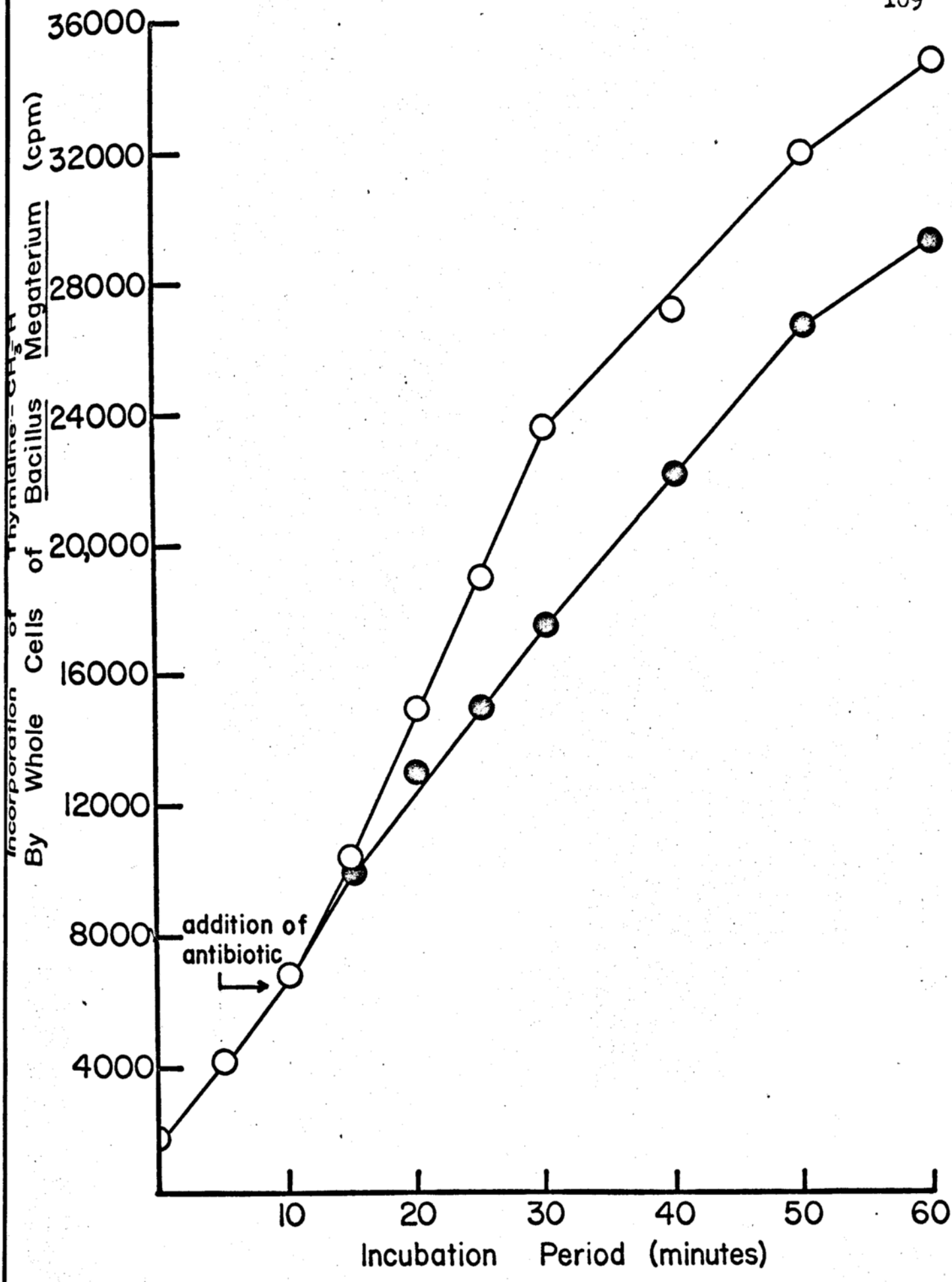
Effect of Antibiotics Produced by Acholeplasma laidlawii B (Tetracycline Resistant) on Incorporation of Thymidine-Methyl-^{3}H by Whole Cells of Bacillus megaterium

