1969

Inhibition of Cell Division, Protein Synthesis and Nucleic Acid Synthesis in Escherichia coli W by Tetracycline Antibiotics

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Inhibition of Cell Division, Protein Synthesis and Nucleic Acid Synthesis in *Escherichia coli* W by Tetracycline Antibiotics

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Thesis

Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Department of Pharmacy at the Medical College of Virginia

Richmond, Virginia

June, 1969

This thesis by Mr. George H. Miller is accepted in its present form as satisfying the thesis requirement for the degree of Doctor of Philosophy

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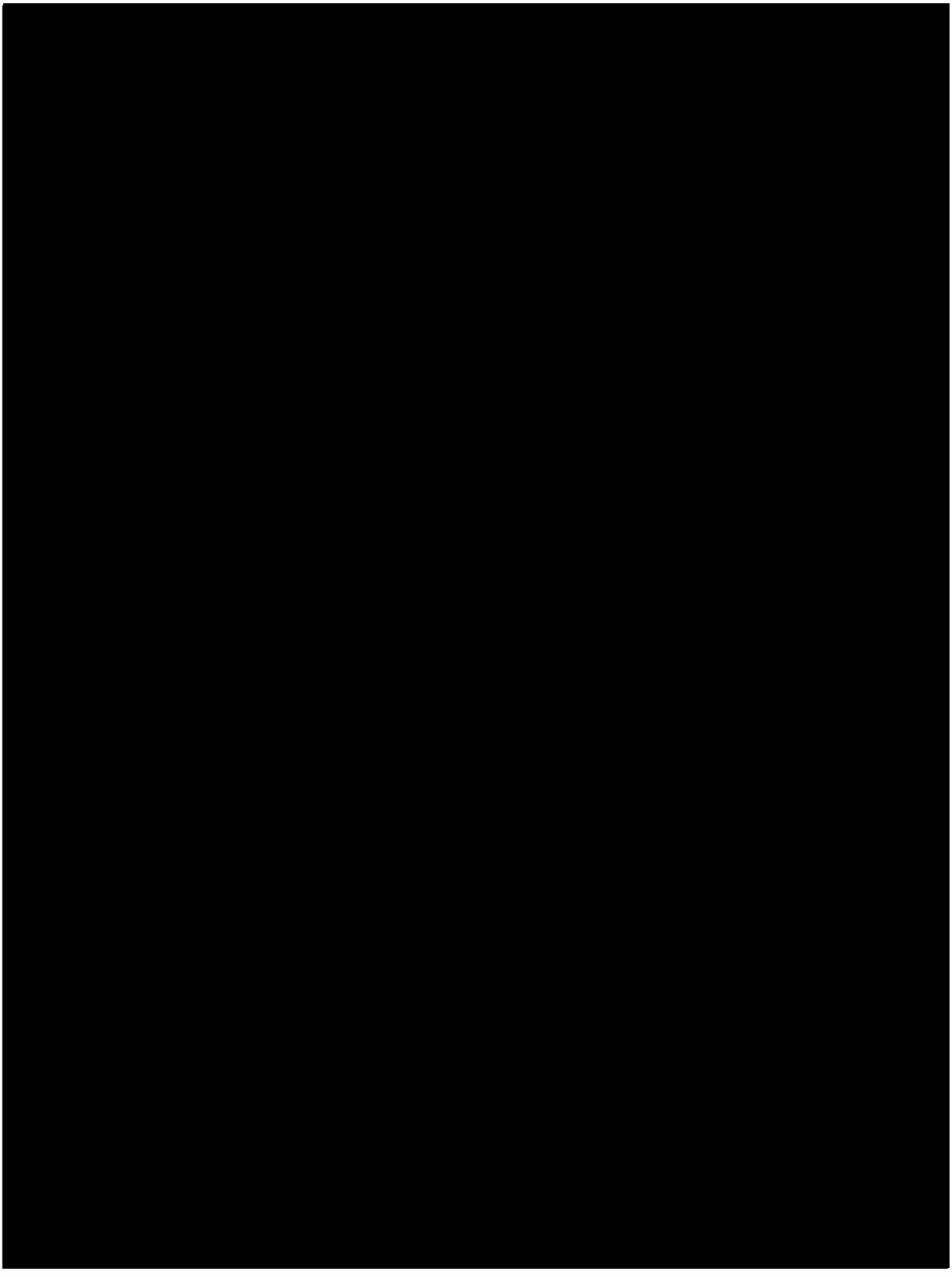

Acknowledgments

The author wishes to express his appreciation and his sincere gratitude to:

Dr. A. N. Martin for providing the opportunity to study and learn in a stimulating and pleasant environment and for his counseling, supervision, patience and especially his expressions of confidence during the course of this work;

Dr. A. A. Cammarata and Dr. F. Peradejordi for the many stimulating and helpful discussions;

Dr. S. A. Khalil for his help and encouragement during the development of the experimental procedures;

Luisa C. Miller for her excellent technical assistance in performing these experimental procedures;

Sung Ho Hahn for his assistance in preparing the materials for the experiments;

John Andrako Jr., William Crouthamel, John Gill, Douglas Martin and Teresa Taylor for their help in the viable cell determinations as well as other experiments;

the members of my Advisory Committee, Dr. E. Kline, Dr. Punch and Dr. Richard in addition to Dr. Cammarata and Dr. Martin for their advice and especially for their reading of this thesis;

the staff of the Computer Center, Department of Biometry, Medical College of Virginia for the prompt statistical analysis of the experimental data and for their assistance in studying the proposed model of tetracycline action;

my parents, Arthur and Priscilla, for their encouragement

Acknowledgments

and sacrifices in my behalf;

my daughter, Claudia, for the inspiration and joy she provided during this work;

my wife, Lilo, for providing the encouragement to undertake this study and for her patience, understanding and sacrifices during its completion in addition to her excellent technical assistance acknowledged above.

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List of Abbreviations

AA	amino acids	
	ala	alanine
	asp	asparagine
	cys	cysteine
	leu	leucine
	lys	lysine
	met	methionine
	phe	phenylalanine
	ser	serine
	thr	threonine
	tyr	tyrosine
	val	valine
DPN	diphosphopyridine nucleotide	
DPNH	reduced diphosphopyridine nucleotide	
EDTA	ethylenediaminetetraacetic acid	
f_1, f_2	ribosomal proteins which are necessary for protein synthesis and which are removed from ribosomes by washing with NH_4Cl	
FMN	flavin mononucleotide	
FAD	flavin adenine dinucleotide	
G	a supernatant enzyme which catalyzes the hydrolysis of guanosine-5'-triphosphate in the presence of ribosomes	
k_{cd}	generation rate constant for cell division	
k_v	generation rate constant for cell division as determined by the viable cell count method	

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kt	generation rate constant for cell division as determined by the total cell count method
k_p	generation rate constant for protein synthesis
k_{na}	generation rate constant for the synthesis of nucleic acid
k^I	the proportionality constant relating generation rate constants to tetracycline concentration; the subscripts v, t, p, na indicate the type of generation constant used, the subscript avg indicates that all generation rate constants were used collectively
LDH	lactic acid dehydrogenase
MDH	malic acid dehydrogenase
na	nucleic acids
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
sRNA	soluble ribonucleic acid
tRNA	transfer ribonucleic acid
AAtRNA	aminoacyl transfer ribonucleic acid
met-$tRNA_f$	methionyl transfer ribonucleic acid which allows the addition of a formyl group to the methionyl amine
N-F-met-$tRNA_f$	N-formylmethionyl transfer ribonucleic acid

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met-tRNA$_m$	methionyl transfer ribonucleic acid which does not allow the addition of a formyl group to the methionyl amine
ATP	adenosine-5'-triphosphate
CTP	cytosine-5'-triphosphate
GDP	guanosine-5'-diphosphate
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
UTP	uridine-5'-triphosphate
poly A	polyadenylic acid
poly c	polycytidylic acid
poly G	polyguanylic acid
poly U	polyuridylic acid
"Nucleic Acids"	the absorbance of an orcinol reaction (4 ml.) which is due to the nucleic acids of E. coli in a 12.5 ml. culture sample
Pm	puromycin
"Proteins"	the absorbance of a Folin-Lowry reaction (6.5 ml.) which is due to the protein of E. coli in a 25 ml. culture sample
T	supernatant proteins which are necessary for protein synthesis

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TCA	trichloroacetic acid
θ	the fraction of total cells in a culture which are found to be viable

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I. INTRODUCTION

Tetracyclines, originally isolated in the late 1940's and early 1950's from the mycelium of Streptomyces, have achieved major importance as therapeutic and prophylactic agents against a wide range of infections in human and veterinary medicine. They have also achieved great importance in agriculture where they are widely used to promote weight gain in livestock. Commercial production, during 1958 in the United States alone was 120 tons with a value of approximately $1 million per ton.

The clinical importance of these compounds has stimulated efforts to define their mode of action as inhibitors of bacterial reproduction. Antibiotics having as many functional groups as the tetracyclines may have many modes of action. The problem facing the research worker is to determine the relative contribution of each mode of action in a given biological system as a function of antibiotic concentration. If all parts of a biological system are exposed to an equivalent antibiotic concentration, one might expect that the degree of inhibition of the various sub-component systems would be proportional to the relative stabilities of the reaction products of the antibiotic and the sub-component system. The most critical reaction from the standpoint of cellular reproduction would then represent the point of the primary biochemical lesion or the site of

action. The type of reaction would be the mode of action for the biological system. For a given biological system it is probable that there is one critical reaction which is most sensitive to the antibiotic, but this reaction may not be the same for every biological system (see Snell and Cheng (1) for an excellent discussion of the difficulties of defining a mode of tetracycline action). Many investigators presently feel that the primary biochemical lesion inflicted upon susceptible bacteria by tetracyclines is a general inhibition of protein synthesis (2,3,4). Therefore it was felt that the study of several measures of inhibition, particularly cell reproduction and protein synthesis, in the presence of several tetracyclines might be a useful way of studying modes of tetracycline action.

Biological activities of a large number of compounds obtained quantitatively under identical conditions and in a precise manner are required to establish structure-activity relationships. Presently available activities for tetracycline antibiotics have been summarized by Barrett (5), Boothe (6) and Plakunov (7). Many of these activities have been obtained under conditions such that the results parallel clinical activities. Thus, some compounds may not have achieved equilibrium with the test system. Some activities have been obtained for the purpose of studying antibiotic resistance while still others have been obtained in widely varying and not easily interrelatable biological test systems. Consequently, quantitative activities suitable for structure-activity relationships have not been reported for most tetracyclines.

The present work was undertaken to obtain activities suitable for structure-activity relationships and which are pertinent to both bacterial reproduction and the proposed mode of action. The activities obtained should include both clinically active and "inactive" tetracycline antibiotics.

II. Literature Survey

A. Tetracyclines - Nomenclature, General Properties and Biological Activity

The isolation in 1947 of the broad spectrum antibiotic, chlortetracycline, from Streptomyces aureofaciens was followed by the discovery of oxytetracycline in 1950 and tetracycline in 1953. The clinical usefulness of these compounds prompted the isolation, synthesis and characterization of a large number of derivatives, degradation products and similar compounds with a wide range of antibiotic properties. The elucidation of the structure of the tetracyclines has been a major achievement in natural product chemistry (8). The structure of chlortetracycline, established on chemical grounds and confirmed by X-ray analysis (9), is shown as (I) in Fig. I. The ring numbering and lettering system shown serve as the basis for the nomenclature of most of the tetracyclines. Several analogs with altered nuclei are also shown in Fig. I.

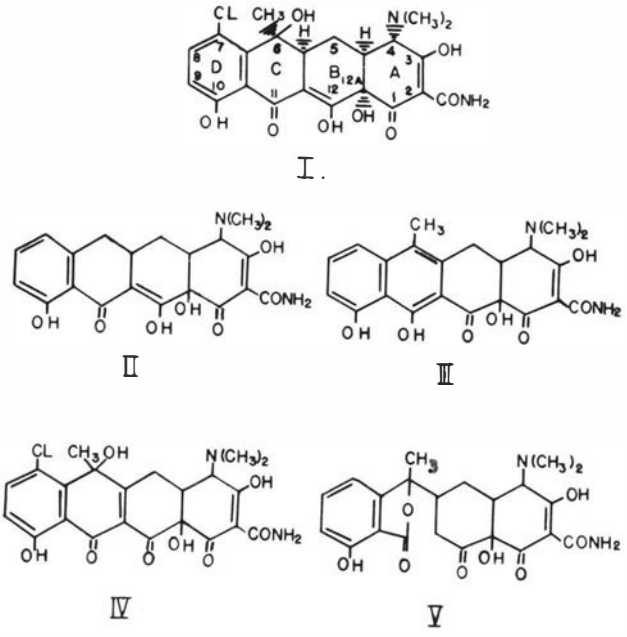

Figure I. The Tetracyclines

(I) 7-Chlorotetracyline

(II) dm-do-tetracycline

(III) 5a(6)-Anhydrotetracycline

(IV) 7-Chloro-5a(lla)-dehydrotetracycline

(V) Isotetracycline

The stereochemistry of the compounds is depicted in (1) of Fig. I. The 4-dimethylamino-group, the 4a- and 5a-hydrogens, the 6-methyl- and the 12a-hydroxy- are all in the alpha orientation. Epimerization by general acid-base catalysis of the 4-dimethylamino-group in neutral solutions occurs rapidly and gives rise to the so-called 4-epitetracyclines (10). Synthetic compounds epimeric at both the 5a- and 6a- position have been reported. The 5a-epitetracycline is essentially inactive while the 6-deoxy-6-epitetracyclines are reported to be only slightly different from the normal 6-deoxy- derivative (6).

A.1. A-Ring Modifications

The 2-carboxamide-group has been modified in several ways. The 2-acetyl-2-decarboxamido-tetracyclines have been prepared biosynthetically and are essentially inactive as antibacterials (5). Dehydration of the carboxamide yields the nitrile, a modification which is also said to result in loss of activity (5). Several compounds which are clinically useful because of their increased water solubility have been prepared by employing the Mannich reaction to form a methylene bridge between the amide nitrogen and various primary amines. Hydrolysis back to the parent compound is probably responsible for the activity of these derivatives (6).

The dimethylamino group at carbon 4 can be epimerized; it can be quaternized by treatment with methyl iodide or other agents and it can be replaced by hydrogen. All of these compounds are reported to have reduced antibacterial activity.

The 4-N-ethyl-methyl-amino as well as the 4-N-methyl-amino derivative have been prepared and are reported to possess biological activity.

The 12a-hydroxy-group may be eliminated by reduction with zinc and ammonium hydroxide or by catalytic reduction of the 12a-formyloxy derivative. In these antibiotics the phenolic diketone moiety is conjugated with the ketone at carbon 1. Such conjugation is said to be responsible for the loss of activity by this compound. The 4a(12a)-anhydrotetracyclines have been prepared by pyrolysis of the 12a-acetoxy derivatives and are reported to have minimal antibacterial activity. The 12a-formyloxy- and 12a-acetoxy- derivatives are reported to possess activity solely because of their hydrolysis to the parent compounds.

A.2. B-Ring Modifications

5-Hydroxytetracycline was the second tetracycline antibiotic isolated from Streptomyces and was the first to have its structure delineated (8). Its clinical utility has been proven throughout the last 20 years. The 5a(11a)-dehydrotetracyclines, (IV) in Fig. I, are also produced biosynthetically and are generally regarded as being inactive (6).

A.3. C-Ring Modifications

In strong acid solutions, tetracyclines are dehydrated to form anhydrotetracyclines, (III) in Fig. I, while in alkaline solutions isotetracyclines, (V) in Fig. I, are formed. Both modifications result in compounds which are traditionally regarded as inactive or as possessing an altered spectrum of

antibacterial activity (5,6). The isotetracycline nucleus has such different conformations available to it that its lack of activity should probably not be utilized in structure-activity relationships. The clinically useful 6-demethyltetracyclines are produced biosynthetically, while the 6-deoxy-6-epitetracyclines are the result of catalytic hydrogenation reactions. Both types of compounds as well as the 6-deoxy-6-demethyltetracyclines, (II) in Fig. I, are potent antibacterial agents. In addition, the 6-methylene tetracyclines have been prepared and are also active antibacterial substances.

A.4. D-Ring Modifications

7-Chlortetracycline, (I) in Fig. I, and 7-bromotetracycline are produced biosynthetically and are among the most potent tetracycline antibiotics. The acid stability of the 6-deoxy-6-demethyl-tetracyclines has allowed the chemical substitution of the aromatic D-ring at positions 7 and 9 and a number of antibacterial substances with a wide range of activities have been reported. The availability of these derivatives made possible our present attempt at quantitative correlations of structure and activity.

A.5. Structure-Activity Generalizations

Modifications of the A-ring which are not readily reversible by simple aqueous hydrolysis lead to drastic reductions in biological activity. The most notable exceptions are the 4-dedimethylamino-tetracyclines which retain about 50% of the biological activity of their parent compound. In contrast, the 4-epitetracyclines are essentially inactive. Plakunov (7)

studied several tetracyclines with altered A-ring systems as well as several compounds structurally analogous to the A-ring of tetracycline and concluded that this ring is the most important part of the tetracycline nucleus. He found that alterations of it may produce compounds with a different antibacterial spectrum which are probably exerting an effect by a different mode of action (vitamin K antagonism is proposed).

Modifications of the substituents at carbons 5, 6, 7 and 9 produce derivatives with quantitatively different activities but do not produce the extreme differences in activity seen with A-ring modifications. These compounds would seem to offer the best possibility for structure-activity correlations and Colaizzi, Knevel and Martin (11) have reported limited correlations of biological activity with Hammett-sigma values for these derivatives.

The oxygen functions at positions 10, 11 and 12 appear to be important for biological activity since alterations of this chromophoric group lead to very inactive compounds. These groupings are also one of the most likely sites for metal ion chelation, a phenomena which has always been expected to play a large role in the activity of these antibiotics (12).

A. 6. Acidity Constants of the Tetracyclines

The normal tetracycline nucleus, (I) and (II) in Fig. I, has three measurable pKa values in the vicinity of 3, 7.5 and 9, Table I, see page 67. Stephens et. al., (13) have defined three ionizable groups, (A), (B) and (C) corresponding to these values as indicated in Fig. II. This assignment of

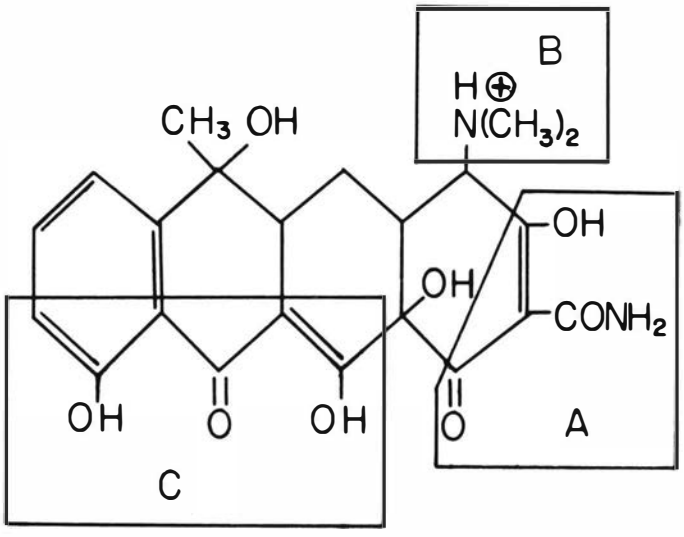

Figure II. The Ionizable Groups of Tetracycline

(A) Tricarbonylmethane

(B) Ammonium Cation

(C) Phenolic diketone

the observed pKa values to specific functions has been the subject of some controversy (14, 15), but it is now generally agreed that the first pKa is attributable to the tricarbonylmethane system (A), the second to the phenolic diketone moiety (C) and the third to the ammonium cation (B). It is recognized that in many compounds considerable overlap may exist between the ionization of the second and third groups (16).

B. Tetracyclines - Biochemical Basis of their Antibiotic Activity

It is generally assumed that all the tetracyclines have similar modes of action. This view is based upon the similarity of their structures, the fact that the bacteriostatic effects of the most active compounds occur at similar concentrations and the common occurrence of cross-resistance. Nevertheless, investigators have occasionally noted dissimilarities, particularly in the nitro-reductase system and in cell wall synthesis. Cross-resistance is seen with a great many other structurally dissimilar antibiotics and it probably does not have any bearing on the mode of bacteriostatic action. Also, there is a large difference in the concentrations at which bacteriostatic effects are seen in some less common tetracyclines. The resolution of these problems, undoubtedly, depends on the test system.

B. 1. Effect on Rate of Cell Reproduction

The antibacterial activity of the tetracyclines has been

evaluated in various media against many pathogenic and non-pathogenic organisms. The data has usually been presented as the minimum concentration of drug necessary to prevent visible growth during a specified time period. These values, the so-called MIC or μ values, have been tabulated by Laskin (17). The wide range of MIC values obtained for a given tetracycline by different investigators is the reason why these activities are not useful in making structure-activity correlations.

The effect of the antibiotics on the rate of cell division has been studied by turbidimetric, viable plate and total cell count methods. Rates of growth measured by turbidimetric methods form the basis for the biological activities compiled by Boothe (6), Barrett (5), Plakunov (7) and Benbough and Morrisson (18). These methods were criticized by Hash et. al., (19) since they are sensitive to cell volume in addition to cell number. They claim that cell volume may change during tetracycline inhibition. The Coulter Counter can be used to observe changes in cell volume. Cell volume changes, other than those normally associated with the exponential growth cycle, however, were not seen in cultures inhibited with low tetracycline concentrations (20). Jones and Morrisson (21) have pointed out that their turbidimetric data were correlated with cell weight, which to them is the most meaningful and only exponential growth parameter. They believe that changes in cell numbers are only exponential because of exponential changes in cell mass and that therefore turbidimetric measures

were more meaningful. Data to be presented later make it clear that measurements of culture turbidity and cell numbers may be different during normal and inhibited cell growth.

Ciak and Hahn (22), Jones and Morrisson (21) and Garrett and Brown (23) demonstrated that exponential growth is maintained in cultures containing low tetracycline concentrations. They proposed kinetic equations which expressed the dependence of the growth rate constants upon tetracycline concentration. Changes in this dependence at higher antibiotic concentrations have been interpreted as indicating a change in the predominant mode of action.

B. 2. Effects on Oxidation and Respiration

Eagle and Saz (24), Snell and Cheng (1) as well as Laskin (17) reviewed the effects of the antibiotics on respiration and on the oxidation of various substrates. The pronounced inhibition in most cultures of these parameters was one of the first areas studied. Cheng and Snell (25) reported 50% inhibition of Q_{O_2} in resting Escherichia coli and Staphylococcus aureus by as little as 2×10^{-6} M oxytetracycline. At the same concentration several tetracycline analogs : 5a(11a)-anhydro-5-hydroxy-tetracycline, 4-dedimethylamino-oxytetracycline and 2-acetyl-2-decarboxamido-oxytetracycline, compounds not normally considered bacteriostatic, caused inhibitions of 46, 59 and 23%, respectively. Crema and Baroli (26) report that oxytetracycline was almost without effect on the respiration of S.curcio when the organism was in the resting state, while as little as

0.1 to 1.0 mcg./ml. of drug inhibited respiration by 50 to 60% at the beginning of the log phase. This observation was confirmed by Wong, Barban and Ajl (27) who report 90% inhibition of respiration in proliferating cultures, by 0.13 mcg./ml of oxytetracycline.

A large number of reports documenting inhibition of oxidation of compounds involved in carbohydrate metabolism have been reviewed (17, 24). These effects are now thought to be due to an inhibition of enzyme formation or to the appearance of an additional mode of action due to the extremely high concentrations of antibiotic employed. Since inhibition of several of these systems, pyruvic "hydrogenlyase", amino acid decarboxylase, α-amylase, alanine dehydrogenase and polynucleotide phosphorylase, occur in in vitro systems with preformed enzymes, the inhibitions can not be solely ascribed to faulty protein synthesis. Laskin (17), however, has concluded that "in no instance is there compelling evidence to suggest that the primary site of action is involved among these effects on oxidation and respiration."

B. 3. Effects on Protein Synthesis in Cultures

In 1950, Gale and Paine (28) reported that chlortetracycline inhibited protein synthesis in S. aureus. This study showed that the antibiotic inhibited "combined glutamate" formation, but not free glutamate accumulation in washed cell suspensions. Hahn and Wisseman (29) noted that adaptive enzyme formation was inhibited in E. coli by both chlortetracycline and oxytetracycline. They attributed this to a general inhibition of protein synthesis.

