STREPTOMYCIN AND CERTAIN ASPECTS OF PROTEIN SYNTHESIS IN *SERRATIA MARCESCENS*

A Dissertation Presented

to

the Faculty of the Department of Biology

University of Houston

in Partial Fulfillment of

the Requirements for the Degree

Doctor of Philosophy in Biological Sciences

by

Mohammed Abdul Quadeer Siddiqui

June 1967

ACKNOWLEDGMENTS

The author acknowledges with deep appreciation the guidance and counselling by Dr. Philip J. Snider as the thesis advisor. The author is also grateful to Dr. Allen H. Bartel, Dr. John E. Evans, and Dr. D. Weber for providing some equipments and laboratory facilities.

Special thanks are due to Mr. D. Nicholson for his work on electrophoresis, Mr. Fredrick Landa for the art work, Mrs. Dianna Pledger for her dedicated service as typist, and Dr. C. Keswani for his criticism. Above all the author appreciates the incalculable encouragement and help of his wife throughout the conduct of this research.

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ABSTRACT

The characteristics of streptomycin action on protein synthesis in Serratia marcescens are described for both in vivo and in vitro systems. For in vitro studies polynucleotide-directed protein synthesis is demonstrated and characterised. The basic requirements are the same as those described for E. coli. Streptomycin inhibits poly-U-directed phenylalanine and poly-C-directed proline incorporation into protein by cell-free extracts of a streptomycin-sensitive strain, while extracts from a resistant strain are relatively unaffected. The sensitivity to streptomycin resides on the 30s subunit of the ribosome. Upon electrophoretic separation of ribosomal proteins, a noticeable difference was observed between the banding patterns of ribosomal proteins from streptomycin-sensitive and resistant strains. Streptomycin, at 5 ug/ml in the growth medium, does not reduce total protein synthesis nor the growth of the sensitive cells. However, ribosomes isolated from cells grown in this sub-lethal concentration of streptomycin show partial reduction of amino acid incorporation in vitro, in the absence of added streptomycin. Evidence suggests that both subtle structural defects, induced during ribosomal formation in the presence of the antibiotic, and binding of streptomycin, probably one to several molecules per ribosome, together account for the defective ribosomal functioning during protein synthesis in vitro.

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Chapter One

INTRODUCTION AND LITERATURE REVIEW

From the vast number of studies on streptomycin a variety of effects appear to result from exposure of bacterial cells to streptomycin: damage to cell membrane causing permability changes (Dubin et al., 1963), inhibition of protein synthesis, breakdown of RNA (Hancock, 1964), impairment of respiratory mechanism (Cginsky, 1953). The comparative extent of these effects depend upon the relative concentration of the antibiotic and of the susceptibility of the bacterial species or strains. Streptomycin affects protein synthesis through an interaction with the ribosomes of sensitive cells. This is undoubtedly one of the principal actions of streptomycin, although it is not certain that this effect explains fully the bacteriocidal action of this antibiotic. Streptomycin action on protein synthesis has been elucidated mainly by experiments in cell-free bacterial extracts. The purpose of this thesis is to examine the effects of sub-lethal concentrations of streptomycin on intact cells and on cell-free extracts of streptomycin-sensitive and resistant strains of the bacterium Serratia marcescens. Such study could contribute toward understanding either specific action of streptomycin or the functioning of ribosomes. The problem required demonstrating and utilizing protein synthesis in vitro for this organism.

During the past few years the understanding of protein synthesis has gained clarity and percision through ingenious experimentation that has made it possible to study the nature of streptomycin action on this process. Some of the most significant previous contributions will be presented in this chapter. The following essential features of protein synthesis are widely accepted (Watson, 1964; only contribu-- tions more recent than Watson's review are specifically cited):

1) All the information for making protein is contained in a linear sequence of nucleotides in double-stranded DNA, one strand of which serves as the template for the synthesis of single-stranded messenger RNA (mRNA), an intermediate used to convey the genetic information from DNA to the site of protein synthesis.