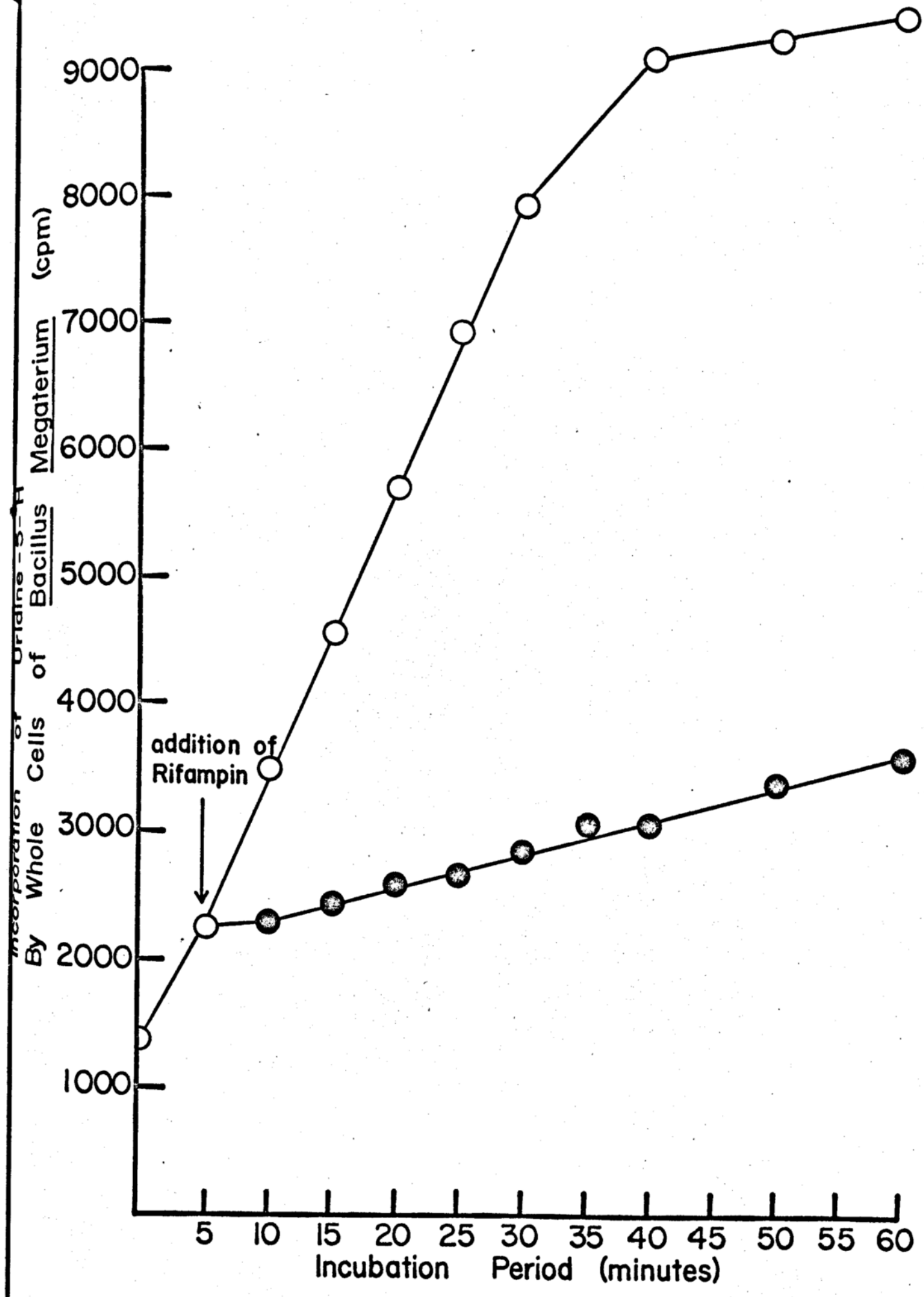
Incorporation of Thymidine-Methyl-^{3}H by Whole Cells of Bacillus megaterium		Antibiotic* Inhibition of Incorporation of Thymidine-Methyl-^{3}H by Whole Cells of Bacillus megaterium	
Time (minutes)	cpm	Time (minutes)	cpm
0	1778	0	1778
5	4193	5	4193
10	6879	10	6879
15	10420	15	9800
20	15348	20	13134
25	19028	25	15096
30	23528	30	17566
40	27298	40	22324
50	32100	50	26850
60	34755	60	29467