Gale and Folkes (30) confirmed that washed suspensions of S. aureus do not incorporate glutamate into protein in the presence of growth inhibitory concentrations of tetracycline; a 90% inhibition of rate of incorporation at 1 mcg./ml. of either chlortetracycline or oxytetracycline was observed. They also noted that inhibition of nucleic acid synthesis, rate of free glutamate accumulation and rate of glucose fermentation took place at higher antibiotic concentrations and that the two antibiotics differed quantitatively in their effect on these parameters. Park (31) and Sazykin and Borisova (32) in short communications, confirmed the preferential inhibition of protein synthesis. Park also noted that inhibition of cell wall synthesis required higher antibiotic concentrations, 50 mcg./ml. Nevertheless, Cheng and Snell (1), unaware of Park's report, have claimed that the use of D,L-Glutamate by Gale and co-workers had not eliminated the possibility of a preferential inhibition of D-Glutamate into cell wall material. Hash, Wishnick and Miller (19) have reported that cells treated with tetracycline show increased cell wall, hexosamines and ribonucleic acid (RNA) but decreased protein and deoxyribonucleic acid (DNA) as compared with control cells. Similarly, the incorporation of C^{14}-labeled lysine, alanine and glutamic acid into the cytoplasmic protein, isolated in a manner so that cell wall protein was eliminated, was greatly inhibited by tetracycline at 0.1, 0.5 and 1.0 mcg./ml. and chlortetracycline at 1.0 mcg/ml. Incorporation of C^{14}-alanine into cell wall material was not inhibited at

1.0 mcg./ml. of chlortetracycline, but was inhibited at 50 mcg./ml.; tetracycline had no effect on this incorporation at 50 mcg./ml. The kinetics of incorporation of the amino acids into cytoplasmic protein revealed that inhibition was immediate. Morrisson et. al., (18, 21) provided further evidence in support of the theory that the major effect of the tetracyclines is an impairment of protein synthesis. Černý and Haberman (33) have made kinetic measurements of the inhibition of protein and nucleic acid synthesis in cultures of E. coli C at tetracycline concentrations of 20 to 50 mcg./ml. They found an immediate cessation of protein synthesis followed by a latter, complete inhibition of nucleic acid synthesis. These concentrations are sufficient to cause cell death and it is unfortunate that these workers did not employ additional lower antibiotic concentrations. Holmes and Wild (34), during the course of investigations of the synthesis of abnormal ribosomal RNA, have reported also at high chlortetracycline concentrations, results similar to those of Černý and Haberman. They observed an immediate cessation of protein synthesis and continued RNA synthesis. Several additional tetracycline analogs have also been studied and similar findings reported (35, 36). Their measurements were completed approximately within the time of one culture generation cycle and they did not observe the inhibition of mucleic acid synthesis which occurs at longer growth times. These authors seem to feel that ribosomal precursor RNA accumulated under antibiotic treatment is not only the result

of an inhibition of protein synthesis, but also due to a specific stimulatory effect of the tetracyclines.

Numerous investigators have reported that the tetracyclines inhibit the formation of adaptive enzymes. Snell and Cheng (1) as well as Laskin (17) have listed these investigations and have emphasized the low concentrations of antibiotic needed to inhibit these phenomena. Sörm et. al., (37) in addition to Hahn and Wisseman (29), have tried to interpret their data as meaning that tetracycline caused an impairment of the overall protein synthesizing ability. These authors showed that the inhibition of glutamic acid decarboxylation was related to the synthesis of the enzyme and not to a lack of necessary co-factors.

Snell and Cheng (1) have cautioned against drawing such general conclusions from this type of data. They believe that an inhibition of adaptive enzyme formation might not be sufficiently critical to account for bacteriostasis. They were also not sure at that time, 1961, that an inhibition of protein synthesis would be able to cause the effects observed in bacterial cultures. They, however, felt that blocking a reaction in the electron transport system or an impairment of cell wall synthesis would be sufficiently critical. An overall inhibition of protein synthesis is presently thought to be critical enough to cause bacteriostasis.

B.4. Effects on Cell-Free Nitro-reductase Systems

Early reports indicated that high concentrations of chlortetracycline were capable of uncoupling oxidative phosphorylation in normal mitochondria (38) as well as in rat liver

and brain slices (39). An interference with orthophosphate uptake was confirmed in the studies of Van Meter et. al., (40). The inhibition by chlortetracycline in rat liver mitochondria at low adenosine triphosphate (ATP) and phosphate concentrations was shown to be reversed by added Mg ions. Similar inhibitions and metal ion reversal were reported for oxytetracycline and tetracycline by Brody, Hurwitz and Bain (41). These reports suggested that the inhibition of protein synthesis reported by Gale and Paine (28) might be due to disruption of the electron transport system, although a similar inhibition of nucleic acid synthesis might have been expected. Inhibition was postulated to occur because of interactions with metallo-enzymes known to be important in these systems. Miura et. al., (42) showed that oxytetracycline was able to reduce incorporation of P^{32} into acid soluble phosphorus in intact resting cultures of S. aureus at growth inhibitory concentrations. However, these experimental results in addition to being highly non-specific, could be interpreted as due to the improper use of controls. Control cells were allowed to enter the log phase of growth while inhibited cells did not leave the stationary phase of growth. In a review of oxidative phosphorylation, Brody (43) reached the conclusion that the uncoupling caused by these antibiotics was non-specific and was most probably the result of removal of essential metal ions. Hunter and Lowry (44) in a later review disagreed with this viewpoint because of the large Mg ion/tetracycline ratio required to reverse the inhibition. However, they believed that inhibition of these mammalian

systems by tetracyclines occurs at such high concentrations as to make unlikely the postulate that this type of activity is responsible for the compound's antibiotic activity. Gomazkov (45), Santi (46) and Morikawa et. al., (47) have recently studied these inhibitions and also noted that the high antibiotic concentrations required for inhibition make this an unlikely mode of action in bacterial cells.

The possibility suggested by this early work, that tetracyclines function as inhibitors by chelation of essential metallo-enzyme systems, prompted Saz and co-workers to search for other similar bacterial systems. Saz and Maramur (48) reported the inhibition of a cell-free nitro-reductase system by chlortetracycline in 1953. The reversal of this inhibition by manganese ions was reported by Saz and Slie (49, 50).

These extracts were capable of mediating the reduction of a large number of aryl compounds (51) as well as the reduction of nitrate to nitrite. The aryl nitro reduction was inhibited by chlortetracycline, but not oxytetracycline and neither compound interfered with nitrate reduction.

In addition to chlortetracycline, several of its degradation products were active inhibitors of the reductase, i.e. aureone amide, aureomycinic acid, anhydrotetracycline, isochlortetracycline and 4-dedimethylamino-aureomycinic acid. Several of these compounds were also active in reductase experiments performed with whole cells, although they are not all bacteriostatic agents.

Further studies (52) with more purified nitro-reductase systems showed that they required ATP, diphosphopyridine nucleotide (DPN) and a dicarboxylic acid as well as cysteine and manganese ions. If reduced diphosphopyridine nucleotide (DPNH) was used in place of DPN and L-malate, the reduction of aryl nitro compounds was no longer inhibited by chlortetracycline.

As a result of these experiments, Saz and co-workers proposed that chlortetracycline was an inhibitor of the formation of DPNH from L-malate and DPN. They thought that it accomplished this inhibition by preventing further metabolism of oxaloacetic acid, the major product of the DPN-malate reaction. It was known that oxaloacetic decarboxylase has a manganese ion requirement and this enzyme was the presumed site of action. The site of action was proposed to be specific for the chlorine containing tetracyclines as oxytetracycline or tetracycline did not inhibit the system. The ability of the inactive as well as active chlorine containing tetracyclines to inhibit the system made it a poor choice for the site of antibacterial action of these compounds.

Saz and associates next directed their attention to purified extracts obtained from both sensitive and resistant E. coli. These more highly purified extracts retained some but not all of the original nitro-reductase activity. While the extracts were no longer able to reduce DPN to DPNH, i.e., they were devoid of malic dehydrogenase activity, they were

still able to reduce chloramphenicol or p-nitrobenzoic acid. Since this reduction of p-nitrobenzoic acid was inhibited by chlortetracycline they were forced to conclude that their previously proposed mechanism was not correct for the new reductase (53).

Extracts obtained from sensitive cells were shown to be greatly stimulated by flavin mononucleotide and manganese ions while those from resistant cells were not stimulated. Kinetic experiments demonstrated that the extracts from sensitive cells oxidized stoichiometric amounts of DPNH in the reduction of FMN and the production of p-aminobenzoic acid. Saz and Martinez (54) interpreted these results to mean that flavin and manganese were loosely associated as co-factors with enzymes in sensitive cells while they were more tightly bound in resistant cells. Plakunov (55) in a study of resistant strains has, however, reported that the differences are more likely ascribable to differences in the relative amounts of FMN and flavin adenine dinucleotide (FAD) found in resistant and sensitive strains of S. aureus.

The hypothesis that tetracyclines inhibit by interfering with flavins has been studied by Yagi et. al. (56). They showed that the mode of action of these compounds in the inhibition of D-amino acid oxidase was by complexation with FAD. They found that chlortetracycline formed highly stable complexes with riboflavin, FMN and FAD. They also showed that the kinetics of inhibition of the oxidation of D,L-alanine by the enzyme-FAD preparation could be explained by assuming the

reversible formation of an inactive chlortetracycline-FAD complex.

Continued purification of the reductase system by Saz and Martinez (57, 58) has not demonstrated that it is the site of bacterial resistance to chlortetracycline. They isolated a protein-stabilized enzyme preparation which catalyzed the reduction of 2,6-dichlorophenolindophenol. This enzyme preparation was inhibited by chlortetracycline when prepared from sensitive but not when prepared from resistant strains. However, chlortetracycline inhibited the reduction of ferricyanide and stimulated the reduction of cytochrome c by extracts prepared from both sensitive and resistant strains. Villanueva (59) in a study of the nitro-reductase of extracts from both sensitive and resistant Nocardia has recently reached the conclusion that there is no difference in reductase activity.

Krćmery and associates (60) have continued work on the hypothesis that development of tetracycline resistance as well as a possible mode of action lies in the electron transport chain. They have shown that triphenyltetrazolium chloride reduction by whole cells of sensitive strains but not by whole cells of resistant strains is inhibited by oxytetracycline. The degree of inhibition varied markedly with the type of carbon source in the growth media. Further studies (61) showed that malate and lactate dehydrogenase (MDH, LDH) activities differed in sensitive and resistant strains. Resistant cells (62) were also not able to reduce tellurite (K_2TeO_3) while

the sensitive strains had this ability, but were inhibited by oxytetracycline. Crude cell-free preparations which catalyzed MDH, LDH and NO_3^- reduction showed similar differences among sensitive and resistant strains. Recently (63) these workers have reported that cytochrome oxidase activity in sensitive strains of S. aureus, E. coli and Brucella abortis are inhibited by short exposure to oxytetracycline. Resistant strains are not affected under the usual aerobic conditions but are inhibited only under anaerobic conditions. These workers believe that oxytetracycline interferes with microbial enzyme systems involved in electron transport and that resistant strains have developed alternate pathways. Several other workers believe, however, that resistance to tetracyclines is associated with an altered permeability of the cells (64, 65, 66).

Colaizzi, Knevel and Martin (11) used mammalian cytochrome C oxido-reductase system to obtain biological activities for use in structure-activity correlations. While many of the active tetracyclines were inhibitors of this system, several tetracyclines which are not considered bacteriostatic were equally good inhibitors. The authors felt that this might be explained by differences in ability to permeate cell walls. However, Saz and co-workers (51) have previously noted that such reasoning was not valid in explaining differences in activity in their nitro-reductase system. The high concentrations (10^{-3} M) of antibiotic necessary for inhibition of this system are typical of results obtained with mammalian systems.

B.5. The Nature of Cell-Free Protein Synthesizing Systems - Translation of the Genetic Code

The purpose of this section is to summarize the existing knowledge of cell-free protein synthesizing systems. In this way, the reader may have a better understanding of the effects of tetracyclines upon these systems and can judge whether these effects are sufficient to account for the inhibition of growth of whole cells. In addition, several recent experiments that have led to new and detailed models of this system will be discussed.

Translation refers to the process in the synthesis of proteins by which a particular sequence of nucleotide bases in messenger RNA (mRNA) determine a specific sequence of amino acids in a polypeptide chain. Translation is accomplished by the interaction of three classes of macromolecules: mRNA, the specified amino acid covalently linked to transfer RNA (tRNA) and a complex ribonucleoprotein called a ribosome. Several excellent review articles have appeared recently which describe existing knowledge of the system in detail (67, 68, 69, 70).

The basic components of a simple cell-free protein synthesizing system are:

1. Ribosomes
2. mRNA
3. Aminoacyl tRNA (AAtRNA)
4. Ribosome associated initiation factors, f_1 and f_2
5. Non-ribosome associated (supernatant) enzymes, T and G

6. Guanosine-5' Triphosphate (GTP)

In addition to these basic components, a cell-free system usually includes a buffer, pH 7-8; monovalent inorganic salts; divalent salts or a polyamine polypeptide; and a sulfhydryl reducing agent.

More complex systems, or alternately more crude systems, may generate within the systems AAtRNA and these systems require additionally ATP, pyruvate kinase (PK), phosphoenol pyruvate (PEP), amino acids (AA), soluble RNA (sRNA) and aminoacyl synthetases. A few experiments have been performed with systems that generate mRNA simultaneously and these require DNA, DNA-dependent RNA polymerase and appropriate nucleotides.

B. 5.a. Ribosomes

Bacterial ribosomes are complex aggregates which consist of 60-65% RNA and 30-35% protein. They serve as a template for the assembly of peptide bonds between AAtRNA molecules held on the ribosomal surface. In addition to this inactive role, it is thought that peptide synthetase enzymes as well as initiation enzymes may be an integral part of the normal ribosomal surface. Because the site of action of tetracyclines is presumed to be on the ribosomal surface, a detailed description of ribosomal structure will be presented.

Ribosomal particles participate in a series of reversible association reactions which are illustrated in Fig. III. The species actively associated with protein synthesis has been shown to be the 70 S ribosomes (71), which have a

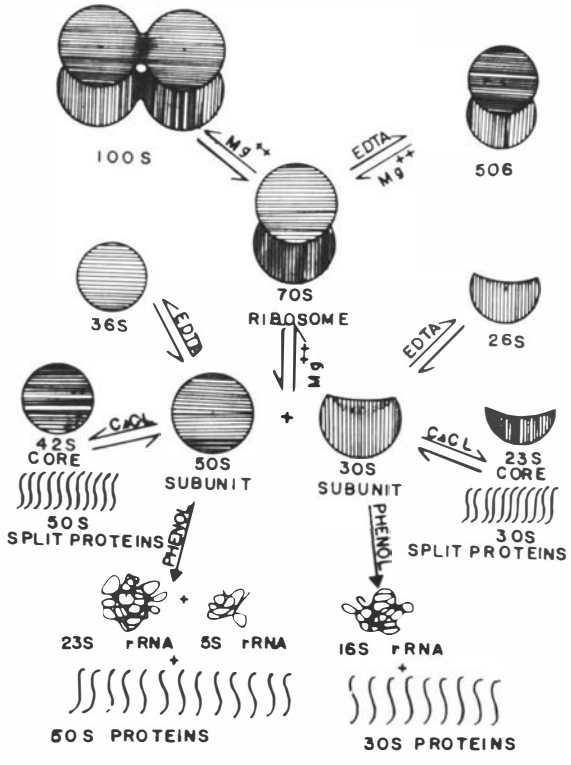

Figure III. Ribosomes, Components, Subunits, Association and Dissociation Reactions. Shaded circles depict the ribosomal particles, scrambled lines depict the ribosomal ribonucleic acids and s-shaped lines depict the ribosomal proteins for which the indicated S values refer to the sedimentation values of the original ribosome subunits.

molecular weight of approximately 2.7×10^6 and a diameter of about 200 Å. They dimerize in solutions of high Mg ion concentration in an apparently non-physiological reaction, they also undergo an apparent shape change in EDTA solutions. The dissociation of 70 S ribosomes to 50 S and 30 S subunits occurs at Mg ion concentrations similar to those found in bacteria (70, 72). The dissociation of 70 S ribosomes to subunits is inhibited by sulfhydryl blocking agents or polycationic polyamines as well as high Mg ion concentrations (73, 74). Moore (75), in a study of the effects of chemical reagents on subunit association, has concluded that hydrogen bond formation either between nucleotide bases or between a nucleotide base and a phosphate group are responsible for this association.

The 30 S subunit consists of a single RNA molecule, 16 S rRNA, with a molecular weight of approximately 0.6×10^6, and at least 15 specific proteins (76). The 50 S subunit consists of 2 molecules of RNA, one a 23 S rRNA and the other a 5 S rRNA, as well as at least 19 specific proteins (77).

The 5 S species of rRNA was first isolated in 1964 by Rossett et. al. (78) and was shown to be distinct from sRNA which has a similar sedimentation coefficient. The entire nucleotide sequence of 5 S rRNA from KB carcinoma cells has been determined by Sutherland, Carrier and Setlow (79), and also that of E. coli, by Brownlee and Sanger (80) and Brownlee, Sanger and Barrell (81). This ribosomal RNA like 16 S and 23 S rRNA contains no unusual nucleotides.

The secondary structure of this relatively new rRNA is also thought to be similar to that of both 16 S and 23 S rRNA. It consists partly of double stranded helical regions and partly of non-hydrogen bonded single strands (82). Extensive studies on the physical and chemical properties of rRNA have been reviewed (85).

The 5' terminal nucleotide sequences of E. coli 16 S and 23 S rRNA have been determined by Takanami (83, 84). These determinations show that the two rRNA's are distinct species.

The protein components of ribosomes have been studied in considerable detail. The large number of proteins indicated in Fig. III, have been isolated and studied by: end-group analysis (86), ultracentrifugation (87, 88), starch gel electrophoresis (86), polyacrylamide gel electrophoresis (76, 77, 87, 90, 91) and chromatography on carboxymethylcellulose (91) and Sephadex (91). They are unique proteins with distinct amino acid analysis (89, 91, 92) and are not the result of aggregates formed during preparation. The large number of ribosomal proteins argues against the theory that rRNA could serve as a mRNA for their synthesis. Ribosomal RNA does not contain enough trinucleotides to synthesize this many large, distinct proteins (70).

It seems likely that ribosomal proteins play distinct and non-interchangeable roles (93, 94, 95). Their functional specificity has been investigated by a degradation - recombination technique. In these experiments the proteins are partially split from the ribosomal subunits with CsCl (Fig. III)

and the liberated core particles and split proteins are separated, isolated and identified. Normal ribosome function can be achieved by mixing the isolated components. Ribosomes reconstituted in this manner but lacking one or more split proteins have been shown in some cases to lack one or more, but not all, ribosomal functions (94). This technique has been used to show that streptomycin sensitivity, resistance and dependence lie with the 23 S core particle and not with the split proteins (96, 97). Sensitivity and resistance have, however, been shown to lie in the protein fraction of the core particles and not in the rRNA (98).

EDTA treated ribosomes contain all known ribosome components but probably because they have different hydrodynamic properties they lack certain specific ribosomal functions (99).

Treatment of ribosomes with LiCl causes a loss of protein components similar to that which is produced by cSCl. LiCl-ribosomes are now known to be identical to the particles found in bacterial cultures inhibited by chloramphenicol (100). These ribosomal particles are probably the same as those found in tetracycline inhibited cultures (34, 36). Such ribosomes are thought to be normal precursor particles.

The effect of Mg ion concentration on the association-dissociation of ribosomes is outlined in Fig. III. It has recently been shown that Mg ion is required to neutralize the phosphate groups in the ribosomal RNA. "Normal" 70 S ribosomes exist in an ionic environment in which the ratio of

ribosomal-bound Mg ion to ionized ribosomal phosphate is 0.5 (72, 101, 102, 103). Cationic groups, such as Ca^{++}, NH_4^+, spermidine or putrescine, in the proper concentrations are also capable of catalyzing the formation of functional ribosomes (72, 102, 104, 105, 106, 107).

B. 5.b. Messenger Ribonucleic Acid

The existence of a species of rapidly synthesized, unstable RNA, called mRNA, was predicted in a review by Jacob and Monod (108) in 1961. The demonstration of the existence of such a species has been accomplished by experiments based on two phenomena: its rapid rate of synthesis and turnover as compared with rRNA or sRNA and also the synthesis of this type of RNA in phage-infected cells. The predicted function of mRNA was that it carried the information of the genetic code from DNA to the ribosomal template, where it directed the sequence of addition of amino acids into protein. Evidence in support of this prediction has been reviewed by Simpson (109) and also by Singer and Leder (110). Convincing evidence for this role has been found in the demonstration of mRNA in peptide-synthesizing polysomes (ribosome-mRNA aggregates) (111), in the synthesis of phage proteins of known sequence by bacterial systems directed by phage mRNA (112) and in the coincidence of amino acid sequence and nucleotide sequence of synthetic polyribonucleotides (113).

Translation of the code is not accomplished by a direct interaction of an amino acid with a specific trinucleotide sequence (codon) of a mRNA, but rather through an intermediate

adaptor molecule, tRNA, covalently linked to an amino acid. The tRNA contains a specific trinucleotide sequence (anticodon) that recognizes the codon. The codons which direct the incorporation into protein of particular amino acids have been determined by studies of the binding of AAtRNA's to ribosomal-codon, where the codon was either a trinucleotide (114) or a polyribonucleotide of known sequence (115). Similarily incorporation of AAtRNA's into protein in systems directed by polyribonucleotides of known sequence (116) has also been used to determine the codons which direct the incorporation of particular amino acids. The four common nucleotide bases when utilized in a trinucleotide code can be arranged in 64 possible combinations. Since there are only 20 amino acids, degeneracies might be expected and indeed are found in the code. The isolation of several different tRNA's capable of accepting a given amino acid has led to the speculation that there may be a particular tRNA corresponding to each codon. Different leucine-tRNA species have been shown to selectively bind to different codons (117). However, it is unlikely that this is the case for all tRNA molecules (118).

To account for the known degeneracies in the code, Crick has introduced a theory of codon - anticodon pairing, called the "Wobble Hypothesis" (119). This theory states that the binding of the first two bases of the codon (5' to 3') with the appropriate bases of the anticodon (3' to 5') follows strict Watson-Crick hydrogen bond base pairing. The interaction of the third base of the codon with the third base of the anticodon

is not specific, but has certain allowable exceptions. These exceptions are not random but may be predicted by a consideration of the bond distances associated with the non-classical hydrogen bonding of codon to anticodon. The single exception to the known code degeneracies occurring in the third base of the codon is in those sequences capable of coding for the initiator, N-formyl-methionyl-tRNA (N-F-met-tRNA$_f$) where degeneracies in the first base of the codon exist (120). Interpretation of this phenomena has not been made.

Since it is known that hydrogen bonding between trinucleotides is not sufficiently strong to stabilize mRNA-AAtRNA complexes, the stablilizing role of the ribosome must be emphasized.

B. 5.c. Transfer Ribonucleic Acid

The adaptor molecule, tRNA, is a relatively small molecule, 4-5 S, which forms an ester with activated amino acids in the presence of amino acid synthetase. This reaction is a relatively well understood area of biochemistry; it is depicted in schematic form in Fig. IV and has been described in several reviews (121, 122). That tRNA functions as an adaptor molecule has been shown by demonstrating that cysteine-tRNA, chemically converted to alanine-tRNA, leads to the incorporation of alanine in place of cysteine into protein (123).

The nucleotide sequences of at least four tRNA molecules have been determined completely (124, 125, 126, 127). A common feature of these and all other tRNA's which have been studied is a terminal ...pCpCpA nucleotide sequence. It is this part

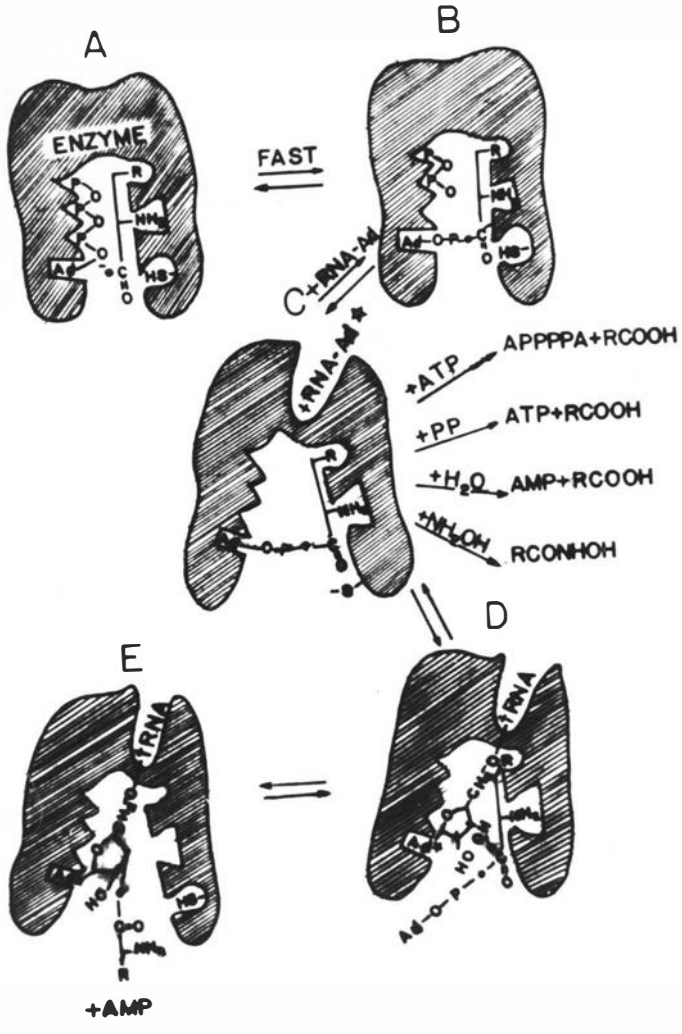

Figure IV. The Formation of Aminoacyl Transfer Ribonucleic Acid. (A) Binding of ATP and an amino acid to the enzyme. (B) Formation of an "activated amino acid." (C) Binding of tRNA to the enzyme. (D) Displacement of the original adenylate bound to the enzyme by the terminal adenylate of the tRNA. (E) Formation of the ester, AAtRNA.

of the molecule which accepts the amino acid (see Fig. IV) and which is probably involved in the binding of the AAtRNA to the 50 S ribosomal subunit (128).