2) mRNA attaches itself to ribosomes and directs the formation of proteins by determining the linear order in which amino acids are incorporated into the polypeptide chain.

3) Amino acids, prior to incorporation into protein, are attached to a specific molecule of RNA, tRNA, which selectively binds to a specific region of mRNA on ribosomes.

4) Structural analysis of ribosomes revealed that they are made up of two sub-units, 50s and 30s particles, and mRNA binds to the 30s sub-unit of ribosomes while the growing

chain of polypeptide is attached through a tRNA molecule to the 50s sub-unit.

5) Ribosomes provide the site of synthesis for all cellular proteins and are therefore genetically unspecific.

6) The polypeptide chains grow by stepwise addition of single amino acids, starting with the amino-terminal amino acid. The growing polypeptide chain remains attached to the terminal tRNA bound to its ribosomal site.

7) Initiating the translation of codons is thought to depend upon N-formylmethionyl-tRNA. AUG or GUG and UUG have been suggested as chain-initiating codons (Clark and Marcker, 1966) and UAG, an amber codon, and UAA, an ochre codon, are believed to be relevant to the chain-termination mechanism of protein synthesis (Stretton and Brenner, 1965; Brenner et al., 1965).

8) It is believed that the polypeptide chains begin to attain their three dimensional configuration before they are released from the ribosomes (Khira et al., 1961)

Messenger RNA:

The concept that the information transmitted from DNA to ribosomes through a class of metabolically unstable molecule, mRNA, was first derived by Jacob and Monod (1961). The indication of short-lived mRNA fractions were already available in experiments with phage-infected E. coli. This small fraction of RNA was found to undergo a rapid turn-over and was later

characterized by Astrachan and Volkin (1958) by pulse-labeling of RNA. Nomura, Hall and Speigelman (1960) observed that the pulse-labeled mRNA had a sedimentation value of approximately 8s and in high Mg^{++} concentration, above 5×10^{-3} M, the RNA fraction was associated with ribosomes. Conclusive evidence for mRNA and its role in protein synthesis came from the experiments of Brenner, Jacob and Meselson (1961). They showed that RNA synthesized after phage-infection of E. coli has a base composition corresponding to that of phage DNA and becomes attached to ribomes during protein synthesis. The experiments indicated that the association of mRNA with ribosomes is a functional one and not merely the result of an affinity between the RNA fraction and ribosomes.

Messenger RNA in bacteria is metabolically unstable with an average life varying from 1/20 to 1/10 the cell generation time (Watson, 1964). The mRNA-destroying enzymes are active in cell-free extracts under conditions where protein synthesis is usually studied. The activity of mRNA can be assayed by the in vitro system of protein synthesis as described by Matthaei and Nirenberg (1961).

Transfer RNA:

It has been known for a long time that tRNA plays a central role in the organization of amino acids into polypeptide chains during protein synthesis. Hoagland et al. (1958)

isolated this species of RNA from ascite tumor cells. It is known that tRNA is a small macromolecule with a sedimentation constant of about 4s, corresponding to a molecular weight of approximately 25,000, and has a chain length of about 70-80 nucleotides. A base sequence of -CCA occurs in the last three nucleotides at the end bound to the amino acid of all tRNA species, and guanosine phosphate is common to other end of all tRNAs. Individual tRNAs combine with an amino acid to produce an aminoacyl-tRNA complex. Formation of the complex utilizes one ATP molecule and activates the amino acid. Aminoacyl-tRNA carries the amino acid to the surface of the ribosome, where polypeptide bond formation occurs. The specificity of tRNA for its amino acid was demonstrated by resolving tRNAs into fractions and subsequently attaching a single tRNA fraction with a single species of amino acid (Weisblum et al., 1962; Sueoka and Yamane, 1962). However, it was shown recently that more than one tRNA fraction binds specifically to a single amino acid (Goldstein et al., 1964). These fractions of tRNAs require different codons of mRNA for the incorporation of the particular amino acid into protein. Soll (1967) showed that while there is a strict specificity of tRNA for the first two letters of codon, one tRNA can often recognize several codons differing only in the third letter.