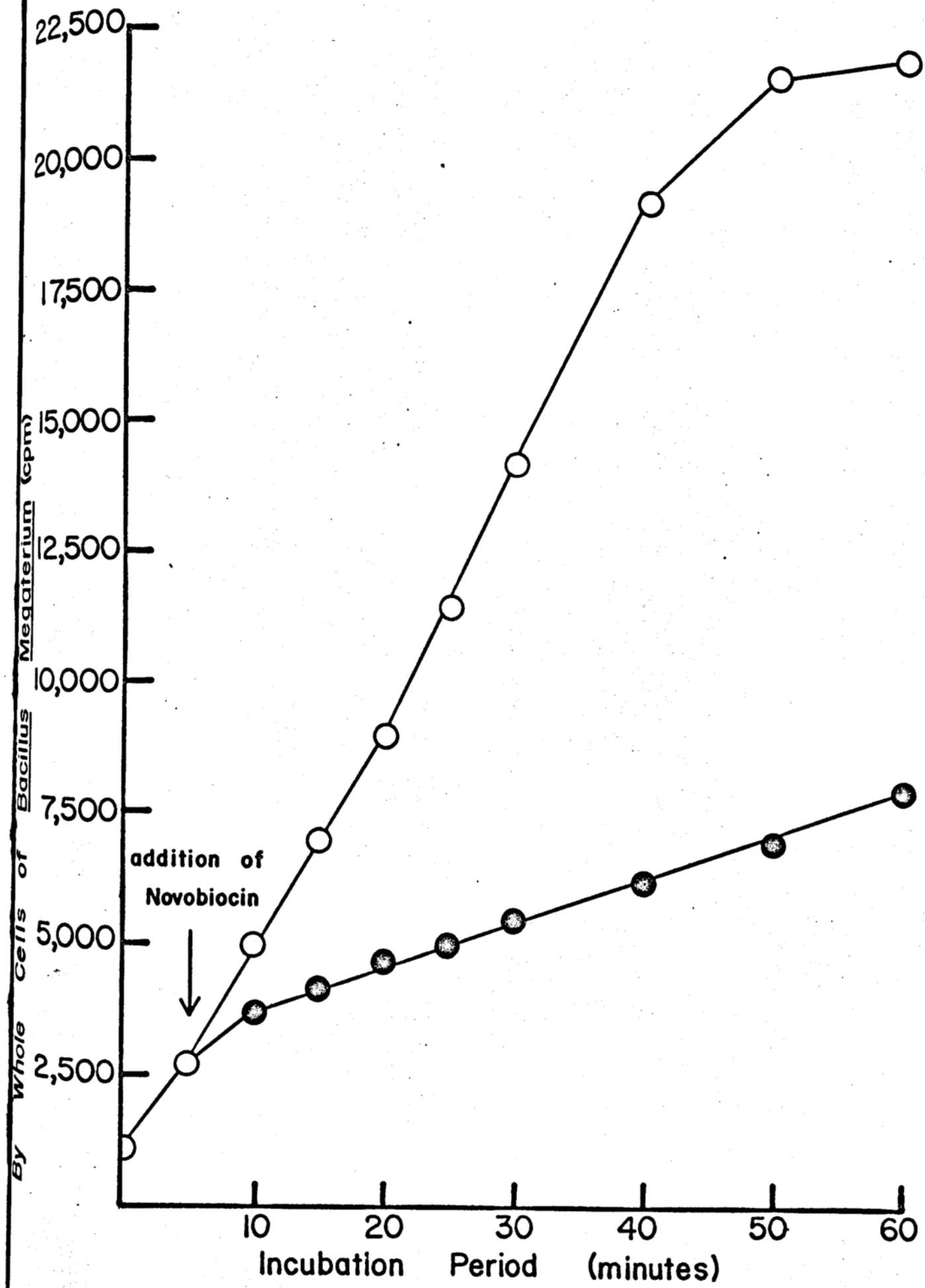
Incubation flasks contained 5 μC of thymidine-methyl-^{3}H in 5 ml of media.

*0.066 Units/ml of antibiotic from Acholeplasma laidlawii B added after 10 minutes.