In addition to the above mentioned amino acid accepting site, each species of tRNA must contain a region which enables it to accept, exclusively, the proper amino acid (recognition site) and also a region which contains the appropriate anticodon for the amino acid accepted. Regions in tRNA which might contain these sites have been predicted on the basis of a clover-leaf secondary structure postulated for tRNA. This is shown in a general manner, following Fuller and Hodgson (129) in Fig. V. This structure was first proposed by Holley et. al. (124) for yeast ala-tRNA. It is based on studies of hydrogen bonding and the results of X-ray diffraction studies which have shown that tRNA's exist in partly helical and partly non-helical conformations. A possible codon - anticodon interaction, shown in Fig. VI has been given by Fuller and Hodgson (129).

B. 5.d. A Model of Cell-Free Protein Synthesis

The sequence of events in a cell-free protein synthesizing system is shown in Fig. VII. They may be outlined as follows:

Step 1. - Binding of a 30 S ribosomal subunit to a mRNA.

Step 2. - Formation of the initiation complex.

a. - Alignment of the codon for N-F-met-$tRNA_f$ on the ribosomal surface so that it may bind to the anticodon, i.e., creation of the N-F-met-$tRNA_f$ or peptidyl binding site.

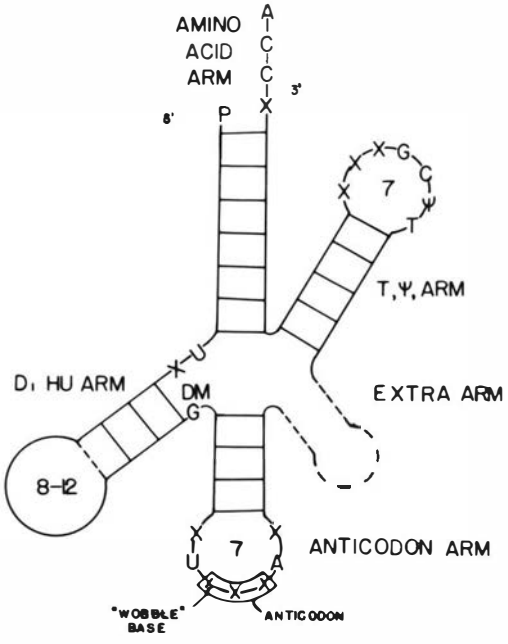

Figure V. Schematic Diagram of the Proposed Clover-Leaf Structure of Transfer Ribonucleic Acids. Hydrogen bonds are indicated as lines linking the strands. The number of nucleotides in each single stranded loop is indicated within the loop. X indicates a nucleotide which varies with the tRNA species, Ψ is pseudouridylic acid, T is thymidylic acid, DMG is dimethylguanylic acid.

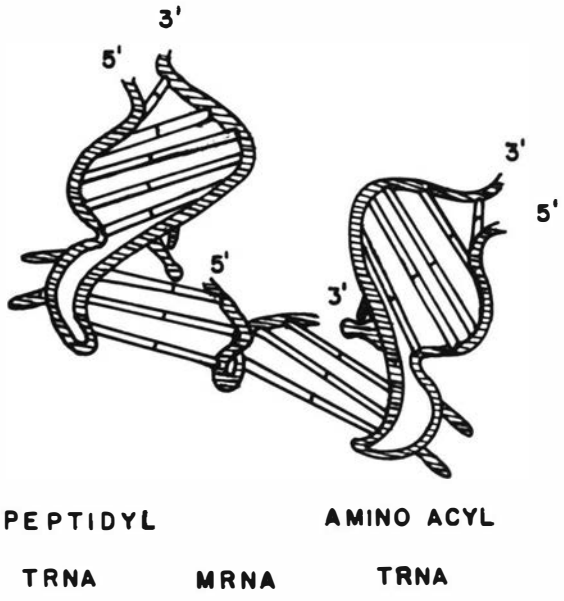

Figure VI. Codon - Anticodon Interaction. Hydrogen bonds are indicated by double lines linking the ribonucleic acids; depicted by shaded lines. A helical relationship is implied between the anticodon loops of the tRNA's and the mRNA. Only the anticodon arms of the tRNA's are shown, see Figure V.

Figure VII. A Model of Cell-Free Protein Synthesis. Shaded half-circles depict 30 S ribosomal subunits, shaded circles depict 50 S ribosomal subunits, a wavy line depicts mRNA and tRNA's are depicted by the symbol,  , the s-shaped curve depicts a protein.

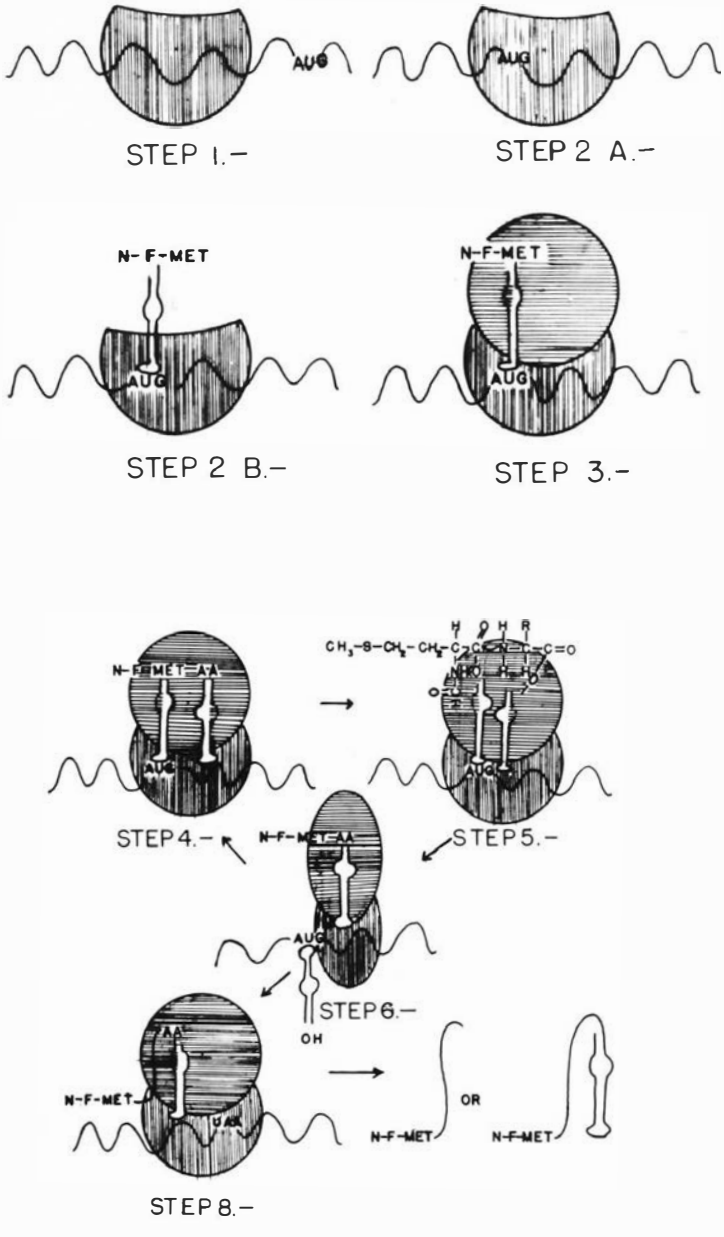
AUG
STEP 1.-
AUG
STEP 2 A.-
N-F-MET
AUG
STEP 2 B.-
N-F-MET
AUG
STEP 3.-
N-F-MET-AA
AUG
STEP 4.-
STEP 5.-
N-F-MET-AA
AUG
STEP 6.-
OH
AA
N-F-MET
UAA
OR
N-F-MET
N-F-MET
STEP 8.-

Figure VII.

b. - Binding of N-F-met-$tRNA_f$ to the ribosome-codon in a reaction which is stimulated by and may require the initiation factors and GTP to form the so-called initiation complex.

Step 3. - Attachment of the 50 S subunit to the initiation complex to form a monosome. This binding simultaneously creates a binding site for AAtRNA. The aminoacyl binding site corresponds to the 3' end of the messenger.[1]

Step 4. - Binding to the aminoacyl site on the 70 S ribosome. Either AAtRNA alone or the complex AAtRNA-GTP-T may fill this site, where T is a supernatant protein.

Step 5. - Formation of the peptide bond. N-F-met-AAtRNA would be the first peptide formed on the ribosome. This reaction may be catalyzed by a tightly bound ribosomal peptidyl synthetase.

Step 6. - Pulsation (Movement) of the ribosome.

a. Removal of $tRNA_f$ from the peptidyl site, either to an exit site or directly into the surrounding solution.

---

[1]If attachment of a 70 S ribosome to mRNA occurs in Step 1 rather than a 30 S subunit, then both peptidyl and aminoacyl binding sites may be created at that time. Alignment of the codon may be such that N-F-met-tRNA may bind to either site. If binding to the aminoacyl site occurs, then translocation of N-F-met-tRNA is required before the next step. Step 3 is then, of course, not necessary. Steps 1 to 3 may be repeated to form a polysome with different ribosomes attaching to the same mRNA after incorporation of about ten amino acids into a polypeptide.

b. - A simultaneous shift of the mRNA and N-F-met-AAtRNA on the ribosome surface so that a new codon is aligned under the aminoacyl site and the peptidyl tRNA occupies the peptidyl site.

Step 6 is accompanied by hydrolysis of GTP catalyzed by G, a ribosome-dependent GTPase.

Step 7. - Steps 4, 5 and 6 are repeated to form an increasingly longer peptide chain.

Step 8. - Chain termination and release.

a. - Either repetition of Step 4 is prevented by alignment of a nonsense codon with the aminoacyl site or Step 5 is prevented by occupation of the aminoacyl site by a non-functional sRNA bound by the nonsense codon. (Such a nonfunctional sRNA which binds to ribosome-nonsense codon has not as yet been found).

b. - Hydrolysis of the peptidyl-tRNA on the ribosome followed by ejection of the peptide and tRNA or removal of an intact peptidyl-tRNA from the ribosome and subsequent hydrolysis to peptide and tRNA.

Several steps in the proposed model have been studied in great detail and their nature is at least partially understood. A lack of specific techniques has prevented thorough investigation of some of the other steps.

Step 1. - Binding of a 30 S subunit to mRNA.

Attachment of mRNA to the 30 S subunit rather than the 50 S subunit was demonstrated by Okamoto and Takanami (130). Binding of mRNA to 70 S ribosomes has been presumed to occur through the attachment to the 30 S subunit and not because of the formation of some new site which does not exist on either subunit alone. Moore (131, 132) has presented evidence that there is only one binding site for mRNA on 70 S ribosomes and that poly C and poly U compete for this site. The attachement of mRNA's to ribosomes is thought to involve only single stranded regions of the mRNA, since double stranded mRNA's do not bind (133). This observation accounts for the strong binding of poly U to ribosomes since it exists as a random coil above 4°C. The length of mRNA bound to ribosomes has been estimated to be about 27 nucleotides. This estimate is based upon the number of nucleotides which are protected from ribonuclease hydrolysis by their attachment to ribosomes. Moore (75) has used chemical modifications of ribosomes and/or mRNA to study their interaction. He found that the most likely groups involved in the binding were amine groups in the rRNA and the phosphate backbone of the mRNA.

Step 2. - Formation of the initiation complex.

Since ribosomes directed by natural mRNA's synthesize complete proteins there must be control mechanisms whereby initial reading of mRNA by ribosomes is restricted to a specific site. Such control might result from attachment of ribosomes at a specific site on mRNA. This mechanism would not be sufficiently

strict to allow proper phasing of the mRNA readout, since the only requirement for binding is that the mRNA be single stranded. (Phasing is the alignment of the first base of a trinucleotide codon whose translation is desired on the ribosome, so that it specifies the readout. When alignment is such that the second or third base specify the code readout then the reading of the mRNA is said to be out of phase.)

In 1961, Waller (134) discovered that methionine was the NH_2-terminal group in at least 45% of all E. coli proteins. Following this report, Marcker and Sanger (135) observed that one species of met-tRNA could be formylated (met-$tRNA_f$) while other species could not be formylated (met-$tRNA_m$). A likely formyl donor was shown to be N^{10}-Formyl-tetrahydrofolic acid (136). They showed that formylation occurred only after formation of the AAtRNA and that an enzyme distinct from methionyl synthetase was involved. Clark and Marcker (137) observed that N-F-met from N-F-met-$tRNA_f$ was incorporated into the NH_2-terminal position of proteins synthesized by a cell-free extract. Later (138) they showed that cell-free extracts directed by poly UG and capable of formylating met-$tRNA_f$ produced proteins in which more than 50% of the NH_2-terminal groups were N-F-met. This finding seemed to explain Waller's early report (134).

Adams and Capecchi (139) showed that N-F-met-ala-ser-asp--phe-thr- was the initial amino acid sequence in R 17 phage coat protein produced by an extract directed by R 17 messenger. Since the identical sequence without the initial N-F-met was

found in endogenous coat protein, these workers proposed that N-F-met-tRNA$_f$ was the universal bacterial chain initiator, but that in some cases either the terminal N-Formyl or the N-F-met groups were removed by hydrolysis. Webster, Englehardt and Zinder (140) reported similar findings. Capecchi (141), Weisbach and Redfield (142) and Lugay and Aronson (143) have studied the hydrolysis of NH_2-terminal formate in cell-free extracts and have attempted to isolate the responsible enzyme. Viñuela, Salas and Ochoa (144) found that all viral proteins as well as the coat proteins synthesized in systems directed by MS-2 phage RNA had N-F-met as the NH_2-terminal group.

In addition to demonstrating that N-F-met from N-F-met-tRNA$_f$ was the first amino acid incorporated into protein by cell-free extracts. Clark and Marcker (137, 138) proposed that the difficulty in assigning a codon to met-tRNA might be due to the existence of two different codons, one codon for met-tRNA$_f$ and another for met-tRNA$_m$. Sundararajan and Thach (145) have established that binding of met-tRNA$_f$ to ribosomes is best when AUG is the codon, but other triplets also code for it at high Mg ion concentrations. Met-tRNA$_m$ is only coded for by AUG. They showed that N-F-met-tRNA$_f$ was capable of phasing the reading of synthetic mRNA's, such as AUGG(U)$_n$. In the presence of N-F-met-tRNA$_f$ binding of val-tRNA in response to the second triplet, GUU, was stimulated. Out of phase binding of other AAtRNA's in response to UGG or GGU was suppressed. Binding experiments with other synthetic mRNA's, including several with the AUG codon not at the 5' termini, gave similar results. Binding

above background did not occur at low Mg ion concentrations if the AUG codon was located at the 3' end of the mRNA. Thach, Dewey, Brown and Doty (146) reported similar results when incorporation of amino acid into protein was used rather than ribosome-codon binding. They noted that if an AUG codon appeared in a mRNA at a position internal to another AUG, this second codon always directed the incorporation of met-$tRNA_m$. They felt that alignment of the AUG codon at the peptidyl site specified chain initiation by N-F-met-$tRNA_f$, while alignment of the codon at the aminoacyl site specified chain continuation with met-$tRNA_m$. Leder and Bursztyn (147) have confirmed these findings on phasing of readout by using $AUG(U)_n$ as a mRNA and measuring the incorporation of met, val and phe. They also showed that N-F-met-tRNA bound during binding experiments was not displaced from ribosomes by the addition of other AAtRNA's. This finding in conjunction with later results of Nomura, Lowry and Guthrie (148) show that binding of N-F-met-$tRNA_f$ has relevance in peptide synthesis.

A solution to the puzzling enigma that cell-free extracts directed by synthetic mRNA's require higher Mg ion concentrations than those directed by endogenous mRNA was suggested by Clark and Marcker (137). They noted that met-$tRNA_f$ binds to ribosome-codon at much lower Mg ion concentrations than those which are necessary for binding met-$tRNA_m$. This observation was also reported by Sundararajan and Thach (145). Nakamoto and Kolakofsky (149) have shown that the Mg ion optimum of a system incorporating phe-tRNA but directed by a mRNA which

allows incorporation of N-F-met-$tRNA_f$ was greatly lowered by including the latter AAtRNA in the reaction mixture. Kolakofsky and Nakamoto (150) studied the Mg ion dependency of extracts directed by MS-2 viral RNA. They found a lowered Mg ion optimum and a large stimulation of overall incorporation when a formyl donor system was added to the extract. The synthesis of coat protein and also other viral proteins was shown to follow this pattern.

Clark and Marcker's original supposition (137) that N-F-met-$tRNA_f$ was capable of initiating protein synthesis because the formamide bond resembled a peptide bond was shown to be false by these same workers (138, 151). They demonstrated that met-$tRNA_f$ as well as N-F-met-$tRNA_f$ could act as an initiator, Leder and Bursztyn (147) and also Salas et. al., (152) confirmed this result. Leder and Bursztyn (147) suggested that formylation might take place after attachment to the ribosome-codon. They showed that this type of ribosomal formylation was possible. Economou and Nakamoto (153) have studied the kinetics of the formylation reaction and concluded that the reaction is so fast that met-$tRNA_f$ would be formylated before binding to ribosomes in any extract capable of performing the reaction. It must, however, be concluded that the unique ability of N-F-met-$tRNA_f$ to initiate protein synthesis lies in the $tRNA_f$ portion of the molecule and not in the formamide bond itself.

Incorporation of met- from met-$tRNA_m$ into internal portions of peptides synthesized by cell-free extracts was reported by Thach et. al., (146). Clark and Marcker (151) confirmed this

finding in systems directed by bacteriophage RNA, Stanley et. al., (154) and Salas et. al., (155) reached similar conclusions using synthetic mRNA's containing the AUG codon.

In summary, these results have been interpreted as reflecting the existence of two binding sites on the ribosome. This theory states that N-F-met-tRNA$_f$ can uniquely bind to the peptidyl site in response to the AUG codon, while met-tRNA$_m$ is bound when the AUG codon is aligned with the aminoacyl site. Matthaei and Voigt (156) have demonstrated the existence of binding sites on the ribosome-codon with two different Mg ion optima, one of which binds N-F-met-tRNA$_f$ and the other which binds met-tRNA$_m$.

The reactivity, in the absence of supernatant factors, of ribosomal bound N-F-met-tRNA$_f$ with puromycin (Pm) is consistent with the conclusion that N-F-met-tRNA$_f$ occupies the peptidyl site (151, 158). Other ribosomal-codon bound AAtRNA's do not have this reactivity in the absence of supernatant. Hershey and Thach (159) have reported that the Pm reaction in their extracts, as well as the formation of the dipeptide N-F-met-phe was strongly stimulated by GTP and supernatant enzymes. They propose that N-F-met-tRNA$_f$ is initially bound to the aminoacyl site of the ribosome and is then transferred in a GTP-dependent step to the peptidyl site -this has been termed the single entry site model. Alternately, binding may occur directly with the peptidyl site but a GTP-dependent chemical activation of the bound met-tRNA$_f$ must then occur before dipeptide formation - this has been

termed the peptidyl site activation model.

Stanley et. al., (154) and Salas et. al., (155) have isolated two protein components, f_1 and f_2, from salt washings of E. coli ribosomes. These proteins are required for the translation of natural messengers, but not for synthetic mRNA, unless they contain the AUG codon. Translation of synthetic mRNA's which contain AUG is greatly stimulated by these factors. This stimulation results from an increased incorporation of met-$tRNA_f$ but not met-$tRNA_m$. These factors stimulate incorporation only at low Mg ion concentrations and do not promote the binding of other AAtRNA's. Andersen, Bretscher, Clark and Marcker (160) and Thach, Dewey and Mykolajewycz (161) and Leder and Nau (162) have substantiated these results and have shown that the binding promoted by these factors is stimulated by addition of GTP. It seems likely that in the presence of these factors and GTP, N-F-met-$tRNA_f$ is bound either directly to, or rapidly transferred to, the peptidyl site. Binding, in their absence, may be to the aminoacyl site.

Similar factors have been isolated by Eisenstadt and Brawerman (163, 164) and Revel and Gros (165, 166). These authors have also noted the possible existence of a third factor, retarded on DEAE-cellulose, which may stimulate or may be an essential requirement for the binding of ribosomes to mRNA in the presence of DNA.

Step 3. - Attachment of the 50 S subunit to the initiation complex.

Binding of AAtRNA's to 30 S subunits has been studied by Kaji, Suzuka and Kaji (167) and Pestka and Nirenberg (168).

They have found that at high Mg ion concentrations, specific binding of AAtRNA's occurs with one site of the subunit. The addition of 50 S subunits (169) causes a 2-3 fold increase in the observed binding, presumably by creating an additional binding area. This may be an extra point of attachment of the tRNA at the same binding site or an entirely new binding site. Since binding in the presence of 50 S subunits has different metal ion cofactor requirements (168), it most likely reflects a new binding site. Matthaei and Milberg (170) have interpreted their results on the kinetics of AAtRNA binding in terms of the creation of a new site upon addition of 50 S subunits. Igarashi and Kaji (171) have shown that phe-tRNA bound to 30 S subunit-codon is dissociated by addition of 50 S subunits and that such prebound AAtRNA's are not incorporated into peptides. This result indicated that binding of AAtRNA to 30 S subunits is non-functional and extraneous.

Recently, however, Nomura and Lowry (172) have reinvestigated the area. They showed that f-2 phage RNA directs the binding of N-F-met-$tRNA_f$ to 30 S subunits at low Mg ion concentrations and that such binding is not stimulated by the addition of 50 S subunits. This binding was stimulated by the initiation factors, f_1 and f_2, and by GTP when salt-washed ribosomal subunits were studied. The viral RNA did not direct the binding of phe-, leu-, lys-, tyr-, or val-tRNA or met-$tRNA_m$ to 30 S subunits. These AAtRNA's were bound however to 30 S subunits at high Mg ion concentrations,

when appropriate synthetic polyribonucleotides were used. This binding was stimulated by the addition of 50 S subunits as had been previously reported (168, 169). These results indicated that binding of N-F-met-tRNA$_f$ to 30 S subunits is different from the binding of other AAtRNA's. Nomura, Lowry and Guthrie (148) in a similar contrast, have reported that the addition of 50 S subunits to N-F-met-tRNA$_f$ bound to 30 S subunit-codon does not cause dissociation of the bound AAtRNA; rather the N-F-met-tRNA$_f$ is now bound to 70 S ribosomes.

Nakada and Kaji (173) had shown earlier that native (as opposed to dissociated) 30 S and 50 S subunits do not combine in 10^{-2} M Mg ion, but are able to synthesize polyphenylalanine in the presence of poly U. The ribosome found associated with poly U in these experiments is a 70 S ribosome. These results indicate that mRNA catalyzed the association of native subunits. Eisenstadt and Brawerman (174) have found similar association of native subunits in the presence of f-2 phage RNA and believe such monosomes are more active in protein synthesis than 70 S ribosomes. They believe that 70 S ribosomes must dissociate prior to participating in peptide synthesis.

Mangiarotti and Schlessinger (175) developed very gentle extractive techniques to study polysome distribution in E. coli. With these techniques, they showed (176) that E. coli do not contain free 70 S ribosomes but rather have a pool of dissociated 30 S and 50 S subunits. Kaempfer, Meselson and Raskas (177) confirmed the existence of such a subunit pool by

growing E. coli in heavy and light isotope media. They found that ribosomes produced in heavy media dissociated and reassociated in very short time periods with ribosomal subunits synthesized subsequently in light media. Subunits, however, once synthesized did not equilibrate with rRNA or ribosomal proteins.

Mangiarotti and Schlessinger (176) and Nomura and Lowry (172) were led by their quite different experiments to propose that protein synthesis is initiated by attachment of a 30 S subunit to mRNA after which a 50 S subunit is added.