The importance of understanding the role of tRNA was

realized when information on its structure was made available. The report of Holley and his colleagues on the complete primary sequence of an alanine-tRNA from yeast is one of the most significant advances in recent years (Holley et al., 1965). Several unusual bases were found throughout the molecule except in the region near the amino acid accepting end. The nature of the base sequence indicates several double stranded regions are present in tRNA. Similar studies on other tRNA have shown significant differences and similarities in the structures of tRNA species for different amino acids.

Ribosomes:

Ribosomes are of universal occurrence in microorganisms and in higher organisms. In electronmicrographs ribosomes appear as dense granules about 200 A units long and about 150 A or more in width. Usually ribosomes are identified by their characteristic sedimentation pattern in the analytical ultracentrifuge. Most bacterial ribosomes are the 70s type, which is considered as the metabolically active particle. The 50s and 30s are the result of dissociation of 70s particle at low Mg^{++} concentrations. Two 70s particles associate to give a 100s unit. By very gentle opening of the cell, aggregates of higher order, polysomes, or ergosomes, containing several 70s units attached to mRNA have been isolated.

Careful studies of ribosomal RNA (rRNA) from E. coli was made by Littauer (1961), Spirin (1960) and Kurland (1961). When extracts of ribosomes or ribosomal sub-units were examined in the ultracentrifuge, both 16s and 23s rRNA were found in the 70s ribosomes, but only 16s rRNA, and not 23s, was part of 30s sub-unit. These two RNAs have approximately the same base composition with high guanine content. This constance in size and composition was found in a large variety of organisms. In intact ribosomes the rRNA is combined with protein and held in stable configuration. E. coli ribosomes were found to contain about 60% RNA and 40% protein (Tissieres et al., 1959). Ribosomal RNA can be separated from ribosomal protein by protein denaturation (with much agents as sodium dodecyl sulfate, phenol, or guanidine) and precipitation with LiCl or $MgCl_2$. Urea also causes dissociation and the rate of dissociation depends upon salt concentration and temperature. Protein thus seperated from intact ribosomes of E. coli was found to be somewhat different in amino acid composition than most proteins (Spahr, 1962). The basic amino acids particularly lysine and arginine are in excess. The molecular weight of ribosomal protein is approximately 25,000. Ribosomal protein, however, is not a single molecular species. Waller (1963) has shown more than a dozen bands of ribosomal protein in starch-gel electrophoresis and Leboy et al. (1964) and several other workers

more recently have obtained about thirty different bands in polyacrylamide gel electrophoresis. It is not definite, however, that each of these components is a different polypeptide or that all are essential constituents of the functional ribosomes. Also it is not certain that all ribosomes from one clone of cells are identical. Moller and Widdowson (1967) characterized ribosomal protein in detail by column chromatography and gel electrophoresis. They fractionated the total ribosomal protein into four classes of chemically different protein and all but one class appeared to be composed of several different proteins.

One approach to study the function and structure of ribosome is to dissociate ribosomes into smaller fractions and re-associate them to make native particles. Both association and dissociation reactions depend upon the ionic environments and appear to involve the reversible binding of Mg^{++} to the phosphate group of ribosomal RNA. Magnesium seems to be an essential cation for the structural integrity and functioning of ribosomes. Complete removal of Mg^{++} causes degradation of ribosomes. In addition to Mg^{++} several other dibasic metallic ions were found to be associated with ribosomes. Polyamines which are normal cellular constituents are strongly bound to ribosomes in E. coli; these include diaminopropane, putrescine, cadaverine and spermidine (Cohen, Lichtenstein, 1960). Further dissociation of ribosomal sub-

units was reported first from Meselson's laboratory (Meselson et al., 1964). The 50s sub-unit yeilds a 40s "core" particle, while the 30s sub-unit yeilds a 23s particle. The proteins free of these "core" particles were called "split" proteins. Mixing split proteins with core particles results in the formation of native ribosomes, active in protein synthesis (Staehelin and Meselson, 1966). The 23s core proteins are physically and functionally different from the split proteins.