Incorporation of Uridine-5-³H By Whole Cells of Bacillus Megaterium (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
addition of antibiotic
10
20
30
40
50
60
Incubation Period (minutes)


Incorporation of Thymidine-CH_3-H By Whole Cells of Bacillus Megaterium (cpm)
36000
32000
28000
24000
20,000
16000
12000
8000
4000
addition of antibiotic
10
20
30
40
50
60
Incubation Period (minutes)


Incorporation of Uridine-5-H
By Whole Cells of Bacillus Megaterium (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
addition of
Rifampin
5
10
15
20
25
30
35
40
45
50
55
60
Incubation Period (minutes)


By Whole Cells of Bacillus Megaterium (cpm)
22,500
20,000
17,500
15,000
12,500
10,000
7,500
5,000
2,500
addition of
Novobiocin
10
20
30
40
50
60
Incubation Period (minutes)

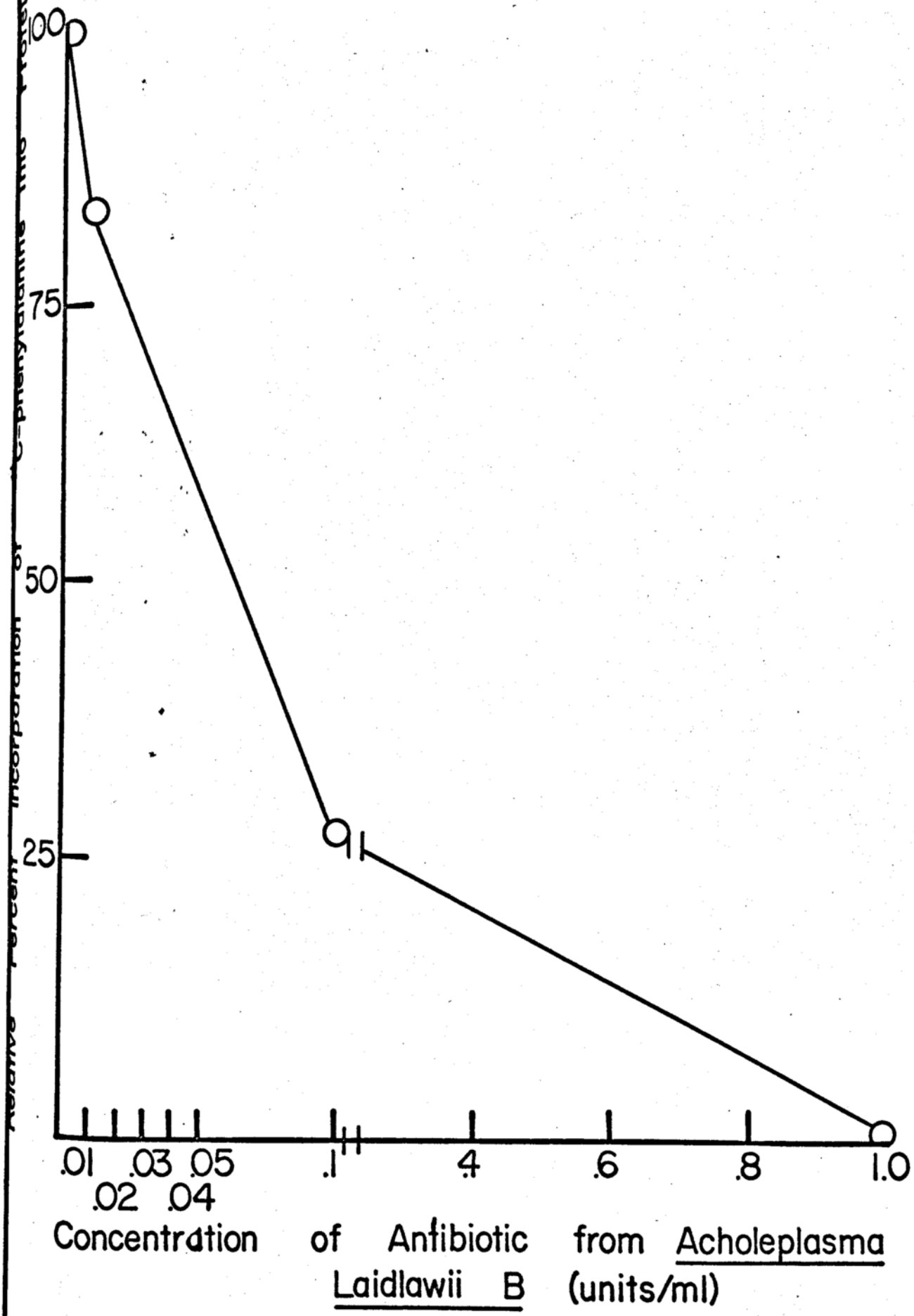

Relative Percent Incorporation of C¹⁴-phenylalanine into Protein
100
75
50
25
.01
.02
.03
.04
.05
.1
.4
.6
.8
1.0
Concentration of Antibiotic from Acholeplasma Laidlawii B (units/ml)