Hille, Miller, Iwaki and Wahba (178) have confirmed the binding studies of Nomura and Lowry. They also showed that N-F-met-tRNA bound to 30 S subunits - codon (in the presence of f_1, f_2 and GTP) was able to react with Pm when 50 S subunits were added. Ghosh and Khorana (179) have studied the binding of N-F-met-$tRNA_f$ to 30 S subunits in the presence of poly $(AUG)_n$ or poly $(UG)_n$. They confirmed the binding, the requirement for initiation factors and GTP in the binding and were able to isolate the ternary complex by sucrose density gradient centrifugation. The isolated complex was able to form a dipeptide, N-F-met-met, when 50 S subunits and met-$tRNA_m$ were added to it. A similar complex containing 70 S ribosomes instead of the 30 S subunit was also capable of forming the dipeptide. They also showed that binding of cys-, val-tRNA or met-$tRNA_m$ to 30 S subunits occurred only at high Mg ion concentration. A complex formed at high Mg ion concentration with 30 S subunits, poly $(AUG)_n$, N-F-met-$tRNA_f$

and met-tRNA$_m$ was also isolated. This complex was not able to form a dipeptide upon the addition of 50 S subunits. These results support the conclusion that only N-F-met-tRNA$_f$ binds in a functional manner to 30 S subunits - codon.

Ohta, Sarkar and Thach (180) have observed a lower Mg ion optimum for GTP-f_1 - f_2 stimulated binding of N-F-met-tRNA$_f$ to 70 S ribosomes (5m$\underline{M}$) than to 30 S subunits (8 - 9m$\underline{M}$). They postulate that this might indicate that the 70 S ribosome is attached to codon easier than the 30 S subunit. They are aware, however, that extractive procedures and storage conditions play a role in ribosomal Mg ion dependency and that the difference in the two optima is not large.

Step 4. - Binding to the aminoacyl site on the 70 S ribosome.

The binding of AAtRNA's to ribosomes is a well known phenomena which has been reviewed by several authors (128, 181, 182). Experiments designed to test for this binding have been used to establish the genetic code (114, 183, 184). Gilbert (71, 185), Cannon, Krug and Gilbert (186) and Bretscher (182) were among the early workers to establish that AAtRNA's were associated with ribosomes during protein synthesis in bacterial extracts. Nirenberg and Leder (114) showed that such specific binding occurred in the absence of protein synthesis and developed a convenient assay method to test the binding. This method, which involves adsorption of the complex on cellulose ester filters, has been widely used in binding investigations. Igarashi and Kaji (171) showed that

AAtRNA's bound to 70 S ribosomes in the absence of protein synthesis were incorporated in the NH_2-terminal position of peptides when protein synthesis was initiated. Wettstein and Noll (187) have employed radioactive tRNA to determine that 2 or 3 AAtRNA's were bound per ribosome during protein synthesis. Igarashi and Kaji (171) have obtained a similar result with saturation binding curves. They also showed that the aminoacyl site of 70 S ribosomes was more readily occupied than the peptidyl site by phe-tRNA in the absence of GTP and enzymes. Matthaei and Milberg (170) obtained similar results by studying the kinetics of AAtRNA binding.

Arlinghaus, Schaeffer and Schweet (188) have demonstrated the existence of two types of AAtRNA binding to rabbit reticulocyte ribosomes. One mode is non-enzymatic, non-GTP-dependent, while the other requires the presence of GTP and enzymes. The existence of an enzymatic and GTP-dependent binding of an AAtRNA other than N-F-met-tRNA or N-acetyl-phe-tRNA (189) has been demonstrated in E. coli extracts, very recently (190, 191, 192). Previous failures to do so may reflect differences in chain initiation or may be the result of inadequate ribosome purification techniques.

Moldave et. al., (193, 194, 195) have isolated complexes of the supernatant enzyme, Transferase I, from rat liver preparations and showed that this enzyme bound GTP and AAtRNA. They suggested that this complex was involved in the binding of AAtRNA's to ribosomes. Allende, Monro and Lippman (196) have reported the isolation from E. coli of 2 or 3 proteins, T_u, T_s and G, from the non-ribosomal (supernatant) portion of

extracts which are distinct from the components of the AAtRNA synthesizing system and which are necessary for peptide bond synthesis. Allende and Weissbach (197) identified a complex similar to that reported by Moldave between GTP and E. coli initiation factor preparations. GDP was able to replace GTP in this complexation but ATP, CTP, UTP AND GMP were not able to compete with GTP. The heat instability and the chromatographic behavior of the protein involved in the formation of the complex suggested that the binding was with the supernatant enzyme T. Allende, Seeds, Conway and Weissbach (198) have now reported that the original initiation factor preparations contained traces of T and that the removal of this protein caused a loss of binding activity. Separately isolated T enzyme was shown to possess the original complexation ability. They isolated a T - GTP complex on cellulose ester filters.

Ravel, Shorey and Shive (199) independently observed similar complexation in mixtures of GTP, phe-tRNA and supernatant factor preparations. They showed that GTP was initially hydrolyzed to GDP by binding to the protein and that following this step, GDP was further bound in a complex with AAtRNA.

Gordon (200) was able to isolate a complex of GTP which was formed by T in the presence of AAtRNA. Later, he proposed a two step binding scheme of GTP to T, followed by formation of a ternary complex with AAtRNA (201). Lucas-Lenard and Haenni (191) have isolated this same ternary complex and report that it is capable of binding to the aminoacyl site

of 70 S ribosomes. When highly purified ribosomes are employed, binding of this complex to ribosomes is much greater than binding of AAtRNA alone. GDP is not able to substitute for GTP in ribosomal binding, although it forms a ternary complex with T and AAtRNA. They have also shown that AAtRNA bound to the aminoacyl site in the presence of T and GTP is able to form a dipeptide if the peptidyl site has previously been occupied.

Step 5. - Formation of the peptide bond.

The formation of the peptide bond between peptidyl-tRNA and the α-amine of the AAtRNA is shown as given by Watson (67) in Fig. VII. Monro and Marcker (202) and Monro (203) have recently shown that N-F-met is bound to the 50 S subunit when it is esterified with the terminal hexanucleotide fragment of $tRNA^f$. They demonstrated that N-F-met bound to 50 S subunits in this way is capable of forming the model dipeptide, N-F-met-Pm. This Pm reaction is not enhanced by GTP and the binding must be carried out in ethanolic solution. The 50 S subunits utilized were as free of initiation and supernatant enzymes as it is presently possible to prepare (4 X NH_4Cl washed) and the Pm reaction catalyzed by these subunits constitutes good evidence that the peptide synthetase enzyme is a part of the ribosome.

Lucas-Lenard and Haenni (191) report that dipeptide but not tripeptide formation occurs on 70 S ribosomes (4 X NH_4Cl washed) between prebound N-Acetyl-phe-tRNA and added phe-tRNA. Formation of this dipeptide is not enhanced by the supernatant

enzyme G. In this study phe-tRNA was added as the ternary complex between phe-tRNA, GTP and supernatant enzyme T.

Step 6. - Pulsation (Movement) of the ribosome.

Wettstein and Noll (187) have proposed that their data indicated that tRNA from the peptidyl site was transferred to a loosely attached "exit" site after formation of a peptide bond. Their proposal was based on the number of tRNA molecules found associated with the ribosome during protein synthesis. Further evidence concerning the existence of such a site has not been presented to date. Indeed, evidence that a specific tRNA leaves the ribosome is not available and probably will not be available until very highly purified preparations of tRNA are obtained. Our present picture of these steps is based on the proposals of Watson (67).

Lipmann et. al., (204, 205) have proposed that the most likely function of their ribosome-dependent GTPase, G, is to cause the pulsation or contractile movement of the ribosome. They have shown that one molecule of GTP is hydrolyzed for each peptide bond formed (203) and Lucas-Lenard and Haenni (191) have shown that this GTP hydrolysis occurs after dipeptide formation.

Step 8. - Chain termination and release.

It is well known that cell-free systems directed by natural mRNA's release their peptides into the supernatant (70) while systems directed by synthetic homopolymers retain the product peptide on the ribosome (71). It has been shown

that nonsense codons produced in bacteriophage mutants cause the release of incomplete proteins (206). These codons, UAA (ochre), UAG (ambre), and UGA, lead to termination of peptide chain growth in systems directed by mRNA's from the mutant strains or phages. Synthetic mRNA's which contain these codons also terminate protein synthesis (206). Last et. al., (207) have shown that the mRNA AUG-UUU-(A)n synthesizes the peptide met-phe-polylysine while AUG-UUU-UAA-$(A)_n$ which is phased to read the chain termination codon UAA, directs the synthesis of polylysine only. Similar results were obtained with other synthetic mRNA's containing termination codons. These workers proposed that the absence of met-phe-tRNA in the supernatant in the experiment utilizing AUG-UUU-UAA-$(A)_n$ indicated that hydrolysis of peptidyl-tRNA occurred on the ribosome. That is, the chain termination codon, UAA, not only terminated peptide synthesis but caused ribosomal hydrolysis of the completed peptide. Unlike AAtRNA's (208), peptidyl-tRNA's, e.g. polylysyl-tRNA (209), are relatively stable in the supernatant of cell-free systems. Therefore, it seems likely that hydrolysis of the ester may occur on the ribosome. Kuriki and Kaji (210) have shown that tRNA obtained by hydrolysis of peptidyl-tRNA is no longer capable of accepting new amino acid.

Capecchi (211) has isolated an R 17 phage mutant whose RNA terminates peptide formation in its coat protein prematurely. By controlling the supply of amino acids to a cell-free system directed by this mRNA he isolated a protein, R factor, which

is necessary for the rejection of the peptide from ribosomes. This R factor does not catalyze hydrolysis of peptidyl-tRNA in the absence of ribosomes. The existence of an R factor has not been shown in other preparations and it is not known whether it is of general importance. Bretscher (212) has used Capecchi's system to search for a nonsense tRNA. Such a tRNA is one which would be capable of interacting with the nonsense codon and thereby cause chain termination. He demonstrated that chain termination occurs in systems which are utilizing highly purified tRNA's and proposes that it is very unlikely that such a nonsense tRNA exists.

B. 6. The Effect of Tetracyclines on Cell-Free Protein Synthesizing Systems

In an early study of the mode of chloramphenicol action Rendi and Ochoa (213) mentioned, without supporting data, that oxytetracycline inhibited amino acid incorporation in their cell-free system. They were using a cell-free system directed by synthetic polyribonucleotides. Franklin (214) reported experiments which showed that chlortetracycline inhibited leucine incorporation in rat liver and in E. coli cell-free systems. Inhibition of the system derived from E. coli was greater than that observed with the system from rat liver. Chlortetracycline caused a 90% inhibition of leucine incorporation in the E. coli system at a concentration of 4×10^{-4}M. Chlortetracycline did not affect the formation of leu-tRNA by either extract but did prevent incorporation of preformed leu-tRNA into protein. Similar results were

obtained with oxytetracycline and tetracycline. Laskin and Chan (215) have shown that cell-free systems directed by poly U were inhibited equally by tetracycline, chlortetracycline, oxytetracycline and 6-demethyl-7-chlortetracycline but that 4-dedimethylamino-5-hydroxytetracycline was only about 1/10 as potent. They also reported that tetracycline did not affect the formation of phe-tRNA. Suarez and Nathans (216) and Day (217) have reported similar inhibitions of phe-tRNA incorporation. Suarez and Nathans (216) reported that inhibition was reversed by the addition of ribosomes - poly U but not by the addition of phe-tRNA or supernatant extract. Hierowski (218) has shown that lysine, proline and phenylalanine incorporation is inhibited in systems directed by synthetic polyribonucleotides. Okamoto and Mizuno (219) and Maxwell (220) have reported inhibition of cell-free systems in which incorporation of algal protein hydrolyzate was directed by endogenous mRNA. Rifino et. al., (221) have reported that several tetracycline derivatives are active inhibitors of poly U directed phenylalanine incorporation but noted that isochlortetracycline, an inactive antibacterial, was an inhibitor of endogenous mRNA directed incorporation.

Because of these reports it has been assumed that tetracyclines cause a general inhibition of amino acid incorporation by cell- free systems, Turley, Thomas and Snell (222) have recently investigated the incorporation of 20 different amino acids into protein by a cell-free system from E. coli directed by endogenous mRNA. They found that incorporation of 18 of

these amino acids was inhibited at low levels of oxytetracycline. Cysteine and tryptophan incorporation were not inhibited at low levels of oxytetracycline and their incorporation was stimulated at high levels of oxytetracycline. The authors postulated that this stimulation may be due to induced codon misreading or that these amino acids might be incorporated into protein by a non-ribosomal system.

B. 6.a. Effects of Tetracyclines in terms of the model of Cell-Free Protein Synthesis

Interference with Step 1. - Binding of a 30 S subunit to mRNA.

Hierowski (218) has reported that the inhibition by tetracyclines of the incorporation of AAtRNA's into protein is more pronounced in systems in which polyribonucleotides serve as the messenger than in systems directed by endogenous mRNA. She noted that tetracycline did not change the Mg ion optima of these system. Polypeptide synthesis directed by poly C in the presence of chlortetracycline (45 μM) was inhibited by 75%. Less inhibition was observed when poly U (70%), poly A (56%), poly U_5A_1 (50%) or poly UC (25%) were used as messengers. It was mentioned that the binding of poly U to ribosomes was not affected by tetracycline. Suarez and Nathans (216) confirmed this result in an experiment using ^{14}C-poly U. Connamacher and Mandel (233) have also reported that tetracycline did not prevent the formation of ribosome - codon complexes. They showed that tetracycline was, however, able to bind to poly U in EDTA solutions. Day (217) has reported binding to

poly U and poly A in the absence of Mg ion as well as at several Mg ion concentrations. Kohn (224) has reported that metal ions are necessary for binding to DNA. Maxwell (225) has recently shown that cultures of B. megaterium inhibited by tetracycline are able to form polysomes. These results constitute a body of evidence that tetracyclines do not interfere with the attachment of mRNA's to ribosomes, but that the particular mRNA used in a cell-free system does affect the degree of inhibition produced by tetracyclines.

Interference with Step 2. - Formation of the initiation complex.

Experiments designed to test the hypothesis that tetracycline inhibits the formation of the initiation complex have not been performed. However, experiments have been performed which suggest that tetracycline does not interfere with binding to the peptidyl site on 70 S ribosomes. Gottesman (226) has recently studied the binding of polylysyl-tRNA to ribosomes. It is believed that this peptidyl-tRNA either binds directly to or is rapidly translocated to the peptidyl site on 70 S ribosomes. Ribosomal bound polylysyl-tRNA is capable of reacting with puromycin (Pm) to form polylysyl-Pm (226, 227) and this product is released from the ribosome under certain circumstances. Chlortetracycline does not prevent the binding of this peptidyl-tRNA to ribosomes (226, 228, 229) nor does it prevent the reaction or release of bound polylysyl-tRNA with puromycin. If ribosomes with prebound polylysyl-tRNA are mixed with lys-tRNA in the absence

of GTP and supernatant enzymes, a single lysine is added to the polylysyl on the ribosome (191). Tetracycline prevents this addition while chloramphenicol does not prevent this single peptide bond formation. Similarly, ribosomes containing prebound N-acetyl-phe-tRNA (a presumed initiator of poly U directed systems) react with Pm and release N-acetyl-phe-Pm into the supernatant. Chlortetracycline does not prevent either the reaction or the release. Chlortetracycline, however, does partially prevent the addition of phe-tRNA to ribosomes containing prebound N-acetyl-phe-tRNA. The phe-tRNA which is bound to the ribosomes is able to form a dipeptide with the N-acetyl-phe-tRNA.

The above results are consistent with the general idea that tetracyclines do not affect AAtRNA binding to the peptidyl site but rather they inhibit binding of AAtRNA's to the aminoacyl site. Direct evidence for this type of inhibition is presented in the following sections.

Interference with Step 3. - Attachment of 50 S subunits to the initiation complex.

This step has not been studied directly, but Day (217) has shown that isolated 30 S subunits containing bound tetracycline are able to form 70 S ribosomes upon the addition of 50 S subunits.

Suzuka, Kaji and Kaji (169) and Vazquez and Monro (230) have shown that tetracycline prevents the binding of AAtRNA's to 30 S subunits - codon and also prevents the stimulation of binding observed when 50 S subunits are added. Binding of

AAtRNA's to 30 S subunits - codon has now been shown to be non-functional unless the AAtRNA is N-F-met-tRNA$_f$. It is however, generally agreed that the stimulation of binding observed when 50 S subunits are added is due to the creation of the aminoacyl site.

It seems unlikely that tetracyclines interfere with step 3 if indeed such a step is involved in protein synthesis.

Interference with Step 4. - Binding to the aminoacyl site of ribosomes.

Hierowski (218) used sucrose density gradient centrifugation to show that binding of phe-tRNA to 70 S ribosomes-codon is partially inhibited (60%) by chlortetracycline. These studies were conducted at 12 m$\underline{M}$ Mg ion and it is probable that phe-tRNA is capable of entering the peptidyl site as well as the aminoacyl site under these circumstances. Suarez and Nathans (216) have shown that the binding of N-acetyl-phe-tRNA at 20 m$\underline{M}$ Mg ion is inhibited by chlortetracycline. The maximum inhibition obtainable at very high tetracycline concentrations was 50%. Clark and Chang (231) observed similar inhibition (40%) of AAtRNA binding to rabbit reticulocyte ribosomes in the presence of the transfer enzymes. They also showed that tetracycline did not interfere with the puromycin reaction in systems synthesizing protein. Vazquez and Monro (23) have noted inhibition of poly A directed lys-tRNA (51 to 61%) as well as poly U directed phe-tRNA binding (38 to 52%).

Because of these reports, it has been generally assumed that tetracyclines prevent binding of AAtRNA's to only one site on the ribosome - codon. Since these experiments were carried out under conditions which favor binding to the aminoacyl site, these investigators have proposed that this is the site of tetracycline action. The recent reports of Gottesman (226) and Lucas-Lenard and Haenni (191), mentioned earlier, have substantiated this conclusion. It should be noted, however, that Wolfe and Hahn (232) were only able to demonstrate a very small inhibition of phe-tRNA binding to ribosomes - poly U by sucrose density gradient centrifugation. Similarly, Laskin (16) has stated that he was only able to demonstrate a small inhibition (30%) of this binding in experiments utilizing the technique of cellulose ester filtration. He emphasized the poor quantitative nature of these experiments. It would seem, that the conclusive experiment has not as yet been performed.

Interference with Step 5. - Formation of the peptide bond.

In the presence of tetracycline, the ability of prebound polylysyl-tRNA to react with Pm has been demonstrated by Gottesman (226). This result and the ability of prebound N-acetyl-phe-tRNA to react with either Pm or bound phe-tRNA in the presence of tetracycline, demonstrated by Lucas-Lenard and Haenni (191), indicates that step 5 is not inhibited.

Interference with Step 6. - Pulsation (Movement) of the Ribosome.

Since it is thought that release of Pm - peptides occurs by normal ribosomal pulsation and this is not inhibited by tetracycline, it is unlikely that tetracyclines inhibit this step in protein synthesis.

Interference with Step 8. - Chain termination and release. Effects of tetracycline on this step have not been studied.

B. 6.b. Binding of Tetracyclines to Ribosomes. -

The presumed mode of tetracycline action established by these experiments is that of a direct competition with AAtRNA's for the aminoacyl binding site on 70 S ribosomes. It is unlikely that tetracycline inhibition is due to an indirect effect caused by binding of tetracycline to some other ribosomal site, since all other ribosomal functions appear normal.

Direct binding to the aminoacyl site has, of course, not been demonstrated. Connamacher and Mandel (223) have, however, shown by equilibrium dialysis and by sucrose density gradient centrifugation, that tetracycline is bound to 70 S E. coli and B. cereus ribosomes. When experiments were conducted at low Mg ion concentrations, the tetracycline was largely associated with the 30 S subunit. Ribosomes prepared from preincubated extracts, so that endogenous mRNA activity was low, showed a reduced ability to bind tetracycline. Last, Izaki and Snell (233) were unable to show any binding of tetracycline to a ribosome sediment produced by centrifugation in sucrose. This failure may be due to a lack of endogenous

mRNA in their preparations or to their sedimentation technique which may require stronger binding than binding demonstrated by sucrose density gradient centrifugation. Day (234) has substantiated the findings of Connamacher and Mandel (223) that tetracycline is associated with the 70 S ribosomes during sucrose density gradient centrifugation. He showed that tetracycline can bind to either 30 S or 50 S subunits. Tetracycline, that is bound to either of these subunits is able to dissociate and rebind to the other subunit if it is added. All of the sucrose density gradient centrifugations reported by Day were conducted on ribosome - tetracycline mixtures that had been subjected to extensive dialysis prior to centrifugation. Day calculated that approximately 1 mole of tetracycline is associated with 1 mole of 70 S ribosomes and that 1 mole of tetracycline is associated with 2 moles of either 30 S or 50 S subunits. He also showed that ribosomes containing bound tetracycline were not able to bind phe-tRNA or incorporate phe-tRNA into protein after addition of supernatant enzymes. Day (235) later showed that the cellulose ester filtration technique also indicated that tetracycline bound to ribosomes.

Recently, Maxwell (236) has shown that large quantities of tetracycline are found associated with the sediment of undialyzed ribosome - tetracycline mixtures after high speed centrifugation. He found about 300 molecules of reversibly associated tetracycline per 70 S ribosome. It is difficult

to say if the tetracycline associated with the ribosomes under these circumstances is the result of binding or of entrapment. However, if these sediments were subjected to dialysis or an additional centrifugation through sucrose and then binding studied by sucrose density gradient centrifugation, a small amount of tetracycline remained with the polysomes or ribosomes. Sedimentation at low Mg ion concentrations revealed that most of the tetracycline was associated with the 30 S subunits.

In the light of these experiments, it seems likely that tetracycline does bind to ribosomes, probably the 30 S subunit, and that such binding is capable of preventing attachment of AAtRNA's to the aminoacyl site.

III. EXPERIMENTAL

A. Materials and Equipment

A. 1. Tetracycline Derivatives

The antibiotics used in this study were the generous gifts of Dr. James H. Boothe, Lederle Laboratories, Pearl River, N. Y., and Dr. Charles R. Stephens, Charles Pfizer Laboratories, Groton, Conn.. They have been identified by their ultraviolet absorption spectra. The wavelengths of several absorption maxima and the absorptivity at these maxima are given in Table I. The spectra were obtained from solutions buffered at pH 6.65 with phosphate buffer (0.2 M). Solutions of the samples of 7-amino-6-demethyl-6-deoxy-tetracycline and 9-nitro-6-demethyl-6-deoxy-tetracycline had "shoulders" in their absorption spectra at approximately 400 mμ. These solutions were separable into two spots by thin layer silica gel chromatography. The solvent system for this chromatography was a mixture (2:2:3:3) of phosphate buffer (0.2 M, pH 6.65), ethanol, butanol and ethyl acetate. It seems likely that these samples contain trace quantities of 5a(6)-anhydrotetracyclines, however, they were used without further purification.

A. 2. Escherichia coli

Escherichia coli W was obtained as a frozen paste.[2] It was cultured over several growth cycles in broth and on agar plates. An isolate from a single colony was used to inoculate

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[2]General Biochemicals, Chagrin Falls, Ohio.

Table I.- The Tetracyclines.

Tetracycline Analog[a]	pKa Values[b]	Ultraviolet Absorption Spectra[c]	
		max. mμ	Absorptivity x 10^{-4}
7-Nitro-dm-do-tetracycline		360, 265	1.3, 1.6
7-Chloro-dm-tetracycline	3.3, 7.3, 9.3 (237)	367, 275	1.4, 1.5
7-Chlorotetracycline	3.3, 7.4, 9.3 (237)	368, 275	1.1, 1.4
Tetracycline	3.3, 7.7, 9.5 (237)	358, 275	1.5, 1.5
5-Hydroxytetracycline	3.6, 7.4, 9.1 (238)	355, 275	1.4, 1.5
7-Amino-dm-do-tetracycline		400, 340, 278	0.4, 1.1, 1.4
9-Amino-dm-do-tetracycline		344, 282	1.3, 1.2
dm-do-tetracycline	3.5, 7.9, 9.7 (17)	348, 273	1.3, 1.7

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a. dm-do- is 6-demethyl-6-deoxy-, while dm- is 6-demthyl-6-epihydroxy-.
b. Numbers in parenthesis indicate literature reference.
c. Values given were obtained in 0.2 M phosphate buffer, pH 6.65, a Model DK-2 Ratio Recording Double Beam Spectrophotometer, Beckman Instruments, Palo Alto, Calif. was used.

Table I. - (cont.)

Tetracycline Analog	pKa Values	Ultraviolet Absorption Spectra	
		max. mu	Absorptivity x 10^{-4}
7-Bromo-dm-do-tetracycline		350, 272	0.7, 1.4
9-Nitro-dm-do-tetracycline		415, 354, 285	0.4, 0.7, 1.1
4-Dedimethylamino-tetracycline	5.97, 8.56 (13)	360, 272	1.1, 1.3
9-Dimethylamino-dm-do-tetracycline		346, 280	0.8, 0.7
5a(6)-Anhydrotetracycline		425, 268	1.2, 5.0
12a-Deoxytetracycline		450, 330, 265	0.2, 1.3, 1.7
Tetracycline methiodide	3.56, 7.80 (13)	350, 300, 275	1.6, 1.4, 1.6
5a(11a)-Dehydrocnlorotetracycline		408, 270	1.1, 2.1
7-Chloro-isotetracycline	3.9, 6.7, 7.9 (238)	344, 288	0.3, 1.7
2-Cyano-2-decarboxamidotetracycline		360, 275	0.9, 1.0

a nutrient agar slant. This slant was stored at 3°C and maintained by monthly transfers. It served as the source of the test organism. For experiments in dextrose-salts broth another slant was prepared from organisms which had grown through several cycles in this broth.