The role of ribosomes as centers of protein synthesis has been established in several different cell types. However, inspite of numerous studies performed to elucidate the mechanism of protein synthesis, we know very little about the precise function of ribosomes. It is believed that ribosomes are entirely nonspecific as to kind of protein synthesized. The non-specificity of ribosomes was well demonstrated (Brenner et al., 1961) by first incorporating heavy isotopes into ribosomes and then labeling the protein synthesized after phage-infection of E. coli cells. The results showed clearly that phage proteins were formed on old ribosomes, produced before phage-infection. Several in vitro studies have also provided evidence for non-specificity of ribosomes. For example, RNA from TMV, added to a system containing E. coli ribosomes, results in the synthesis of proteins similar to viral proteins (Tsugita et al., 1962), and f_2 phage RNA

stimulates phage-specific proteins formed in vitro in an E. coli system (Loeb and Zinder, 1961; Nathans et al., 1962). Recent studies on the mode of action of streptomycin suggest that the structure of ribosomes plays an important part in the translation of messenger during protein synthesis. A change in the ribosomal conformation may alter the normal meaning of the code (Davies et al., 1964; Gorini and Kataja, 1964).

Protein synthesis:

The flow of genetic information from the sequence of nucleotides results in the formation of a linear arrangement of amino acids in polypeptides. The polypeptide chain folds into secondary and tertiary structures for making biologically functional proteins. It is believed that the secondary and tertiary structure of a polypeptide is determined in a given environment, solely by the amino acid sequence.

Protein synthesis is a complicated process. The essential sub-cellular fractions for the ordered polymerization of amino acids in vitro are (a) the ribosomal fraction and (b) supernatant fraction. The amino acid incorporation is dependent upon ATP and ATP generating system, and it is stimulated by the synthetic messenger RNA, tRNA, mixture of L-amino acids and GTP. The major steps of protein synthesis, as they are known at present, are (a) formation of aminoacyl-tRNA, (b) specific binding of aminoacyl-tRNA to ribosomes in response

to mRNA, or polynucleotide template (Nirenberg and Leder, 1964), and (c) sequential addition of amino acids into the polypeptide chain subsequent to matching of aminoacyl-tRNA to template codons; this step requires the polymerization enzymes (Nathans and Lipmann, 1961). Although the codon recognition step can occur with 30s ribosomes alone, the polymerization reaction requires 50s sub-units also (Pestka and Nirenberg, 1966).

One of the problems in understanding the mechanism of protein synthesis is to know how exactly the three major components, mRNA, tRNA, and ribosomes interact to fulfill their specific functions, such as polypeptide chain initiation and termination, recognition of codons, movement of mRNA on ribosomes and role of enzymes and GTP in the process. Moldave (1965) summarized the general properties of the binding of aminoacyl-tRNA to mRNA-ribosome complex as a non-enzymatic reaction which requires an optimum concentration of Mg^{++} ions and also a monovalent cation, preferable an ammomium ion. Both charged and uncharged tRNAs are attached to ribosomes with non-covalent bonds, but the tRNA bound is specific for the codons of the mRNA used. Arlinghaus et al. (1964) and Warner and Rich (1964) demonstrated that ribosomes contained at least two binding sites for tRNA, one for peptidyl-tRNA and the other for amino acyl-tRNA. Recently, however, it was suggested that ribosomes may contain more than two sites

for binding tRNAs (Wettstein and Noll, 1965). It was further shown that tRNA binds primarily to the 30s ribosomal sub-unit in the presence of poly-U; only a slight binding to the 50s portion was observed, which may be due, in part, to the presence of 30s sub-units in the 50s peak (Pestka and Nirenberg, 1966).