TABLE 9

Antibiotic Sensitivity Pattern of *Bacillus subtilis* Marburg Strains

Antibiotic (mcg or units/disc)	Inhibition Zone Diameter: Parent Strain (mm)	Inhibition Zone Diameter: Resistant Strain* (mm)
Acholeplasma laidlawii B antibiotic (0.25) units	15	0
Tetracycline (12.5) mcg	23	23
Streptomycin (2) mcg	17.5	16
Chloramphenicol (30) mcg	40	40
Erythromycin (0.05) mcg	18	18
Ampicillin (2) mcg	28	27
Neomycin (30) mcg	18	18
Novobiocin (7.5) mcg	26	26.5
Capreomycin (12.5) mcg	14	14
Mikamycin (12.5) mcg	24.5	25
Lincomycin (12.5) mcg	28	28
Staphylomycin (12.5) mcg	23.5	24

*Resistant strain obtained by stepwise exposure of parent culture to *Acholeplasma laidlawii* B (tetracycline resistant) produced antibiotics.

TABLE 10

Heat Stability* of Antibiotics from *Acholeplasma laidlawii* B (Tetracycline Resistant)

pH of Solution	Inhibition Zone Diameter, mm
2	24
5	23.5
7	23.5
9	24
Control (not heated)	24

*Heated in 100°C water bath for 15 minutes.

TABLE 11

Purification of Antibiotics from Acholeplasma laidlawii B (Tetracycline Resistant)

Purification Step	Weight (mg)	Units/mg	Purification	Yield %
Starting material	3750	0.004	----	---
Dowex 50 X2	1250	0.0125	3-fold	100
Dowex 1 X2*	150	0.033	8-fold	41

*(only 12 of 15 units put on column)

FIGURE 16

FLOW SHEET FOR PURIFICATION OF ANTIBIOTIC PRODUCED BY *ACHOLEPLASMA LAIDLAWII* B (TETRACYCLINE RESISTANT)