A. 3. Culture Broths

The culture broth used for activity determinations was a high peptone media containing in a volume of 1 L., 40 g. casein hydrolysate (GBI #20)[2], 15 g. yeast autolysate (GBI #120)[2], 3 g. $K_2H\ PO_4$[3] and 0.7 g. KH_2PO_4.[3] The pH of this broth was constant throughout the experiments at 6.65. The divalent cation content of the broth was estimated to be equivalent to about 10^{-3} M calcium ion by means of a divalent cation electrode.[4] For experiments at pH 6.2 or 7.4, the broth contained in addition to the peptones either 0.5 g. K_2HPO_4 and 2.5 g. KH_2PO_4 or 21.85 g. K_2HPO_4 and 1.7 g. KH_2PO_4, respectively. Similar divalent cation concentrations were observed in these broths.

The dextrose-salts broth (pH 6.7) contained in each liter, 10 g. dextrose, 3 g. $(NH_4)_2SO_4$, 6 g. K_2HPO_4 and 8 g. KH_2PO_4.

All broths were filtered through cellulose ester membranes[5] (0.22 μ dia. pores) and autoclaved prior to use.

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[3]All unspecified chemicals were analytical reagent grade.

[4]Orion Research Inc., Cambridge, Mass. 02139

[5]Millipore Corporation, Bedford, Mass.

A. 4. Culture Turbidity and Colorimetry

A diffraction grating colorimeter[6] equipped with a red sensitive phototube in conjunction with matched 1/2 inch square cuvettes was used. Culture turbidity was measured at 650 mμ. Absorbance of orcinol reaction mixtures was measured at 650 mμ while that of Folin-Lowry reactions was measured at 750 mμ.

A. 5. Total Cell Counts

An electronic particle counter[7] was used. The counter was equipped with a 30 μ orifice and two threshold pulse height analyzers. Instrument settings which gave satisfactory results were:

1/ Aperture Current, 0.707; 1/Amplification, 1/4; Matching Switch, 32 H; Gain, 100; Lower Threshold, 10 and Upper Threshold, off. These settings were established by size frequency analysis of latex suspensions of known particle size as well as suspensions of E. coli W.

Cells were counted in a saline - formaldehyde solution which had been filtered to remove particulate contamination. Background counts in this fluid were 200 to 600 particles per 50 lambda. The formaldehyde concentration in the counting fluid was either 0.2 or 1%. The higher concentration gave constant values for total cells over a 24 hour period while

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[6]Spectronic 20, Bausch and Lomb, Rochester, N. Y.

[7]Coulter Counter Model B, Coulter Electronics, Inc. Hialeah, Fla.

the lower concentration allowed some growth (1 to 5%) in the first ten minutes after samples were removed from the culture.

B. Methods

B. 1. Escherichia coli Growth

A broth culture was allowed to grow for 12 hours at 37.5°C. A dilution of this culture, whose growth was measured turbidimetrically, was allowed to grow into the log phase. When a concentration of 1×10^8 organisms/ml. was obtained, 2 ml. samples were used to inoculate nine replicate 100 ml. volumes of broth contained in loosely capped 500 ml. flasks. The flasks were shaken in a constant temperature water bath[8] at 37.5°C. Freshly prepared antibiotic solutions were added in 1 ml. volumes to 8 of the 9 replicate cultures after 2,700 seconds of growth.

Experiments designed to study time of onset of inhibition were performed with cultures of either 200 ml. per 1 L. flask or 350 ml. per 2 L. flask.

B. 2. Viable Count Method

One ml. samples of the cultures were obtained at 900 or 1,800 second intervals. A minimum of 8 samples was obtained from each culture. Samples were diluted with sterile 0.9% saline solution so that 100 to 200 organisms/ml. would result. One ml. of this suspension was pipetted onto each of 5 agar plates. Agar plates had previously been made by adding

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[8]American Instrument Company, Silver Spring, Md.

approximately 15 ml. of sterile melted Peptone-Casein Agar U.S.P.[9] to sterile plastic petri dishes (100 x 15 mm.). After spreading the bacterial suspension over the surface of the plate, approximately 5 ml. of warm agar was added. When the agar had solidified the plates were incubated at 37.5°C.

The colonies which were visible after 36 to 48 hours of incubation were counted with the aid of an electronic register.[10] The mean number of colonies visible on the 5 plates from each sample was used to calculate the number of viable organisms in the original culture.

B. 3. Total Count Method

One ml. culture samples were obtained at the same time as the samples used for viable cell counts. They were placed in bottles containing appropriate volumes of the saline - formaldehyde counting fluid so that a concentration of 10,000 to 30,000 organisms per 50 lambda was obtained. The mean of 4 counts per sample was corrected for background noise, but not coincidence and used to calculate the total number of cells in the original culture.

B. 4. Nucleic Acid and the Protein Determinations

Appropriate volumes of culture (0.5 to 25 ml.) were filtered under vacuum through 25 mm. diameter cellulose ester membranes (0.22 μ dia. pores) which had previously been treated to extract detergent. Culture samples were obtained at times similar to those at which viable and total cell counts were made.

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[9]Fisher Scientific, Fairlawn, N. J.

[10]American Optical Company, Instrument Division, Buffalo, N. Y.

It was necessary to remove the detergent impregnated in these membranes (239) since it reacted with the orcinol reagent and gave high blank values, if not removed. Detergent extraction was accomplished by suspending filters in cold distilled water (4 L. per 100 filters) and then boiling the suspension for 15 minutes. This process was repeated 3 times with fresh cold water. Filters treated in this manner were stored in distilled water for times no longer than 48 hours. These filters were able to retain E. coli quantitatively and gave minimal and constant blank reactions with the orcinol reagent.

After culture samples were collected on the filters, the retained bacteria were washed with 0.9% saline solution under vacuum. The washed samples were precipitated by layering, without vacuum, 2 ml. of trichloracetic acid (TCA) over the filters for 5 minutes. TCA soluble material was removed by application of vacuum and 2 ml. of 95% ethanol layered over the filters for 5 minutes. After removal of the ethanolic extract, the filters with retained nucleic acids and proteins were placed in screw cap test tubes. Two ml. of 5% TCA was added and the tubes were placed in a boiling water bath for twenty minutes.

Nucleic Acid Determinations

One ml. aliquots of the hot-TCA soluble material were removed from the cooled tubes. Three ml. of the orcinol reagent were added and after heating in a boiling water bath for 20 minutes, the absorption at 650 mμ of the cooled solution

was measured. This absorption was corrected for that produced when an equal volume of sterile broth was applied to the filters instead of culture. It was necessary to prepare separate blank reactions for each lot of filters and each lot of broth. The orcinol reagent was purified, mixed and used according to the methods of Schneider (240). It is a solution of recrystallized orcinol (0.66%), $FeCl_3$ (0.33%) and 5% TCA in concentrated hydrochloric acid.

The quantity reported as "Nucleic Acids" is the corrected absorbance due to a 12.5 ml. culture sample in an orcinol reaction volume of 4 ml. Since only the logarithm of a quantity proportional to nucleic acid concentration was needed to calculate generation rate constants the absorbance was used directly rather than a proportional quantity obtained by reference to a standard curve. A standard curve for the assay procedure was prepared by filtering various volumes of culture. An example is shown in Fig. VIII. It and other standard curves prepared with E. coli extracts were linear throughout the tested ranges. Standard curves were also prepared with highly polymerized yeast RNA[11] and also with salmon sperm DNA[11]. They were found to be linear throughout the tested concentration range (as high as 60 mcg./ml. of either RNA or DNA). The RNA had an absorptivity of 0.023 cm.^{2}/mcg. and a negative intercept of 0.042 while the DNA had an absorptivity of 0.004 cm.^{2}/mcg. Mixtures of DNA and RNA produced absorbances equivalent to those calculated from the

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[11]Calbiochem, Box 54282, Los Angeles, California

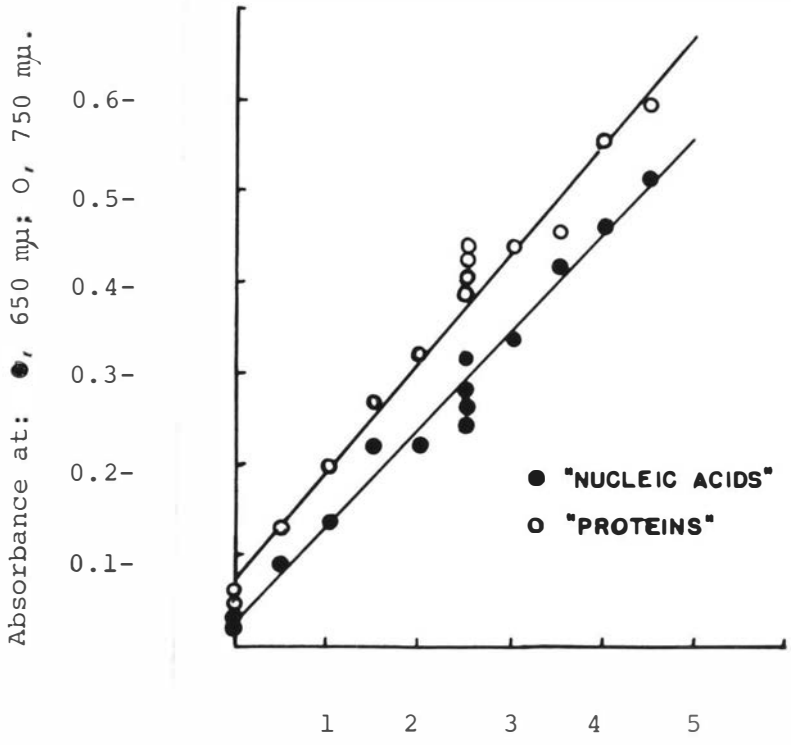

Figure VIII. Calibration Curves for "Nucleic Acids" and "Protein" Determinations. The amounts of "Nucleic Acids" or "Protein" found by the assays are linearly related to the amount of culture filtered.

sum of the two Beer's law equations.

Maaløe and Kjeldgaard (240) have estimated the DNA:RNA ratio in cells as a function of growth rate. They find this ratio to be about 1:10 in rapidly dividing cells. While in slowly dividing cells the ratio of DNA:RNA was reduced to about 1:4. Using these figures and the absorptivities for yeast RNA and salmon sperm DNA given above one can calculate that the contribution of DNA to the quantity called "Nucleic Acids" in this work ranges from about 2 to 5%. Since this is the case it might have been more proper to report the results as "Ribonucleic Acids" rather than "Nucleic Acids" but since there is a contribution to this quantity by DNA in these experiments the more general term was preferred. Another reason for preferring this term is that in tetracycline inhibited cells the DNA:RNA ratio might be different than that found in normal cells. The results of Černý and Habermann (33), however, indicate that this ratio does not change very much.

Protein Determinations

The TCA solution was completely removed from the tubes containing the filters, by vacuum aspiration. The filters with retained protein precipitate were covered with 1.5 ml. of a trypsin solution- (18) 10 mcg./ml in 0.005 M NH_4OH and 0.05 M $(NH_4)_2CO_3$-. The suspended filters were stirred vigorously and allowed to digest for 2 hours (the same results were obtained after as much as 48 hours of digestion). It was

necessary to utilize trypsin digestion rather than strong alkali, because the cellulose ester membranes were hydrolyzed and gave very high blank reactions in the presence of strong alkali. The resulting solution was used as a sample for protein determination by the Folin-Lowry method. The reagents were added directly to the tubes and the absorbance of the colored solutions measured at 750 mμ. Blank reactions were prepared by filtering sterile broth and used to correct the absorbance of the samples. The Folin-Lowry reagents were prepared, standardized and used according to the methods of Layne (242). A solution of cupric sulfate (0.01%), sodium tartrate (0.02%) and sodium carbonate in 0.1 N NaOH was added to the protein solution (5 ml. per tube) and after 10 minutes, 0.5 ml. of 1.0 N Folin-Ciocalteu phenol reagent[12] was added.

The quantity reported as "Protein" is the corrected absorbance due to a 25 ml. sample of culture in a final reaction volume of 6.5 ml. As in the previously mentioned case of "Nucleic Acids" absorbance was used directly rather than a proportional quantity obtained from a calibration curve. A standard curve for the assay procedure was prepared by filtering various volumes of E. coli cultures. An example is shown in Fig. VIII. It and other standard curves prepared with E. coli extracts were linear throughout the tested ranges. It is, however, well known (242) that the Folin-Lowry reaction

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[12]Will Scientific Inc., Baltimore, Md. 21224

does not always follow Beer's law. Standard curves were also prepared with bovine serum albumin[11] and pancreatic trypsin.[11] Adherence to Beer's law was observed in the case of the albumin in the tested concentration range (as high as 45 mcg./ml.) while Beer's law was only followed for trypsin concentrations of less than 25 mcg./ml. The albumin had an absorptivity of 0.008 $cm.^2/mcg.$ while the initial absorptivity of the trypsin was 0.034 $cm.^2/mcg.$ The decision to utilize the absorbance of Folin-Lowry reaction mixtures directly in the calculation of the term called "Protein" was based, therefore, firstly on the fact that the available standard curves with E. coli extracted by the experimental method were linear and secondly on the realization that conversion of absorbance to concentration of some standard protein would be valid only if the standard protein were the same, in respect to non-linearity of absorbance and absorptivity, as the experimentally measured proteins. Such standard proteins were not available.

IV. RESULTS AND DISCUSSION.

A. Effect of Tetracyclines on Rate Constants for Cell Division.

It has previously been shown (22) that the effect of low concentrations of tetracycline on an exponentially dividing culture is to cause a reduction in the generation rate constant, that is:

$$[N] = [N^o]e^{k_{cd}t}, \qquad \text{iff } k^o_{cd} > k_{cd} > 0 \qquad \text{Eq. (1)}$$

When the time, t, is greater than 30 minutes after antibiotic addition and where $[N^o]$ is the concentration of cells in the culture 30 minutes after antibiotic addition, N is the concentration of cells at any later time, k_{cd} is the generation rate constant for cell division in the presence of antibiotic and k^o_{cd} is the generation rate constant in the absence of antibiotic. Generation rate constants for cell division, k_{cd}, of cultures inhibited by 18 tetracyclines have been measured by viable, k_v, and by total, k_t, cell count methods. Typical results of these experiments are shown in Fig. IX and Fig. X. These experiments can all be quantitatively described by Eq. (1). Generation rate constants obtained from the fit of this experimental data to Eq. (1) as well as data to be presented later are given in Tables II and III. Tests for the fit of experimental data to Eq. (1) were found to be highly significant in cases where data obtained at times greater than 30 minutes but less than 4 hours after antibiotic addition were used.

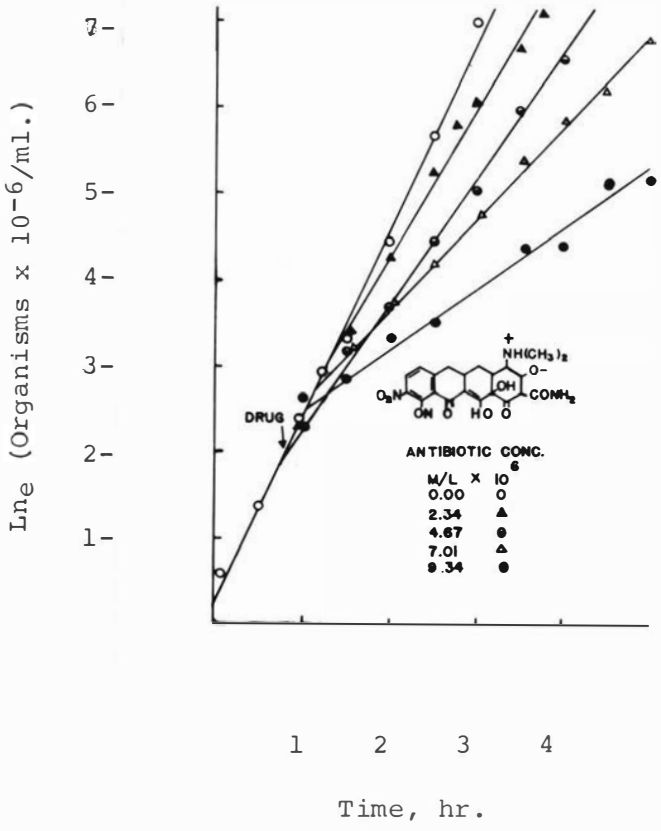

Figure IX. An Example of Viable Cell Generation Rate Curves. The logarithm of the number of viable cells as a function of time of growth in cultures with various concentrations of 9-Nitro-dm-do-tetracycline. The slopes, k_v, are given in Table III.

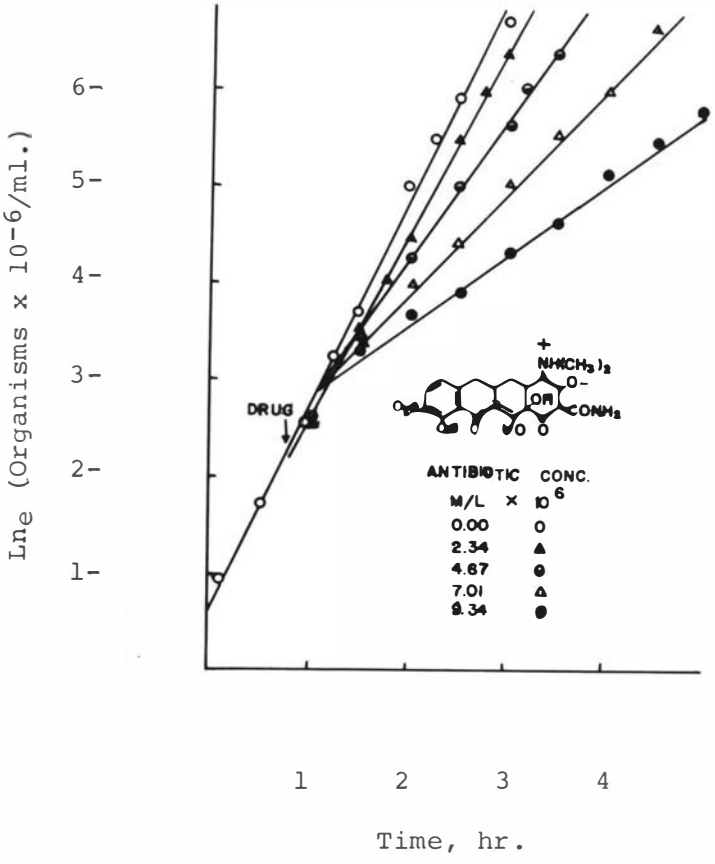

Figure X. An Example of Total Cell Generation Rate Curves. The logarithm of the total number of cells as a function of time of growth in cultures with various concentrations of 9-Nitro-dm-do-tetracycline. The slopes, k_t, are given in Table III.

Table II.- First Order Generation Rate Constants, $sec.^{-1} \times 10^4$, for *E. coli* W Cultures Inhibited by Various Tetracycline Antibiotics, M $\times 10^6$.

M	k_v	k_p	k_{na}
Tetracycline			
0.000	5.57	5.85	5.07
0.599	3.80	4.46	3.85
0.898	3.23	2.97	2.89
1.198	2.27	1.96	2.10
1.497	1.49	1.76	1.45
1.796	1.10	1.25	1.27
7-Chlorotetracycline			
0.000	5.41	5.94	5.80
0.246	4.82	4.51	4.97
0.492	3.48	3.33	3.53
0.788	2.33	2.17	2.14
0.985	2.16	1.84	2.16
1.231	0.52	0.93	0.51

Table II.- (cont.)

M	k_v	k_p	k_{na}
5-Hydroxytetracycline			
0.000	5.75[a]	5.79	5.97
0.537	4.71	3.49	3.75
0.878	4.04	3.47	-
0.878	3.75[b]	-	-
1.073	2.25	2.24	2.14
1.376	2.23	2.24	2.40
1.376	2.20	-	-
7-Chloro-dm-tetracycline[c]			
0.000	5.42	5.02	5.90
0.221	4.99	4.41	5.26
0.441	3.37	3.05	3.68
0.706	1.46	1.13	1.50
0.882	0.55	0.68	1.06
dm-do-tetracycline[d]			
0.000	5.89	5.05	5.25
0.978	4.58	4.55	4.77
1.955	3.65	3.42	3.50
2.972	2.76	2.57	2.47
3.910	1.46	1.57	1.65
4.888	0.84	1.07	0.97

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a. An average value, determined on several different days.

b. Values of k_t which were used in the calculation of the viable inhibitory rate constant, k_v^I.

c. dm- is 6-demethyl-6-epihydroxy-.

d. dm-do- is 6-demethyl-6-deoxy-.

Table II.- (cont.)

M	k_t^e	k_p	k_{na}
7-Bromo-dm-do-tetracycline[d]			
0.000	5.89	5.41	5.89
2.468	4.29	3.78	3.52
4.938	2.70	2.59	2.29
7.406	1.26	1.50	1.50
9.876	-	0.85	0.55
7-Chloro-isotetracycline			
0.000	5.71	5.26	5.40
163.49	5.81	4.74	5.39
322.30	5.71	4.86	5.53
12a-Deoxytetracycline			
0.000	5.71	5.26	5.40
79.14	3.90	3.69	3.59
156.03	1.59	1.57	0.95
5a(11a)-Dehydrochlortetracycline			
0.000	5.71	5.26	5.40
150.68	5.23	4.26	4.55
297.06	4.64	4.03	4.66
2-Cyano-2-decarboxamido-tetracycline			
0.000	5.71	5.26	5.40
33.16	5.94	4.92	5.44
128.88	5.88	4.63	4.72

---

e. Values of k_t were obtained with 0.2 % HCHO in the diluting solution, see Experimental.

Table III.- First Order Generation Rate Constants, $sec^{-1} \times 10^4$, for E. coli W. Cultures Inhibited by Tetracyclines, M x 10^6, and the Fraction of Viable to Total Cells Present at the Start, θ_1, and at the End, θ_5 of the Experiments.

M	k_v	k_t[a]	k_p	k_{na}	θ_1[b]	θ_5[b]
7-Amino-dm-do-tetracycline[c]						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.493	4.93	4.80	4.32	4.72	0.80	0.96
0.985	3.71	3.83	3.36	3.66	0.75	0.63
1.478	2.98	3.05	2.74	2.77	0.68	0.62
1.971	2.41	2.41	1.96	2.02	0.72	0.71

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a. Values of k_t. were obtained with 0.2 % HCHO in the diluting solution, see Experimental.

b. θ was calculated from Eq. (1) using the values k_{cd} and N^o, obtained by regression analysis of the original data.

c. dm-do- is 6-demethyl-6-deoxy-.

Table III.- (cont.)

M	k_v	k_t^a	k_p	k_{na}	θ_I^b	θ_S^b
9-Amino-dm-do-tetracycline[c]						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.474	5.27	5.27	4.61	4.86	0.74	0.74
0.948	4.06	4.59	3.91	4.08	0.87	0.40
1.421	3.79	3.73	3.38	3.44	0.72	0.80
1.895	–	3.06	3.02	3.17	–	–
5a(6)-Anhydrotetracycline						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
4.477	5.00	5.46	4.70	4.90	0.69	0.35
8.953	4.38	4.67	3.79	3.87	0.79	0.52
13.430	4.12	3.88	3.25	3.45	0.64	0.90
17.907	-	3.32	2.56	2.77	-	-

Table III.- (cont.)

M	k_v	k_t[a]	k_p	k_{na}	θ_1[b]	θ_5[b]
7-Nitro-dm-do-tetracycline[c]						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
0.111	5.68	5.50	4.86	5.44	0.67	0.87
0.222	4.20	4.75	3.88	4.32	0.74	0.33
0.333	3.17	3.82	3.11	3.52	0.72	0.28
0.444	2.45	2.82	2.19	2.59	1.00	0.61
9-Nitro-dm-do-tetracycline[c]						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
2.337	4.77	5.37	5.07	5.15	0.97	0.41
4.674	3.93	4.11	3.48	3.54	0.68	0.53
7.011	2.93	2.96	2.82	2.55	0.82	0.77
9.348	1.95	2.02	1.92	1.85	0.69	0.62

Table III.- (cont.)

M	k_v	k_t[a]	k_p	k_{na}	θ_1[b]	θ_5[b]
4-Dedimethylamino-tetracycline						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
3.030	5.27	5.39	4.63	5.01	0.79	0.67
6.060	4.32	4.50	3.96	4.30	0.80	0.53
9.091	3.43	3.80	3.58	3.51	0.86	0.50
12.121	3.17	3.12	2.97	3.04	0.65	0.70
9-Dimetnylamino-dm-do-tetracycline[c]						
0.000	6.25	6.09	5.45	5.40	0.66	0.83
3.467	5.60	5.71	5.01	5.00	0.87	0.74
6.935	4.32	4.69	3.92	3.99	0.70	0.41
10.402	3.34	3.47	3.09	3.51	0.60	0.50
13.870	2.83	2.94	2.40	2.56	0.74	0.63
Tetracycline metniodide						
0.00	6.25	6.09	5.45	5.40	0.66	0.83
63.48	4.87	5.14	4.03	4.26	0.83	0.56
126.95	3.02	3.39	2.78	2.91	0.71	0.42

Generation rate constants for cell division have been obtained by two different methods, by viable counts, k_v, and by total cell counts, k_t. It can be seen in Table III that rate constants for most cultures obtained by the two methods are apparently identical, within experimental error.