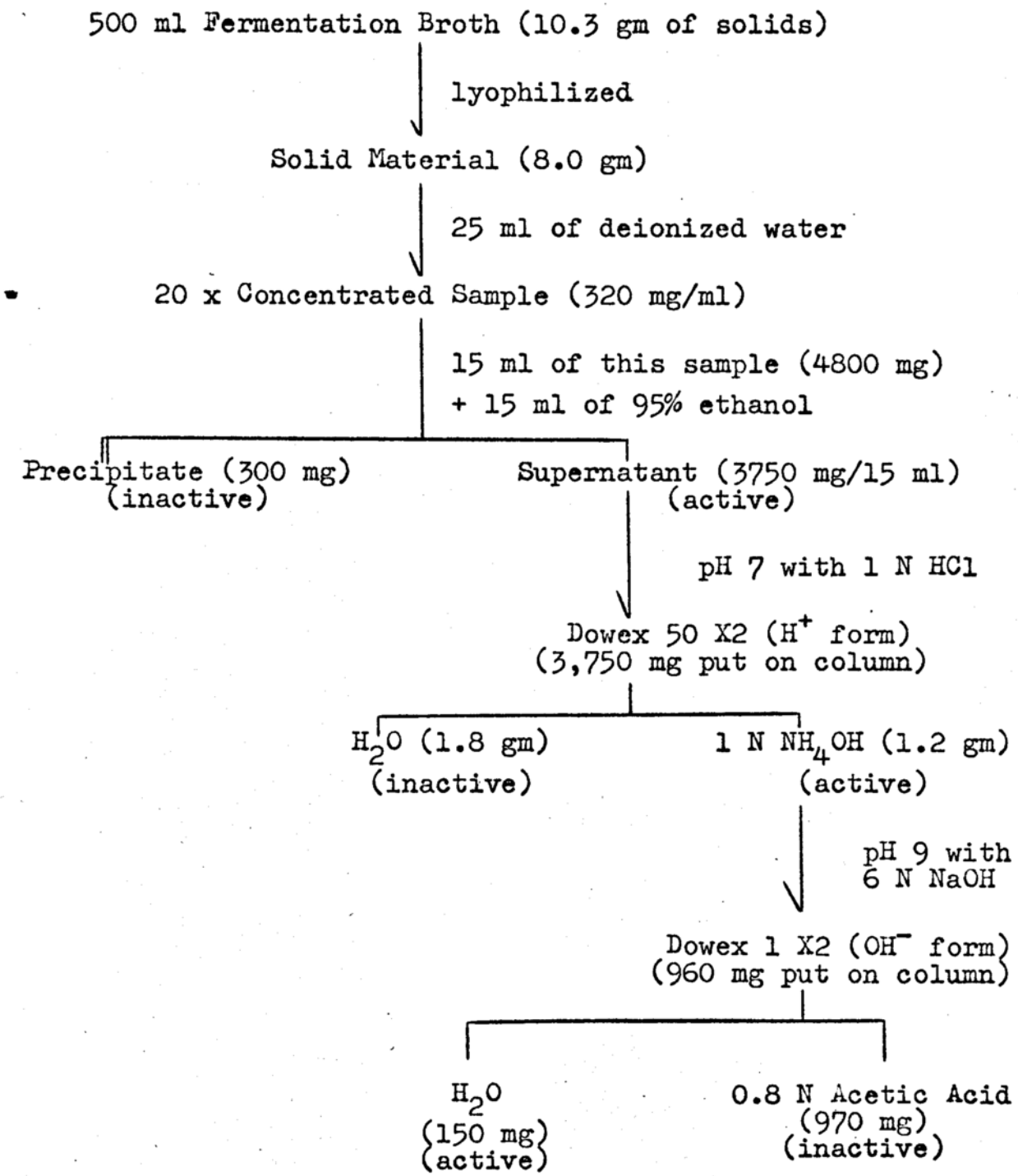


TABLE 12

Paper Chromatographic Behavior of Antibiotic(s) from *Acholeplasma laidlawii* B (Tetracycline Resistant)

Solvent System	R_f
Benzene:Methanol (4:1)	0
n-Butanol:Acetic Acid:Water (5:1:4)	0
n-Butanol:Pyridine:Water (6:4:3)	0
n-Butanol:Ethanol:Water (2:1:1)	0.14
n-Propanol:Pyridine:Acetic Acid:Water (15:10:3:12)	1

SUMMARY

PART I

Tetracycline inhibited the incorporation of ^{14}C-phenylalanine into protein by a cell-free system from French press broken cells of tetracycline-sensitive and tetracycline-resistant Acholeplasma laidlawii B.

When Acholeplasma laidlawii B cells were broken using a Vibrogen mill, the S-30 fraction actively incorporated ^{14}C-phenylalanine into protein with the following requirements:

1) 105,000 x g supernatant
2) ribosomes (105,000 x g precipitate)
3) ATP
4) GTP
5) polyuridylic acid
6) a maximum concentration of 18 μM of magnesium ion in the incubation buffer
7) incubation buffer of pH 7.4
8) incubation time of 30 minutes.

Tetracycline inhibited the incorporation of ^{14}C-phenylalanine into protein by the S-30 fraction from Vibrogen mill broken cells of tetracycline-sensitive and tetracycline-resistant Acholeplasma laidlawii B.

Tetracycline resistance by a mutant of Acholeplasma laidlawii B is due to some mechanism other than synthesis of ribosomes with altered sensitivity to tetracycline.

PART II

A tetracycline-resistant mutant of Acholeplasma laidlawii B produces a mixture of antibiotics.

They have the following biological characteristics:

1) active against Bacillus subtilis Marburg, Bacillus megaterium and Staphylococcus aureus,

2) inactive against Sarcina lutea, Escherichia coli B, Saccharomyces cerevisiae L-24, Acholeplasma laidlawii B, and cultured tissue cells (Eagle's KB, WI-38 and Hela),

3) inhibits protein synthesis in whole cells of Bacillus megaterium and in cell-free preparations of Escherichia coli B,

4) bacteriostatic against Bacillus subtilis Marburg at MIC,

5) a mutant Bacillus subtilis Marburg resistant to 25 x MIC of these antibiotics was not cross resistant to a number of known antibiotic families (macrolide, tetracycline, peptide, aminoglycoside, coumarin, penicillin, and lincomycin).

They have the following chemical characteristics:

1) water soluble

2) behave as basic compounds when treated with charcoal, ion exchange resins, or electrical charges

3) heat stable in acidic and alkaline solution.

Two ion exchange resins can be used to purify the antibiotics 8 x. A 59% loss in units of activity is accompanied with this procedure.

Three separate bioactive compounds have been detected on descending paper chromatography using n-butanol:ethanol: water (2:1:1) as developing solvent.

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ANTIBIOTICS AND *ACHOLEPLASMA LAIDLAWII* B

I. MECHANISM OF TETRACYCLINE RESISTANCE IN *ACHOLEPLASMA LAIDLAWII* B

II. CHARACTERISTICS OF ANTIMICROBIAL AGENTS PRODUCED BY *ACHOLEPLASMA LAIDLAWII* B (TETRACYCLINE RESISTANT)

by CARL CHRISTOPHER FRATERRIGO

(Under the supervision of Professor D. Perlman)

PART I

The mechanism of resistance to the terracyclines has been studied in both Gram-negative and Gram-positive bacteria. Although the exact mechanism of resistance in these organisms has not been totally elucidated, results of labelled tetracycline uptake experiments together with data from inhibition of cell-free protein synthesis have been used by a number of workers to conclude that decreased permeability to the drug is the mechanism of resistance.

In the present studies an active cell-free protein synthesizing system by the S-30 fraction of *Acholeplasma laidlawii* B was obtained by breaking the cells in a Vibrogen mill. The requirements of the system for active incorporation of ^{14}C-phenylalanine into protein were similar to those found in *Escherichia coli* and *Staphylococcus aureus*:

1) ribosomes
2) 105,000 x g supernatant
3) GTP
4) ATP
5) polyuridylic acid

Tetracycline inhibits the incorporation of ^{14}C-phenylalanine into protein by the S-30 fraction from Vibrogen mill broken cells of tetracycline-sensitive and tetracycline-resistant Acholeplasma laidlawii B.

From the above data we concluded that tetracycline resistance in a mutant of Acholeplasma laidlawii B is due to some other mechanism than ribosomes with altered sensitivity to tetracycline inhibition.

PART II

Production of antibiotics by Actinomycetales, fungi and bacteria have been reported. Although the exact relationship between Mycoplasma and bacteria is not known, it is not unlikely that Mycoplasma also produce antimicrobial substances.

A tetracycline-resistant mutant of Acholeplasma laidlawii B was found to produce a mixture of antimicrobial substances when grown in PPLO Broth (Difco) supplemented with yeast extract, sodium acetate and calf or swine serum. These substances were active against Bacillus subtilis Marburg, Bacillus megaterium and Staphylococcus aureus.

They were ineffective against Escherichia coli B, Saccharomyces cerevisiae L-24, cultured tissue cells (Eagle's KB, WI-38 and Hela) and Acholeplasma laidlawii B.

Their mechanism of action was shown to involve inhibition of protein synthesis since inhibition of the incorporation of L-leucine-^{14}C by whole cells of Bacillus megaterium and L-phenylalanine-^{14}C incorporation into protein by a cell-free system from Escherichia coli B was observed. The antibiotics were found to be bacteriostatic against Bacillus subtilis Marburg at the MIC. A mutant of Bacillus subtilis Marburg resistant to 25 x the MIC of the antibiotics showed no cross resistance to other selected known families of antibiotics (macrolide, peptide, aminoglycoside, coumarin, penicillin, tetracycline, and lincomycin).

The basic character of these antibiotics was demonstrated by their adsorption on charcoal at pH 9, retention on Dowex 50 X2 at pH 7, movement toward cathode at pH 6.4 and 1.9 using ionophoresis and increased bioactivity at alkaline pH.

An 8 x purification procedure was accomplished on Dowex 50 X2 and Dowex 1 X2 ion exchange resins. A 59% loss in units of activity was detected after treatment with the Dowex 1 X2 resin.

The antibiotics were heat stable in acidic and alkaline solutions. Three separate bioactive compounds

have been detected on descending paper chromatography using n-butanol:ethanol:water (2:1:1) as developing solvent.

APPROVED Howard Pelham

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