However, in these cultures the concentrations of cells at any time as determined by the two methods were quite different. This is shown in Fig. XI for cultures inhibited by 7-amino-6-demethyl-6-deoxy-tetracycline. Similar results were obtained with the other tetracyclines tested by both methods. The fraction of viable cells to total cells, Θ, would be 1 if there was no difference between the concentration of cells measured by the two methods. This fraction, Θ, has been calculated from the regression fit of the experimental data to Eq. (1) for the tetracyclines tested by the two methods. Values of Θ at two culture growth times: Θ_1, 900 seconds after antibiotic addition and Θ_5, 15,300 seconds after antibiotic addition, are given in Table III. It should be noted that values of Θ calculated in this manner are very sensitive to small differences in generation rate constants. Despite this sensitivity, Θ remains relatively constant in most cultures throughout the time of antibiotic inhibition. In those cases where it does vary, it does not appear to be consistently related to antibiotic concentration. The average value of Θ in these cultures is about 0.6. At higher antibiotic concentrations when generation rate constants obtained by the viable count method are negative, this fraction

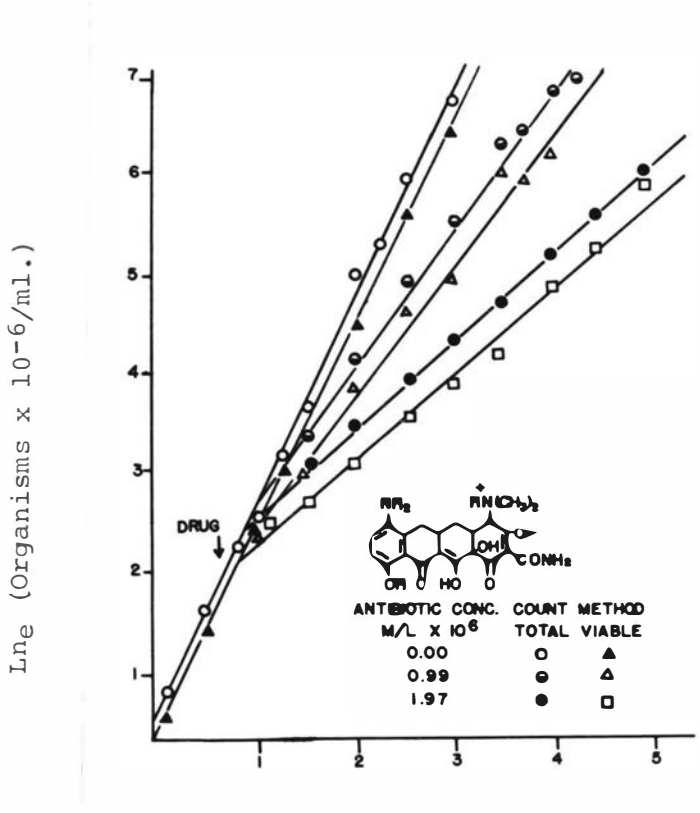


Figure XI. Examples of the Difference Between Total and Viable Cell Generation Rate Curves. The logarithm of either the total number or the number of viable cells as a function of time of growth in cultures with various concentrations of 7-Amino-dm-do-tetracycline. The slopes, k_t or k_v, are given in Table III.

has values of 0.1 or 0.01. It is not possible in the present work to conclude with certainty that the tetracyclines are acting solely as growth inhibitors and not as both bacteriostatic and bactericidal agents. However, if there is an antibiotic induced cell death, it is not large. Rather it seems most likely that the difference in cell concentrations obtained by the two methods is caused by the formation of single colonies by more than one viable cell. Inclusion of a surfactant in the viable cell diluting solution did not, however, greatly affect this fraction. The use of the higher formaldehyde concentration in the diluting fluid for total counts made this fraction, θ, more constant.

Garrett and Brown (22) and Garrett, Miller and Brown (243) have observed that generation rate constants for cell division obtained in the presence of tetracycline obey the relationship:

$$k_{cd} = k^{o}_{cd} - k^{I}_{cd}[Tc], \quad \text{iff } k_{cd} > 0 \qquad \text{Eq. (2)}$$

where k_{cd} may be either k_v or k_t, k^{I}_{cd} is the inhibitory rate constant for cell division and [Tc] is the antibiotic concentration. This equation was obtained by simplification of a more general relationship:

$$k^{o}_{cd}/k_{cd} = K\,[Tc] + 1 \qquad \text{Eq. (3)}$$

where K is a constant. The derivation of these equations will be presented in a later section of this thesis.

The generation rate constants obtained in the present work can be described by Eq. (2) but not by Eq. (3). Typical

examples of the fit of experimental data to this expression are shown in Figures XII, XIII, XIV and XV. Values of the inhibitory rate constant, k^{I}_{cd}, obtained by application of regression analysis to the experimentally determined generation rate constants and the antibiotic concentrations in the cultures are given in Table IV. These values are estimates of the activity of the tetracyclines which should be suitable for use in structure-activity relationships.

B. Effect of Tetracyclines on Rate Constants for Protein and Nucleic Acid Synthesis.

It was mentioned in the literature survey that many investigators have observed that tetracyclines cause an inhibition of protein synthesis in bacterial cultures. The results of two typical experiments in which the rates of protein synthesis in E. coli cultures inhibited by tetracyclines were studied are shown in Fig. XVI and XVII. These results can be described by an equation similar to Eq. (1):

$$[P] = [P^{o}]\, e^{kpt}, \qquad \text{iff } k^{o}_{p} > k_{p} > 0 \qquad \text{Eq. (4)}$$

when the time, t, is greater than 30 minutes after antibiotic addition and where $[P^{o}]$ is the concentration of protein in the cells 30 minutes after antibiotic addition, [P] is the protein concentration in the cells at any later time and k_{p} is the generation rate constant for protein synthesis in presence of antibiotic. The generation rate constant for protein synthesis in the absence of antibiotic, k^{o}_{p}, is identical with the generation rate constant for cell division in the absence of antibiotic, k^{o}_{cd}. Values of k_{p} are given in

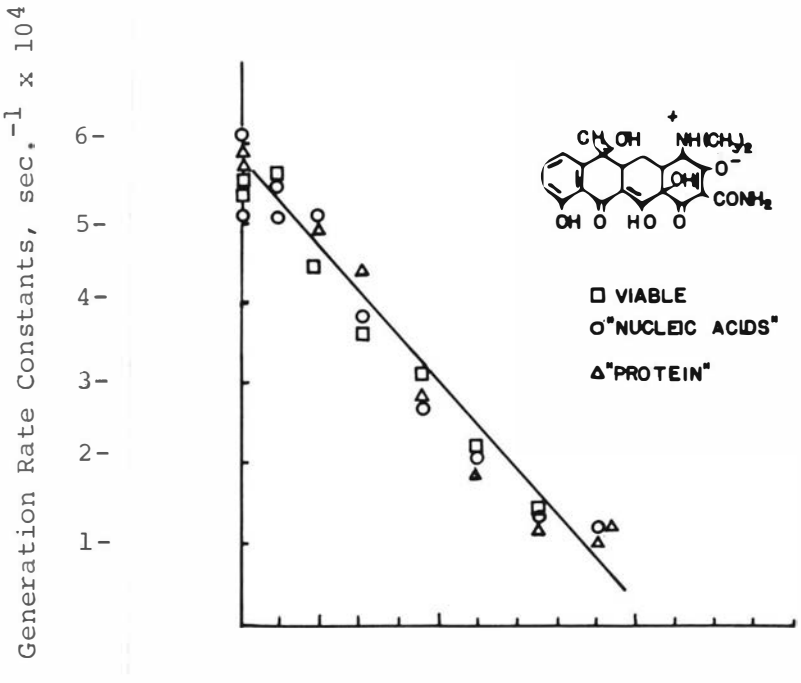


Figure XII. The Linear Dependence of Generation Rate Constants, k_v, k_p and k_{na}, upon Tetracycline Concentrations. The line shown is the average inhibitory rate constant, k^I_{avg}, it and values of k^I_v, k^I_p and k^I_{na} are given in Table IV.

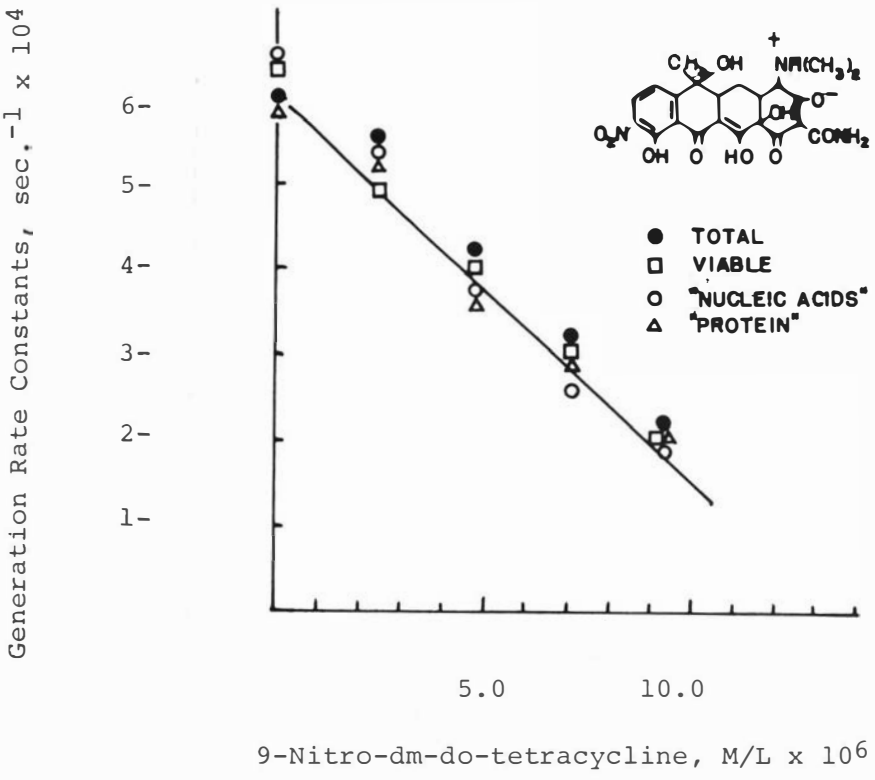


Figure XIII. The Linear Dependence of Generation Rate Constants, k_v, k_t, k_p and k_{na}, upon 9-Nitro-dm-do tetracycline concentration. The slope of the line shown is the average inhibitory rate constant, k^I_{avg}, it and values of k^I_v, k^I_t, k^I_p and k^I_{na} are given in Table IV.

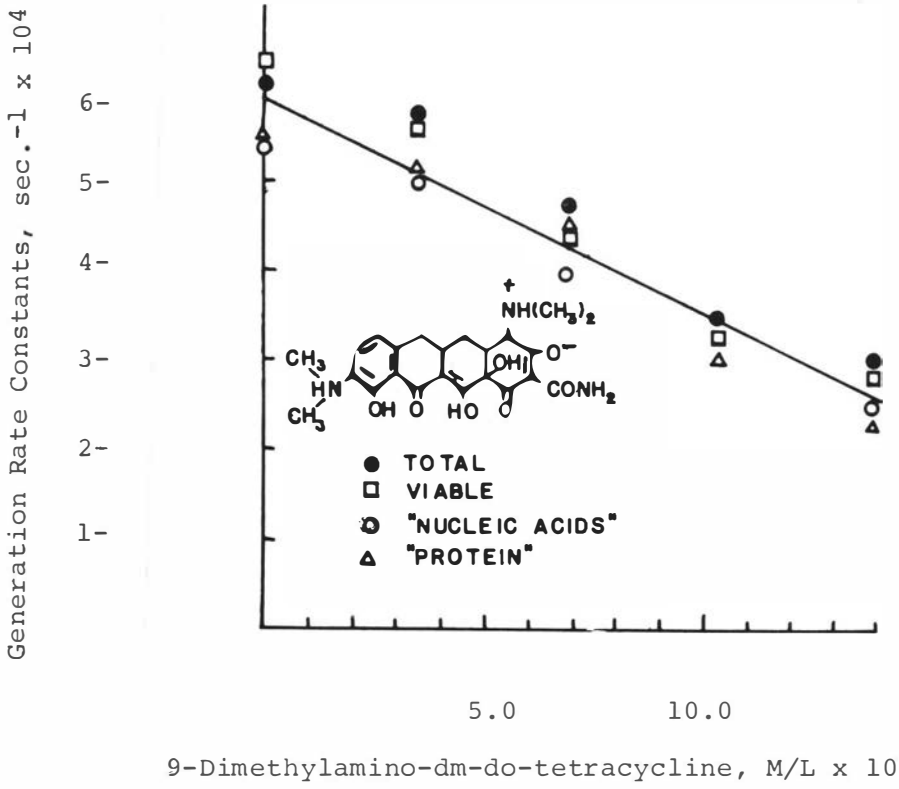


Figure XIV. The Linear Dependence of Generation Rate Constants, k_v, k_t, k_p and k_{na}, upon Concentration of 9-Dimethylamino-dm-do-tetracycline. The slope of the line shown is the average inhibitory rate constant, k^I_{avg}, it and values of k^I_v, k^I_t, k^I_p and k^I_{na} are given in Table IV.

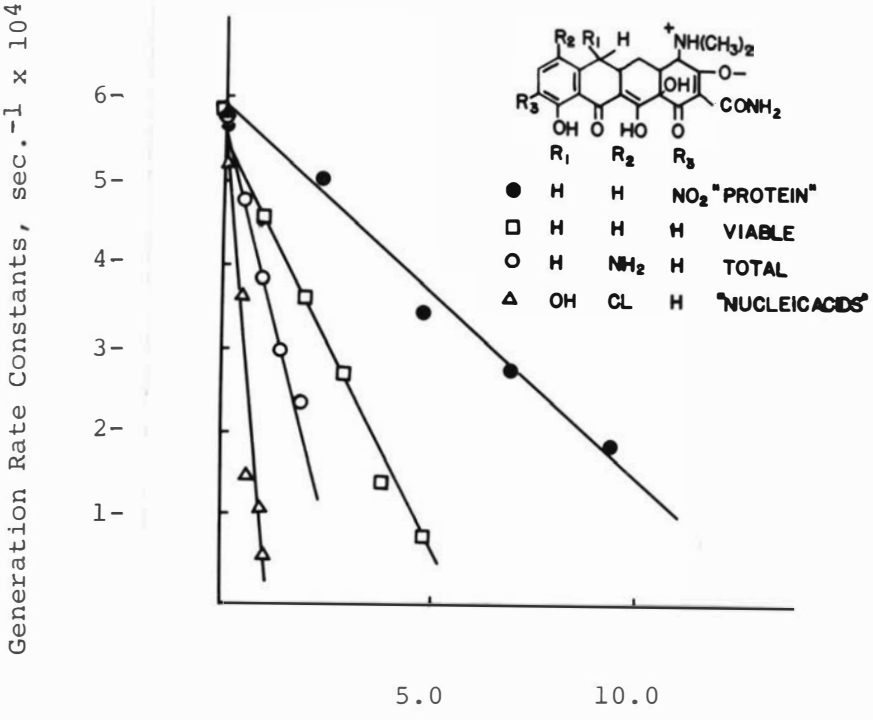


Figure XV. The Linear Dependence of Generation Rate Constants, k_v, k_t, k_p and k_{na}, upon Concentration of several Tetracyclines. The slopes of the lines shown are the average inhibitory rate constants, k^I_{avg}, they and values of k^I_v, k^I_t, k^I_p and k^I_{na} for each antibiotic are listed in Table IV.

Table IV.- Inhibitory Rate Constants, L/M-sec., for Inhibition of E. coli W by Various Tetracyclines.

Tetracycline Analog[a]	$-k^I_v$	$-k^I_t$	$-k^I_p$	$-k^I_{na}$	$-k^I_{avg}$ [b]
7-Nitro-dm-do-tetracycline	833.51	656.76	735.68	769.19	748.78**
7-Chloro-dm-tetracycline	591.86	-	460.25	536.48	517.21
7-Chlorotetracycline	387.77	-	396.14	420.20	401.37
Tetracycline	267.84	-	279.97	267.95	271.50
5-Hydroxytetracycline	275.19	-	254.79	251.83	257.15
7-Amino-dm-do-tetracycline	179.01	175.90	174.08	197.95	181.73**

a. dm-do- is 6-demethyl-6-deoxy-, while dm- is 6-demethyl-6-epihydroxy-.

b. Tested by analysis of covariance to determine if significant differences existed between the inhibitory rate constant determined by using all values of generation rate constants collectively, k^I_{avg}, and individual inhibitory rate constants determined separately. The symbol * indicates significant differences at $P>0.95$, while ** indicates significant at $P>0.975$. See for example, Steel, R. and Torrie, J., "Principles and Procedures of Statistics", McGraw Hill Book Co., Inc., New York, N.Y., (1960) pp. 174.

Table IV.- (cont.)

Tetracycline analog	$-k^I_v$	$-k^I_t$	$-k^I_p$	$-k^I_{na}$	$-k^I_{avg}$ [b]
9-Amino-dm-do-tetracycline	155.53	151.23	128.82	146.13	145.03*
dm-do-tetracycline	103.62	-	86.70	92.93	94.42*
7-Bromo-dm-do-tetracycline	-	62.68	46.09	51.42	51.79
9-Nitro-dm-do-tetracycline	43.40	43.15	42.91	47.86	44.33
4-Dedimethylamino-tetracycline	25.39	23.29	22.24	25.45	24.09
9-Dimethylamino-dm-do-tetracycline	26.22	24.62	23.11	20.73	23.67**
5a(6)-Anhydrotetracycline	12.75	13.81	15.91	17.23	15.48**
12a-Deoxytetracycline	-	2.63	3.85	2.37	2.62[c]
Tetracycline methiodide	2.54	2.12	1.96	2.10	2.18[c]
5a(11a)-Dehydrochlorotetracycline	-	0.36	0.25	0.42	0.34[c]
7-Chloro-isotetracycline	no activity observed				
2-Cyano-2-decarboxamido-tetracycline	no activity observed				

c. Insufficient data available for testing by analysis of covariance.

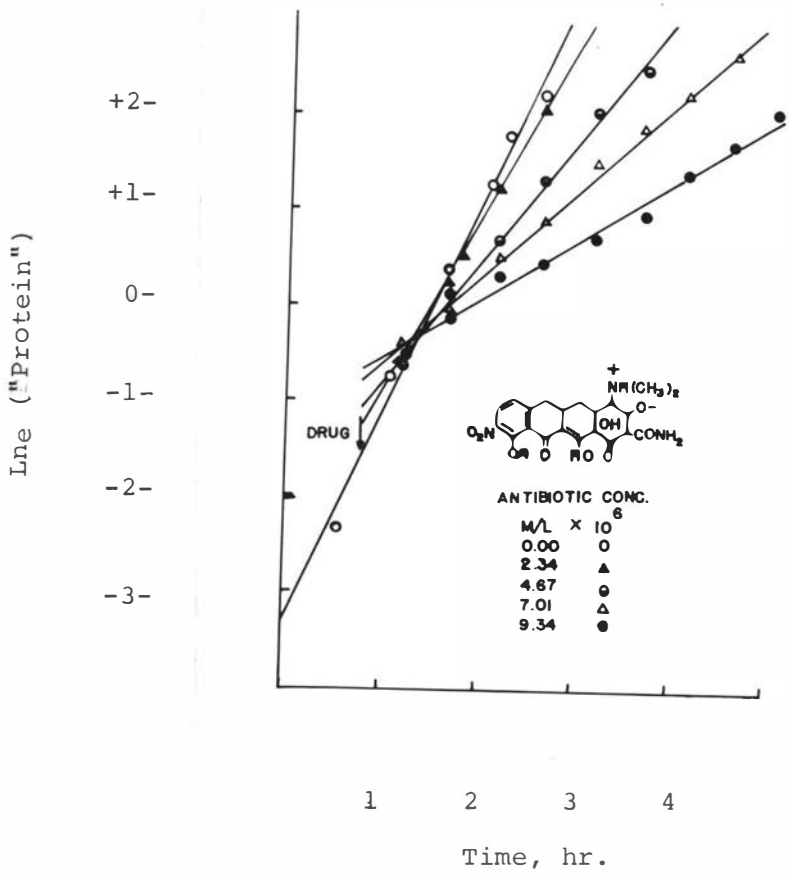


Figure XVI. An Example of Generation Rate Curves for Protein Synthesis. The logarithm of the "Protein" found in E. coli cultures as a function of time of growth in the presence of various concentrations of 9-Nitro-dm-do-tetracycline. The slopes, k_p, are given in Table III.

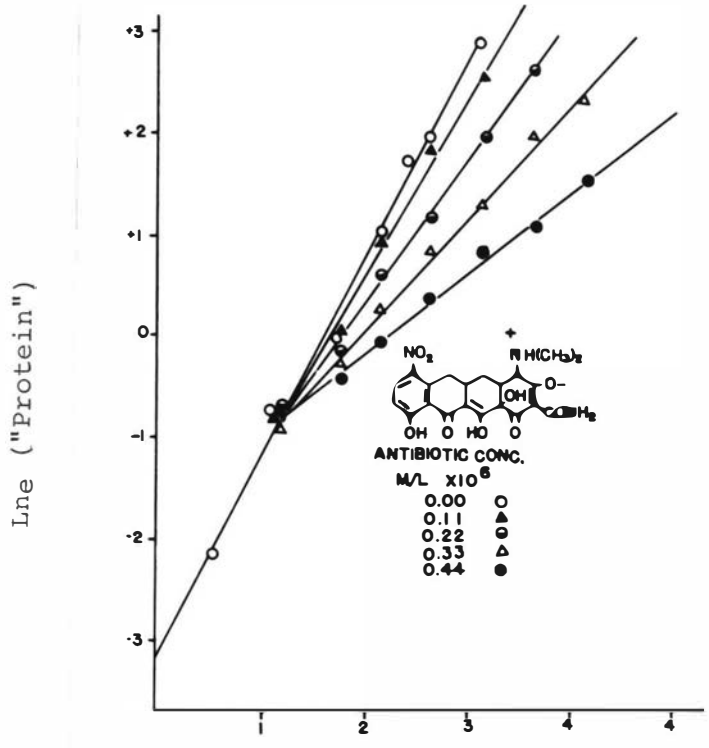


Figure XVII. An Example of Generation Rate Curves for Protein Synthesis. The logarithm of the "Protein" found in E. coli cultures as a function of time of growth in the presence of various concentrations of 7-Nitro-dm-do-tetracycline. The slopes, k_p, are given in Table III.

Tables II and III for the 18 antibiotics studied. It can be seen that these values are either identical with or are very close to the values of k_v or k_t observed in any given culture.

Generation rate constants for protein synthesis obtained by regression analysis from Eq. (4) are in agreement with an equation similar to Eq. (2):

$$k_p = k^o - k_p^I[Tc], \qquad \text{iff } k_{cd} > 0 \qquad \text{Eq. (5)}$$

where k_p^I is the inhibitory rate constant for protein synthesis. Values of k_p do not fit an equation similar to Eq. (3). Fig. XII, XIII, XIV and XV are examples of the fit of values of k_p to Eq. (5). Values of the inhibitory rate constants for protein synthesis are given in Table IV.

The results of two typical experiments in which the rates of nucleic acid synthesis in cultures inhibited by tetracyclines were studied are shown in Figures XVIII and XIX. These experiments did not show an increased rate of nucleic acid synthesis in inhibited cultures as suggested by several investigators (18, 30, 36) but rather showed a decreased amount of nucleic acids correlated to the observed inhibition of cell division. The data from these experiments also fit an expression similar to Eq. (1):

$$[NA] = [NA^o]\, e^{k_{na}t}, \qquad \text{iff } k_{na}^o > k_{na} > 0 \qquad \text{Eq. (6)}$$

when the time, t, is greater than 30 minutes after antibiotic addition and where $[NA^o]$ is the concentration of nucleic acids in the cells 30 minutes after antibiotic addition, [NA] is the nucleic acid concentration in the cells at any later time

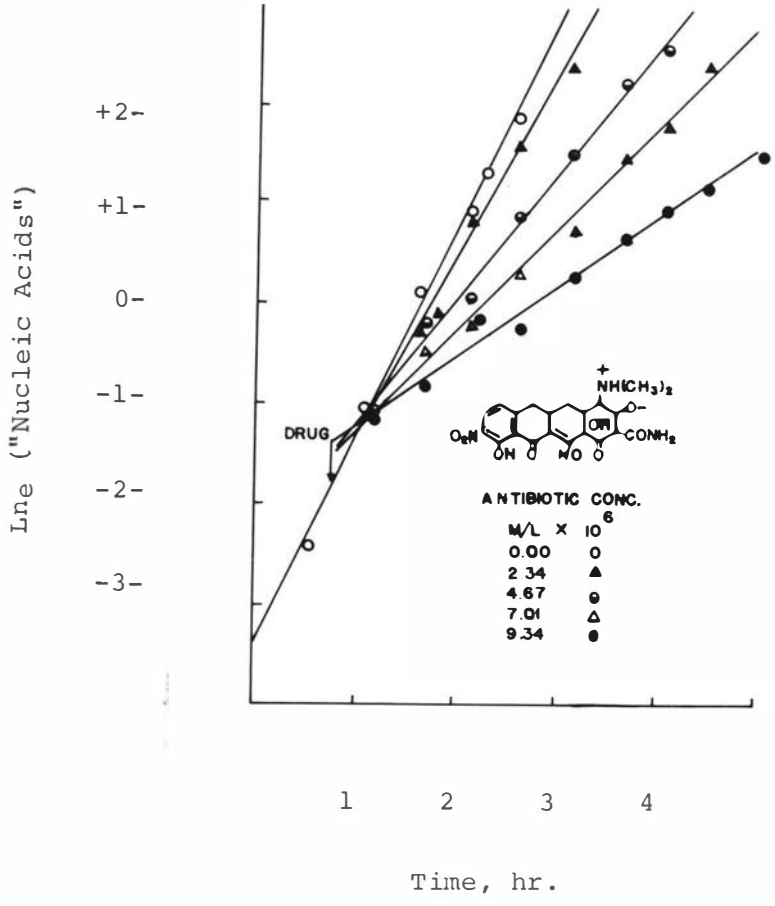


Figure XVIII. An Example of Generation Rate Curves for Nucleic Acid Synthesis. The logarithm of "Nucleic Acids" found in E. coli cultures as a function of time of growth in the presence of various concentrations of 9-Nitro-dm-do-tetracycline. The slopes, k_{na}, are given in Table III.

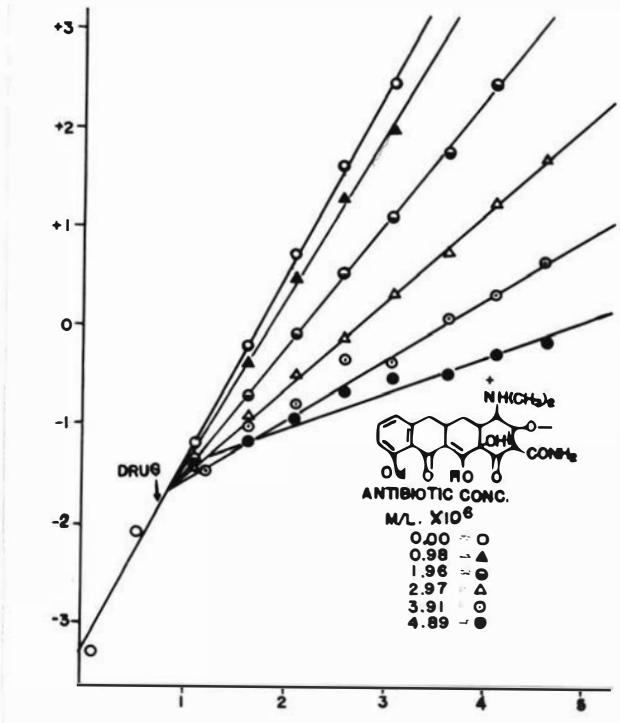


Figure XIX. An Example of Generation Rate Curves for Nucleic Acid Synthesis. The logarithm of "Nucleic Acids" found in E. coli cultures as a function of time of growth in the presence of various concentrations of dm-do-tetracycline. The slopes, k_{na}, are given in Table III.

and k_{na} is the generation rate constant for nucleic acid in the presence of antibiotic. The generation rate constant for nucleic acid synthesis in the absence of antibiotic, k^o_{na}, is identical to the generation rate constant for cell division in the absence of antibiotic, k^o_{cd}. Generation rate constants obtained from the fit of experimental data to Eq. (6) are given in Tables II and III. It can be seen that these values are either identical to or very similar to the values of k_v, k_t or k_p observed in any given culture.

Generation rate constants obtained from Eq. (6) are in excellent agreement with an equation similar to Eq. (2):

$$k_{na} = k^o - k^I_{na}[Tc], \qquad \text{iff } k_{cd} > 0 \qquad \text{Eq. (7)}$$

where k^I_{na} is the inhibitory rate constant for nucleic acid synthesis. Figures XII, XIII, XIV and XV are examples of the fit of values of k_{na} to Eq. (7). Values of the inhibitory rate constants derived from Eq. (7) are listed in Table IV. Values of k_{na} do not fit an equation similar to Eq. (3).

As mentioned above, the values of k_v, k_t, k_p and k_{na} for any given culture are either identical or very similar. Therefore, values of the respective inhibitory rate constants, k^I_v, k^I_t, k^I_p or k^I_{na}, would be expected to be similar. We have tested the hypothesis that these constants are experimentally indistinguishable and find no statistically significant differences between the individual values and an average inhibitory rate constant, k^I_{avg}, for 7 of the antibiotics. Statistically significant differences were found, Table IV, for the 6 other antibiotics tested. Sufficient data was not

available to test the other antibiotics in this fashion. This simple analysis of covariance test did not consider the error involved in the determination of the individual generation rate constants. It seems likely that within present experimental accuracy the 4 generation rate constants, viable and total cell division, protein and nucleic acid synthesis, are the same for all antibiotics. That is a one-to-one relationship, balanced growth, is found among these parameters not only in control cultures but also in antibiotic inhibited cultures.

If balanced growth is indeed maintained in tetracycline inhibited cultures then the average inhibitory rate constant, k^{I}_{avg} in Table IV, would be the best measure of tetracycline activity to use in structure-activity relationships.

C. Time of Onset of Tetracycline Inhibition of Whole Cell Cultures.

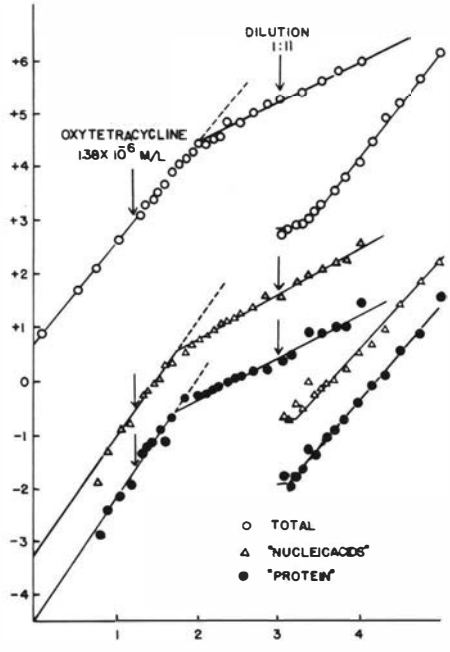
The fact that inhibited cultures exhibit parallel rates of cell division, protein and nucleic acid synthesis is not in accord with the interpretation of previous experiments (18, 30, 34, 36). These experiments were either single point measurements or kinetic experiments which were carried out only during times immediately preceeding antibiotic addition. These experiments reported either decreased protein/cell and increased nucleic acid/cell or inhibition of rate of protein synthesis and stimulation of rate of nucleic acid synthesis. However, Černy and Habermann (33) in a kinetic experiment covering several generations showed parallel rates of protein and nucleic acid synthesis with different times of onset of action. Their work was done at

antibiotic concentrations 15 times higher than those presently employed, which caused complete inhibition and undoubtedly cell death.

The agreement of experimental data with Eq. (L), Eq. ($) or Eq. (¢) was only observed for data obtained approximately 30 minutes after antibiotic addition. During this initial time period cultures appeared to maintain growth rates similar to those observed in controls. This can be observed in the experiments shown in Figures IX, X, XI, XVI, XVII, XVIII and XIX. Similar observations were made in the other experiments not shown. Figure XX shows an experiment designed to document this observation. It can easily be seen that the onset of inhibition for all parameters is considerably beyond the time of antibiotic addition. Viable plate counts, not shown, paralleled those obtained by the total count method. This experiment has been reproduced at a different oxytetracycline concentration with similar results. In addition, Fig. XX shows that the time of onset of recovery from inhibition upon dilution of cultures into antibiotic free media is not immediate either, but also exhibits a lag time. It was thought that both inhibition of and recovery of protein synthesis occurred slightly before the other parameters are affected. In order to test the observation on time of onset of inhibition an experiment was performed in a dextrose-salts broth where the rate of cell division in control cultures was 1/2 that in the high peptone broth. This single experiment, Fig. XXI, suggests that protein synthesis measured by the Folin-Lowry technique and also cell mass as measured by turbidity might be

Figure XX. Time of Onset of and Recovery From Oxytetracycline Inhibition in Peptone Broth Cultures. Generation rate curves for total cells, nucleic acid synthesis and protein synthesis in the presence of 1.38×10^{-6} M 5-Hydroxytetracycline and a one - to - eleven dilution into antibiotic - free media of the inhibited culture. The generation rate curve for protein synthesis has been transposed by subtracting 0.5 from actual values of Ln_e "Protein." The generation rate constants, $sec.^{-1} \times 10^4$, for the undiluted cultures are given in Table II except for values obtained by measuring culture turbidity (not shown) which were 5.63 and 2.19 before and after antibiotic addition, respectively. The values of the generation rate constants in the diluted culture were $k_t = 5.43$; $k_p = 5.09$; $k_{na} = 4.61$; k_v (not shown) = 5.33 and for culture turbidity = 4.76.

The time of antibiotic addition was 4,470 sec. and the time of dilution was 10,850 sec. The times of onset of inhibition and the times of onset of recovery calculated as the intersection of the regression lines, respectively in sec., were: for protein synthesis, 6,200 and 11,800; for turbidity, 6,400 and 11,500; for nucleic acid synthesis, 6,400 and 12,400; and for cell division - total 7,100 and 12,500 and viable, 6,500 and 12,200.



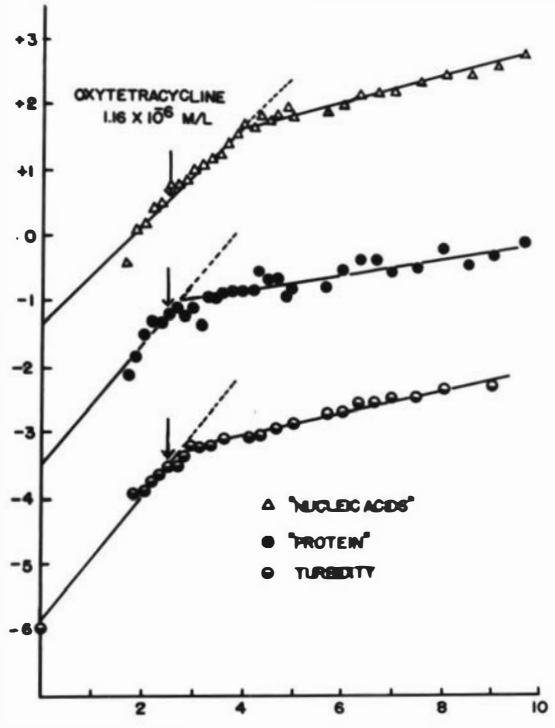
Ln$_e$ (Concentration)

Time, hr.

Figure XX.

Figure XXI. Time of Onset of Oxytetracycline Inhibition of Cultures in Dextrose-Salts Broth. Generation rate curves for nucleic acid synthesis, protein synthesis and culture turbidity in the presence of 1.16×10^{-6}M 5-Hydroxytetracycline. The generation rate curve for nucleic acid synthesis has been transposed by adding 2.0 to the actual value of $\ln_e$"Nucleic Acids." Values of generation rate constants, $sec.^{-1} \times 10^4$, in the absence and presence of antibiotic respectively, were: k_{na} = 2.13 and 0.56; k_p = 2.62 and 0.37; k_t (not shown) = 2.44 and 0.12; and for turbidity 2.54 and 0.37.

The time of antibiotic addition was 8,900 sec. The times of onset of inhibition, sec., calculated as the intercept of the regression lines were: for protein synthesis, 9,900, for turbidity, 10,300; for cell division, 10,750; and for nucleic acid synthesis, 14,200.



Ln_e (Concentration)

Time, hr.

Figure XXI.

inhibited sooner than nucleic acid synthesis. Cell division, not shown, was inhibited at an intermediate time. The existence of these different lag times explains the discrepancy between parallel equilibrium inhibition rates observed in this work and non-parallel amounts of nucleic acid and protein per cell reported by others (18, 30, 34, 36). Experiments performed in culture media which do not allow rapid rates of cell division would be expected to accentuate this difference in times of onset of inhibition.

This order of inhibition and recovery is consistent with the hypothesized primary inhibition of protein synthesis followed by a secondary, indirect inhibition of cell division and nucleic acid synthesis due to a lack of protein.

D. Effect of Broth pH Changes on Tetracycline-Inhibited Cultures.

Jones and Morrisson (20) and Benbough and Morrisson (17) have reported that when the pH of culture media of known free metal ion concentration is varied, the inhibition produced is proportional only to the "molecular", presumably zwitterionic, species of several tetracyclines. Connamacher, Mandel and Hahn (66) have recently reported data on the uptake of tetracycline in B. cereus cultures at pH 6.7 and 7.3 and at two Mg ion concentrations which are in qualitative agreement with the hypothesis of Jones and Morrisson.

Because the slow growth rate characteristics of E. coli W in dextrose-salts broth made it unlikely that the tetracyclines would be sufficiently stable it was not possible to verify these results for other tetracyclines in

a media of known free metal ion concentration. However, experiments in peptone broth are consistent with these results at two broth pH values where free metal ion concentration would be expected to be constant. Figure XXII shows a plot of Eq. (2) for experiments at pH 6.2 and 6.65. The concentration of tetracycline is adjusted to reflect the concentration of zwitterionic form rather than the total concentration. Experiments at a broth pH of 7.4, where a much lower free metal ion concentration would be expected because of the higher $HPO_4^{=}$ concentration, do not fit the single line plotted in Fig. XXII. Activities obtained with 5a(6)-anhydrotetracycline (pH 6.2 and pH 6.65) and 4-dedimethyl-amino-tetracycline (pH 6.65 and pH 7.4) do not fit such a simple concept either, Table V. These tetracyclines have, however, unusual pKa's.

In addition to the growth-rate dependent difference in time of onset of inhibition of the various culture parameters, there is a lag time observed before any parameter is inhibited, Fig. XXI. Two general explanations are possible for this lag time. First, a finite time may be required to establish equilibrium concentrations within the cells. Second, antibiotic may permeate the cell and arrive at the receptor site but require finite time to exert a measurable effect.

Besides causing a change in the degree of equilibrium inhibition produced by a given total antibiotic concentration, broth pH changes are also capable of changing the growth-rate independent lag time for onset of inhibition. Figure XXIII shows an experiment identical to that presented previously,

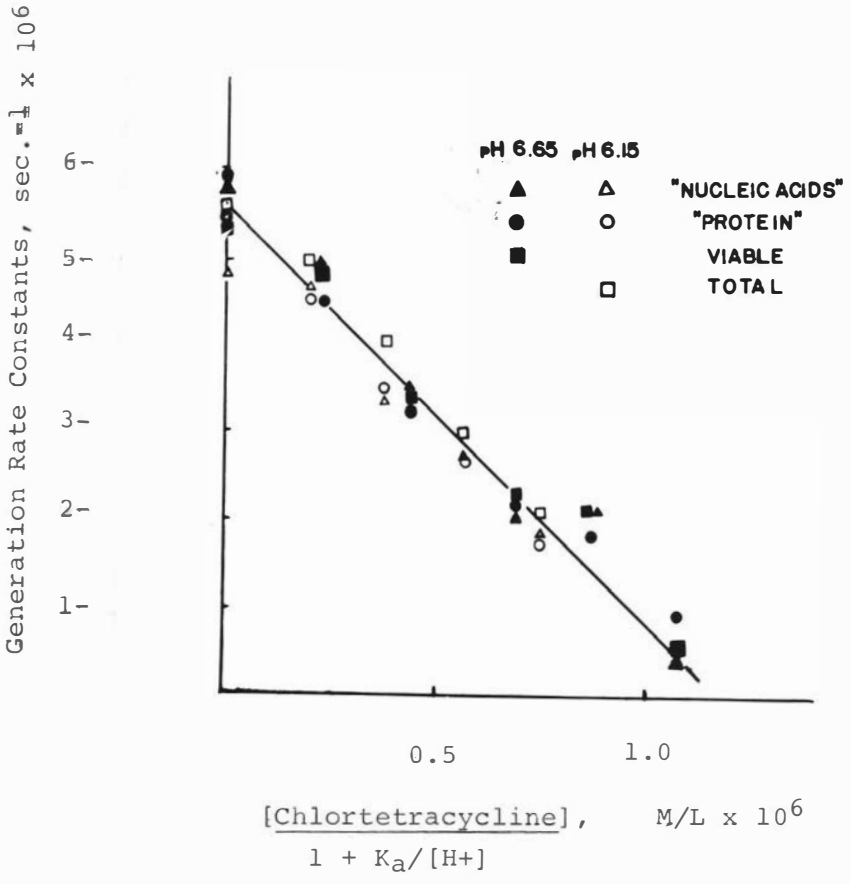


Figure XXII. The Linear Dependence of Generation Rate Constants upon Concentration of Zwitterionic Chlortetracycline. Constants were obtained at two broth pH values, 6.15 and 6.65. Values of generation rate constants obtained at a higher broth pH value, 7.4, and, therefore, a higher $HPO_4^=$ concentration do not fit this single line. The slope of the line shown is 470 L/M-sec.

Table V.- First Order Generation Rate Constants, $sec^{-1} \times 10^4$, and Inhibitory Rate Constants, L/M-sec., for E. coli W in Various Broth Cultures Inhibited by Several Tetracyclines.

10^6 M	k_t	k_p	k_{na}	k^I_{avg}
7-Chlorotetracycline, peptone broth, pH 6.2				
0.000	5.56	5.50	4.88	
0.192	4.95	4.55	4.66	
0.384	4.06	3.50	3.38	
0.575	3.01	2.69	2.71	
0.767	2.10	1.78	1.86	
k^I	-461.11	-484.15	-417.52	-454.28**
5a(6)-Anhydrotetracycline, peptone broth, pH 6.2				
0.00	5.56	5.50	4.88	
4.67	4.71	4.68	3.90	
9.34	3.94	3.23	3.45	
14.01	3.35	2.57	2.92	
18.68	1.73	1.99	2.35	
k^I	-19.27	-19.54	-12.95	-17.25**

** Significant differences between inhibitory rate constants determined by analysis of covariance as explained in Table IV.

Table V.- (cont.)

10^6 M	k_t	k_p	k_{na}	k^I_{avg}
4-Dedimethylamino-tetracycline, peptone broth, pH 7.4				
0.00	4.57	5.22	4.76	
3.34	3.87	5.01	3.96	
6.65	3.33	3.73	3.48	
9.96	2.71	3.03	3.00	
13.24	2.09	2.13	2.54	
k^I	-18.48	-24.70	-16.32	-19.83**
7-Chlorotetracycline, pH 6.7, dextrose-salts broth				
0.000	2.39	2.30	2.18	
0.163	2.07	1.92	2.01	
0.327	0.97	0.84	0.79	
0.490	0.39	0.51	0.50	
0.655	0.22	0.38	0.37	
k^I	-368.34	-315.81	-312.59	-332.25

** Significant differences between inhibitory rate constants determined by analysis of covariance as explained in Table IV.

Figure XXIII. The Effect of Broth pH on Time of Onset of Oxytetracycline Inhibition. Generation rate curves, done on separate days at two peptone broth pH values, for cell division- by the total count method and for culture turbidity in the presence of two different concentrations of 5-Hydroxytetracycline. The generation rate curves for cell division have been transposed by subtracting 3.0 from the actual value of Ln_e (organisms/ml. x 10^{-6}). The generation rate constants, $sec.^{-1}$ x 10^4, in the absence and presence of antibiotic, respectively, for cell division, were 5.16 and 2.20 at pH 6.65 and 4.75 and 2.30 at pH 7.4, and for turbidity were, 5.63 and 2.19 at pH 6.65 and 5.56 and 2.21 at ph 7.4.

The times of antibiotic addition were 4,470 sec. at ph 6.65 and 5,405 sec. at pH 7.4. The times of onset of inhibition, sec., calculated as the intersection of the regression lines were for cell division 7,100 and 6,200 and for turbidity 6,400 and 5,900 at pH 6.65 and 7.4, respectively.

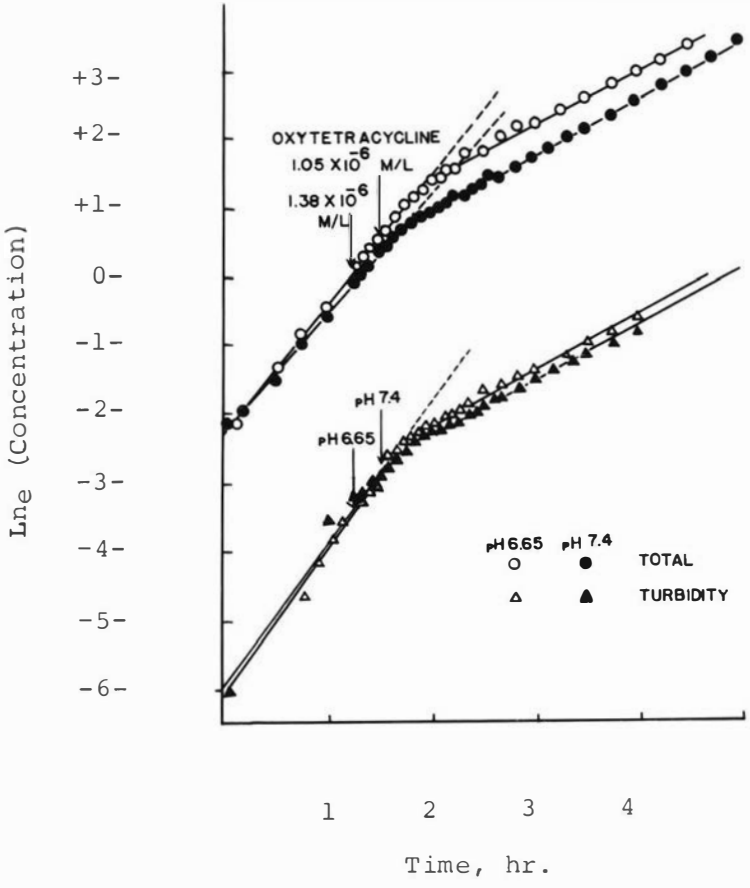


Figure XXIII.

Fig. XX, except that the pH of the broth was 7.4 instead of 6.7. The lag for onset of inhibition of protein synthesis (measured as cell mass) and cell division are seen to be shortened by two-thirds. The equilibrium amount of inhibition achieved by two different total oxytetracycline concentrations was the same in both experiments. If the hypothesis of Jones and Morrisson (20) is correct, then the concentration of zwitterionic species present in the broth must be the same in both experiments. The nature of the initial inhibitory process must be quantitatively different in these experiments. It seems most likely that the process of permeation into the cells is affected by the pH change and that this is the process responsible for the growth-rate independent lag time observed in these experiments.

Arima and Izaki (64, 244, 245) have studied permeation of oxytetracycline in sensitive and resistant cells at high antibiotic concentrations and found that it depends upon an energy requiring process. Franklin (65) has verified a glucose-dependent uptake at low concentrations of tetracycline and chlortetracycline. The condition of the cells in these cultures is so different from the cells in the present work that it is hard to make meaningful comparisons. However, it is clear that the ability to permeate the cells is an important factor in antibiotic activity, whether this process is an inactive or an active diffusion. Either process might be expected to be sensitive to the broth pH.

E. Kinetic Models for Inhibition of Bacterial Cultures by Tetracyclines.

Studies of the kinetics of inhibition of cell growth are capable of providing information of a type other than the estimates of relative potencies or the estimates of order of onset of inhibition which have been mentioned above. Kinetic studies can provide information which is useful in testing proposed models of antibiotic activity. Any proposed model of tetracycline activity should be consistent with several results of the present study.

These are:

a. Exponential growth is maintained in cultures inhibited by low concentrations of tetracyclines; see Eq. (1), (4) and (6).

b. The generation rate constants for cell division, protein and nucleic acid synthesis are the same in any given tetracycline-inhibited culture, $k_{cd}>0$.

c. Rate constants for inhibited cultures are linearly dependent upon the first power of the antibiotic concentration; see Eq. (2), (5) and (7).

d. Antibiotic inhibition is reversed by dilution into antibiotic free media, Fig. XX.

In addition, it seemed likely that:

1. Protein synthesis is inhibited before cell division or nucleic acid synthesis.

2. Permeation of antibiotic into cells is important and might be responsible for the lag in time of onset of inhibition.

A simple model which describes the kinetics of cell division observed in E. coli cultures inhibited by low tetracycline concentrations has been proposed by Garrett, Miller and Brown (243). It assumes equilibrium (K) between free E. coli (E_f) and tetracycline (Tc) to form inhibited E. coli (E_i):

$$E_f + Tc \overset{K}{\rightleftharpoons} E_i \qquad \text{Eq. (8)}$$

and it also assumes that the rate of generation of total E. coli (E_t) is proportional to the concentration of free E. coli:

$$\frac{d\ (E_t)}{d\ t} = k^o_{cd}\ (E_f) \qquad \text{Eq. (9)}$$

When the fraction of inhibited E. coli (Φ) is calculated from Eq. (8):

$$\Phi = \frac{E_i}{E_t} = \frac{K\ [Tc]}{1 + K\ [Tc]} \qquad \text{Eq. (10)}$$

and when substituted in Eq. (9), one obtains:

$$\frac{d\ (E_t)}{d\ t} = k^o_{cd}\ (1 - \Phi)\ E_t = k^o_{cd}\ \left(1 - \frac{K\ [Tc]}{1 + K\ [Tc]}\right) \qquad \text{Eq. (11)}$$

Since it is found experimentally that,

$$\frac{d\ E_t}{d\ t} = k_{cd}\ (E_t) \qquad \text{Eq. (12)}$$

by combining Eq. (11) and Eq. (12), one obtains:

$$k_{cd} = k^o_{cd}\ \left(1 - \frac{K\ [Tc]}{1 + K\ [Tc]}\right) \qquad \text{Eq. (13)}$$

which is a rearrangement of Eq. (3). When K[Tc] is much less than 1, a "limiting case", then Eq. (13) is equivalent to Eq. (2), where $k^I_{c.d.} = k^o_{c.d.}\ K$.

This simple kinetic model is consistent with results outlined as a, c and d. It does not consider the observations outlined in b, 1 and 2. Consequently, it does not lead to Eq. (2) in a biochemically meaningful fashion. Garrett et. al. (243) have tried to explain why this limiting case of Eq. (13) is observed during tetracycline inhibition by postulating that there are differences between rates of protein synthesis and the rates of cell division in inhibited cultures. The present report has shown that this is most probably not true, result b, and it would be helpful if another hypothesis were advanced to explain this adherence to the limiting case of Eq. (13).

A model consistent with the results outlined above has been sought within the proposed biochemical mode of action. This model assumes that tetracyclines bind to the aminoacyl site of ribosomes and thereby prevent the binding of AAtRNA. The evidence for this mode of action has been presented in the literature survey. If the usual type of enzyme-inhibitor kinetics is followed it would be expected that this mode of action would generate an inverse relationship between rate of protein synthesis and inhibitor concentration. However, a growing E. coli culture is not the normal in vitro test system. Such a culture will not have constant concentrations of substrate or enzymes. It was thought, therefore, that a model which allowed these concentrations to vary with the time of growth and also the inhibitor concentration, might lead to results similar to that observed in the experimental cultures.

Conditions have not been found such that the proposed model generates outputs which agree with the experimental data. The model will be presented, however, since it or a simple variation of it may be consistent with the experimental observations when other conditions are tried.

The model, Fig. XXIV, is presented in terms of some of the reactions involved in protein synthesis in a bacterial culture. The simplest model involving the least number of reactions has been postulated as a starting point. Reactants are expressed in terms of concentration in the total culture. The model states that there is a reversible passage of tetracyclines from outside the cells (Tc') into the cells (Tc), Eq. (14). This step has been ignored in initial models. Tetracyclines inside the cells bind to ribosome-RNA (R) and form a complex (R·Tc) which is not capable of synthesizing protein, Eq. (15). Normal ribosomes-mRNA complexes are, of course, capable of binding substrate (S), AAtRNA and forming a peptide bond (P) on the ribosomal surface, Eq. (16). Peptide bond formation frees the aminoacyl site of the ribosome-mRNA complex and allows either tetracycline or AAtRNA to bind.

Two steps, Eq. (17) and (18), have been incorporated into the model with the expectation that they might lead to results different from ordinary enzyme kinetics. The first of these steps is that substrate is synthesized from substrate precursors (S') in a reaction which has been presumed to proceed at a rate proportional to the concentration of protein

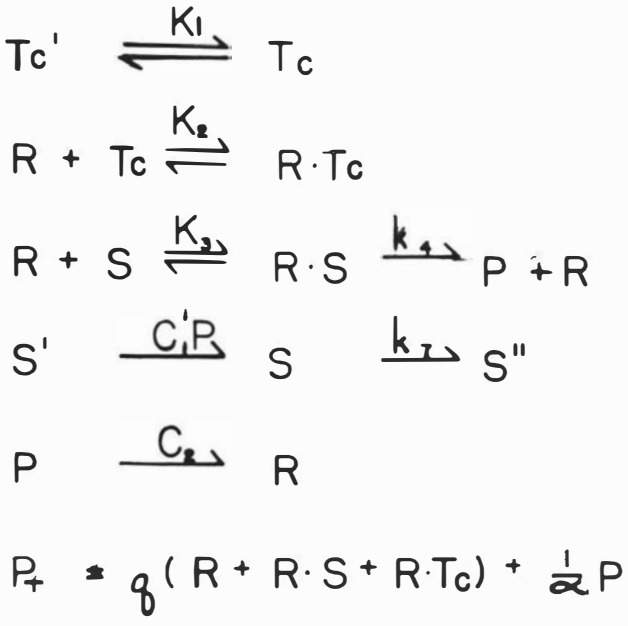


Figure XXIV. Proposed Model of Tetracycline Inhibition of Protein Synthesis in Bacterial Cultures. Tc' is the concentration of tetracycline in the culture broth, Tc is the concentration within the cells, R is ribosomes - mRNA - peptidyl tRNA, S is AAtRNA, P is the peptide bond concentration, S' are AAtRNA precursors, S' are AAtRNA degradation products, P_t is the total protein concentration within the cells, q was 0.4 and α was 20.

in the culture. That is, the rate of this synthesis step has been presumed to be proportional to P. The proportionality constant utilized, C_1, is a pseudorate constant and contains in addition to the concentrations of the precursors, the fraction of the total number of peptide bonds in the culture which are part of the enzymes catalyzing this reaction, i.e. aminoacyl synthetases. Substrate is also lost, non-enzymatically, in this model by hydrolysis, Eq. (17). The second differentiating feature of this model is that it postulates that a fraction of the product peptide bonds are ribosomal precursor proteins and that these proteins give rise to new ribosomes, Eq. (18). The constant, C_2, utilized in this step is also a pseudo-rate constant which contains the concentration of rRNA in the culture as well as the fraction of total peptide bonds which are ribosomal peptides. However, the assumption that C_1 or C_2 are constants, which has been employed, is probably not valid. The concentration of tRNA or rRNA per cell might be relatively constant, but the concentration of these species in the culture is undoubtedly not constant. They are probably increasing at the same rate as the number of cells in the culture. Perhaps there are offsetting changes in other substrate or ribosomal precursors and therefore, this approach, because of its simplicity, was tried first.

The concentration of total protein (P_t) present in the culture has been assumed to be the sum of the ribosomal and non-ribosomal protein, Eq. (19). For these calculations it was assumed that ribosomes were 40% protein (q) and that

there are 20 peptide bonds in the average E. coli protein (α).

The differential equations, Eq. (20) through (26), derived from this model are given in Table VI. They are too complex to be solved by ordinary integration. They do however, lead to a simple expression for the rate of total protein synthesis:

$$\frac{d P_t}{d t} = k_4[R \cdot S] - (1/\alpha - q) [P] \qquad \text{Eq. (27)}$$

This equation stresses the important role played by α and q in determining the nature of the P_t output in the model.

The above model is based upon the concentration of the various reactants in the total culture. If one were to write a similar model based upon the concentrations per cell one might be justified in assuming a steady-state in R.S' in which case:

$$[R \cdot S'] = \frac{k_4[R_t'] \quad [S']}{K_m + [S']} \qquad \text{Eq. (28)}$$

where the superscript (') is used to designate concentrations per individual cell rather than in the total culture and R_t' is the concentration of all forms of ribosomes within the cell. While this equation cannot be substituted into Eq. (27), it indicates that the model might be tested under two general conditions, when S is greater than K_m and when S is less than K_m.

Since ordinary integration methods are not useful, the model has been tested by analog simulation. An unscaled analog computer program is given in Fig. XXV. The model is too big (too many integrations) for the available analog

Table VI.- The Differential Equations from a Model of Tetracycline Inhibition of Protein Synthesis in Bacterial Cultures.

$$\frac{d\,Tc'}{d\,t} = k_{-1}[Tc] - k_1[Tc'] \qquad \text{Eq. (20)}$$

$$\frac{d\,Tc}{d\,t} = k_1[Tc'] - k_{-1}[Tc] - k_2[R][Tc] + k_{-2}[R\cdot Tc] \qquad \text{Eq. (21)}$$

$$\frac{d\,R\cdot Tc}{d\,t} = k_2[R][Tc] - k_{-2}[R\cdot Tc] \qquad \text{Eq. (22)}$$

$$\frac{d\,R}{d\,t} = k_{-2}[R\cdot Tc] - k_2[R][Tc] + k_4[R\cdot S] + k_{-3}[R\cdot S] - k_3[R][S] + C_2[P] \qquad \text{Eq. (23)}$$

$$\frac{d\,S}{d\,t} = k_{-3}[R\cdot S] - k_3[R][S] - k_7[S] + C_1[P] \quad ; \quad C_1 = C_1'[S'] \qquad \text{Eq. (24)}$$

$$\frac{d\,R\cdot S}{d\,t} = k_3[R][S] - k_{-3}[R\cdot S] - k_4[R\cdot S] \qquad \text{Eq. (25)}$$

$$\frac{d\,P}{d\,t} = k_4[R\cdot S] - C_2[P] \qquad \text{Eq. (26)}$$

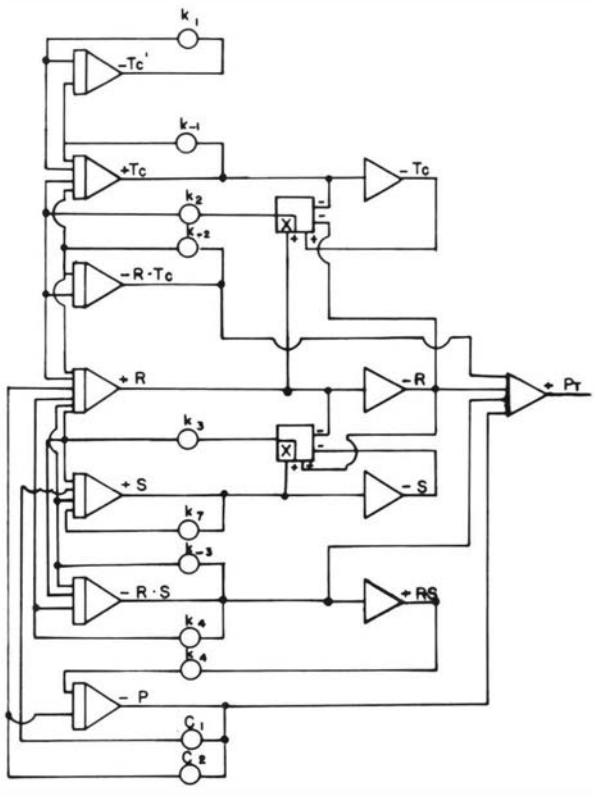


Figure XXV. An Unscaled Analog Computer Program for the Solution of the Differential Equations of the Proposed Model of Tetracycline Inhibition of Protein Synthesis in Bacterial Cultures.

computer[13] and could only be partially tested with this instrument. In addition, most analog computers are only able to perform accurate calculations over about 2 log cycles. These difficulties were overcome by using the International Business Machines 1130 digital computer[14] with associated Continuous Systems Modeling Package. The actual program utilized is given, in Fig. XXVI, in ordinary analog computer symbols. Eighty analog elements were available for use with this package and the output is accurate over several log cycles.

Since the values for initial concentrations of reactants and rate constants which were tried did not lead to outputs which agreed with the experimental data, the values will not be described in detail. The values tried are listed in Table VII. When K_m was greater than S, no conditions could be found where the output was similar to experimental data, even when Tc' and Tc were zero. While it was possible to obtain an exponential increase of total protein synthesis, balanced growth was not maintained. That is, the ratio of (S) to (R) was not constant. These experiments were unfortunately performed before the need to utilize a value of α larger than 1 was realized. Inspection of the results reveals that this would not have changed the results of these experiments, however, other conditions might be found where

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13. TR 20, Electronic Associates Inc., Long Branch, N. J.

14. We are grateful to Mrs. Lillian Kornhaber of the Department of Biometry, Medical College of Virginia, for performing these calculations.

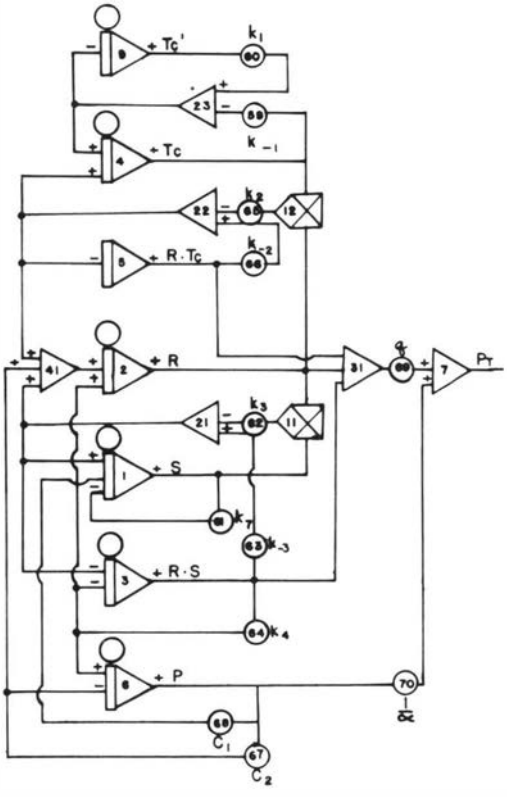

Figure XXVI. The Continuous Systems Modeling Package Program Used in the Search for Solutions to the Differential Equations from the Proposed Model of Tetracycline Inhibition of Protein Synthesis in Bacterial Cultures.

Table VII.- Compilation of Conditions Which Were Employed to Test the Model of Tetracycline Inhibition of Protein Synthesis in Bacterial Cultures.

Identity #	S^o	R^o	P_t^o	k_3	k_{-3}	k_4	K_m	C_1	C_2	k_7
A	3.750	0.075	0.03	0.02	0.004	1.2	60.20	1.4	1.0	0.008
B	3.750	0.075	0.03	0.02	0.004	1.2	60.20	1.4	1.1	0.008
C	0.375	0.075	0.03	0.02	0.004	1.2	60.20	1.4	1.0	0.008
D	0.375	0.075	0.03	0.10	0.020	6.0	60.20	7.0	5.0	0.040
E	3.750	0.750	0.30	0.10	0.020	6.0	60.20	7.0	5.0	0.040
F	0.375	0.075	0.03	1.00	0.020	6.0	6.02	7.0	5.0	0.040
G 1 2 3 4	0.375	0.075	0.03	1.00	0.200	6.0	6.20	7.0	5.0, 5.5, 6.0, 6.5	0.040
H 1 2	0.375	0.075	0.03	1.00	0.200	6.0	6.20	10.0, 15.0	6.0	0.040
I 1 2 3	0.375	0.075	0.03	0.10	0.020	6.0	60.20	1.65, 1.75, 1.85	0.5	0.040
J 1 2 3 4	0.375	0.075	0.03	100.00	20.000	6.0	0.26	2.0, 2.4, 2.5, 3.0	0.5	0.004

Table VII.- (cont.)

I.D. #	S^o	R^o	$R \cdot S^o$	P^o	P_t^o	k_3	k_{-3}	k_4	K_m	C_1	C_2	k_7
K 1	0.375	0.075	-	-	0.030	100.0	20.0	6.0	0.26	12.0	2.5	0.020
K 2	0.150	0.300	0.0225	0.025	0.046	100.0	20.0	6.0	0.26	12.0	2.5	0.020
L 1 2 3 4 5 6	0.150	0.030	0.0225	0.025	0.046	100.0	20.0	6.0	0.26	12.0	2.6, 2.7, 2.8, 3.0, 3.25, 3.5	0.020
M 1 2 3 4 5	0.150	0.030	0.0225	0.025	0.046	100.0	20.0	6.0	0.26	12.0	3.5	0.250, 0.500, 1.000, 2.500, 5.000
N	0.135	0.030	0.0175	0.055	0.074	100.0	20.0	6.0	0.26	2.5	0.5	0.004
O	0.405	0.090	0.0525	0.055	0.112	100.0	20.0	6.0	0.26	2.5	0.5	0.004
P	0.100	0.028	0.1100	0.055	0.018	100.0	20.0	6.0	0.26	2.5	0.5	0.004
Q	0.244	0.071	0.0635	0.239	0.066	100.0	20.0	6.0	0.26	2.5	0.5	0.004

a. For experiments A to Q inclusive values of k_1, k_{-1}, k_2, k_{-2}, Tc', and Tc were not used. For experiments A to O inclusive, was programmed as 1.

Table VII.- (cont.)

Identity #	Tc^o	k_2	k_{-2}	Tc'^o	k_1	k_{-1}
Q 2	1	1000.00	200.000	-	-	-
Q 3	1, 2, 3	100.00	20.000	-	-	-
Q 4	3	10.00	2.000	-	-	-
Q 5	3	1.00	0.200	-	-	-
Q 6	20	0.10	0.020	-	-	-
Q 7	20, 80, 240	0.01	0.002	-	-	-
Q 8	240	0.02, 0.005	0.002	-	-	-
Q 9	240	0.01	0.001, 0.004, 0.010, 0.020	-	-	-
Q 10	240	0.01, 0.10	-	-	-	-
Q 11	-	0.10	0.020	50, 100, 150, 200	1	0.1

b. For experiments Q 2 to Q 11, inclusive, the values of S^o, R^o, $R \cdot S^o$, P^o, P_t^o, k_3, k_{-3}, k_4, K_m, C_1, C_2, and k_7 which were used were the same as those given above under Identity # Q.

this would not be true.

Conditions which allowed an exponential increase of total protein and where (S) and (R) increased exponentially at the same rate were found when (S) was greater than K_m and Tc' and Tc were equal to zero. However, when Tc' or Tc were different from zero, it was not possible to find conditions of Eq. (14) and/or Eq. (15) such that protein synthesis was maintained at a reduced exponential rate. Rather the rate of protein synthesis always decreased initially and then increased to the rate occurring in the absence of antibiotic. It was not possible to find conditions which maintained the initial reduced rate of protein synthesis for times as long as those seen experimentally. In addition, the rate constants evaluated from the initial inhibited protein synthesis, Table VII, did not have a linear dependence upon the total antibiotic concentration.

Several variations of the model have been suggested by the present work. When (S) is less than K_m, values of rate constants were rejected because the rate of protein synthesis was not first-order. These non-exponential results might have been caused by the value of α utilized. These values of rate constants might lead to exponential protein synthesis and (S) and (R) might be produced in proportionate amounts if a larger number were utilized for α.

When (S) is greater than K_m, the model as presently stated appears to be inherently balanced, i.e. after establishment of equilibria between antibiotic and ribosomes it returns

to the rate of protein synthesis prevailing before addition of antibiotic. Perhaps alternate values of rate constants can be found which would prolong the time of establishment of this equilibria. The statement of the equilibria between Tc' and Tc is not exact as expressed in Eq. (14). It has only been used in a very limited manner. Expression of this equilibria in terms of the concentration of tetracycline in the broth (Tc') and the concentration within an individual cell (Tc_i) might be a desirable way of altering the present model such that attainment of equilibria between antibiotic and ribosomes is not achieved.

If these changes are not sufficient, then the effect of variation of the pseudo-rate constants, C_1 and C_2, with time and with tetracycline concentration should be considered. The concentrations of either tRNA or rRNA in the culture which are contained in these "constants" undoubtedly change during culture growth. It seems likely that tetracyclines would indirectly affect these "constants" by virtue of their secondary inhibition of nucleic acid synthesis. The present work has utilized these parameters as constants solely because this was the simplest and easiest procedure. The introduction of such a modification would necessitate a very large number of integrations and is probably not practical. It would require a great deal more knowledge about metabolic events within the inhibited bacterial cells than is presently known in a quantitative manner. It should be realized that the present work as well as others

(18, 30, 33 and 34) has measured effects on synthesis of undifferentiated nucleic acids and not on the individual species of ribonucleic acids.

Another alternative to the present model would be to formulate a similar model on the basis of concentrations per cell and then sum these concentrations over the number of cells which exist at any given time. In this type of a model it should be possible to make certain simplifying assumptions: such as steady-state concentrations of various intermediates, perhaps R·S, tRNA and rRNA. The summation of values over indices which change with time and inhibitor concentration does, however, present unusual problems in analog simulation and has not been utilized in this study.

V. Summary

Sixteen of the 18 tetracyclines under investigation have been shown to inhibit cell division, protein synthesis and nucleic acid synthesis in cultures of exponentially dividing E. coli W. The number of cells in inhibited cultures has been obtained as a function of time of growth by viable and by total cell count methods. Small differences found between the total number of cells and the number of viable cells are not believed to be related to the effect of the tetracyclines in these cultures. Rather these differences are felt to be caused by aggregation of viable cells during the assay for viable cells. A filter technique using detergent-extracted cellulose ester membranes was developed to isolate TCA-ethanol precipitates of the E. coli cells as a function of the time of growth. The hot TCA soluble extract of these precipitates was used to measure nucleic acids by the orcinol reaction. The hot TCA insoluble material remaining on the filter was trypsin-digested and the protein content estimated by the Folin-Lowry assay.

At low concentrations of these tetracyclines, inhibition is produced after an initial time lag during which no effect on the tested culture parameters was seen. After this lag time, all tested culture parameters could be described by first order kinetic expressions, such as; Eq. (1), (4) and (6). Generation rate constants for tetracycline-inhibited cultures, k_v, k_t, k_p, k_{na}, have been obtained from the

least squares fit of the experimental data to these equations. It seems most likely that generation rate constants obtained for all culture parameters are identical for any given culture; that is, balanced growth is maintained. This result, although not strictly in agreement with previous reports is explained by the fact that previous experiments were either only single-point measurements or were not determined over a sufficient number of generations such that equilibrium inhibitionwwas not observed by these workers.

Generation rate constants for all culture parameters were found to be linearly related to the concentration of the tetracycline analog in the culture, Equations (2), (5) and (7). The proportionality constants, k_v^I, k_t^I, k_p^I, k_{na}^I, of these relationships have been determined. When the data for all culture parameters were treated together a single proportionality constant, k_{avg}^I, was obtained. This value is a measure of the antibacterial potency of the tetracycline analog. These potencies are thought to be suitable for use in structure-activity relationships since they are kinetic constants determined by several different assay methods.

The simple model of Garrett, Miller and Brown (243) which attempts to explain the linear relationship of generation rate constants to antibiotic concentration has been presented. The inadequacy of the kinetic explanation proposed by this model has been noted and attempts have been made to derive a more satisfactory model of tetracycline inhibition. A model based on the biochemical mode of action

of the tetracyclines was proposed. This model was investigated to see if it might lead directly to the linear relationship of generation rate constants to antibiotic concentration. It was felt that this might result from the inclusion of reactions leading to the growth of "substrate" and "enzyme" concentrations in addition to the usual enzyme-kinetic reactions. Conditions such that the model generated output consistent with the experimentally observed type of inhibition were not found. Of course, all conditions were not tested. Several modifications of the model have been suggested such that the experimental results might be explained by this type of model but because of the large number of differential equations which need to be solved these have not been tested.

The time of onset of inhibition was approximately 30 minutes (1 1/2 generations) in these cultures. This time and the time of recovery from inhibition upon dilution into antibiotic-free media was observed to be shorter for the inhibition of protein synthesis than for the inhibition of cell division or nucleic acid synthesis. This difference in time of onset of inhibition was especially pronounced when the broth was changed such that the rate of growth was reduced. Under these conditions the time of onset of inhibition of protein synthesis did not vary while the time of onset of inhibition of cell division and nucleic acid synthesis were increased. An increase in broth pH value, achieved by changing total phosphate as well as phosphate

ion ration, decreased the time of onset of inhibition of cell mass and cell division. These experiments can be most easily explained if one assumes that inhibition of nucleic acid synthesis and inhibition of cell division are the result of a prior inhibition of protein synthesis. In addition there is a finite lag time, which is broth pH sensitive, before protein synthesis is inhibited.

This interpretation is consistent with, and these results are further evidence that, the primary mode of action of tetracyclines is to cause an inhibition of protein synthesis. Since all tetracyclines behaved qualitatively the same it seems most likely that they are all acting by the same mechanism. However, this cannot be said with certainty since the differences between time of onset of inhibition of protein synthesis and the times of onset of inhibition of cell division and nucleic acid synthesis were only measured for cultures inhibited by oxytetracycline. Indeed it should be emphasized that these conclusions with regard to the mode of action of oxytetracycline are based on a single experiment, Fig. XXI.

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