There have been a number of studies on the interaction of ribosomes with polynucleotides, in order to understand the role of this complex in protein synthesis. In general, a large variety of polynucleotides can react with ribosomes. The presence of secondary structure, however, prevents such interaction. The discovery of Nirenberg and Matthaei (1961) that synthetic polyribonucleotides can function as messenger in a cell-free system has provided researchers with a new approach to the study of protein synthesis. There was evidence that strongly suggests that the genetic code was a triplet code (Crick et al., 1961). The triplet binding method of Nirenberg and Leder (1964) helped tremendously in allocating codons to specific amino acids, and by 1965 Nirenberg had tested nearly 44 nucleotides for their specific assignments (Nirenberg et al., 1965). With the significant advances in recent years (Jones et al., 1966) the genetic code is now entirely known. The best allocation of the 64 possible codons, based on evidence mainly from E. coli, indicates that most codons correspond to amino acids; however, some codons

serve in other capacities, such as initiation, termination and regulation of protein synthesis.

The individual polypeptides are built up sequentially beginning with N-terminal amino acids (Goldstein et al. 1964). Using synthetic oligonucleotides as templates and purified E. coli ribosomes, Smith et al. (1966) provided conclusive evidence that the direction of code translation is from the 5' to 3' end of the template RNA. The most recent studies on polypeptide initiation implicate a paricular amino acid, N-formylmethionine, as the N-terminal amino acid in all proteins of E. coli. Adams and Capecchi (1966) synthesized N-formylmethionine-RNA and used this in a cell-free system with R17 phage RNA as messenger. The protein coat was shown to contain N-formylmethionine and N-formylalanine. They conclude that coat protein is made with an N-terminal fragment starting with meth-ala-ser- ---. In vivo the N-terminal amino acid is probably split off by an enzyme. AUG (Thach et al., 1966) and GUG or UUG (Clark and Marcher, 1966) have been cited as chain initiation codons. A significant aspect of the chain initiating mechanism is using the correct concentration of divalent cation particularly Mg^{++}. AUG initiator functions best at low concentration of Mg^{++} and the activity of UUG requires rather high Mg^{++} concentration (Thach et al., 1966). It seems that if a particular mRNA does not include an initiator triplet, it can still start by making a mistake

and this is more likely to happen at higher Mg^{++} levels; thus, the optimum value for Mg^{++} concentration for a protein synthesizing system may be artifically high. The problem of polypeptide chain termination is closely related to that of initiation. One mechanism for termination suggest a chain-terminating codon and a corresponding RNA, which cannot form its aminoacyl-tRNA. There is considerable evidence for this kind of chain-terminating mechanism from studies with amber mutants. Stretton and Brenner (1965) suggested that the chain is terminated at the site of a amber mutation in E. coli, which, when suppressed, substitute, another amino acid for the wild-type amino acid. It was proposed that the "amber" mutation is from a sense codon to one for nonsense, a chain-terminating triplet. Several evidences indicate that the chain terminating triplet for amber mutation is UAG. Another amber codon was recently recognized as UGA (Brenner et al., 1967). Another class of chain-terminating mutation called "Ochre", with a triplet code of UAA, as reported by Brenner et al. (1965). It was suggested that both amber and ochre triplets may be used for natural chain termination.

Streptomycin and protein synthesis:

(A) Streptomycin as a cation: The structure of streptomycin shown in the conventional two dimensional configuration obscure, the fact that separate portions of the molecule can interact in interesting ways. The differences in pro-

perties between streptomycin and dihydrostreptomycin appears to be related to differences in their structures. Streptomycin and dihdrostreptomycin showed striking differences in precipitating nucleic acids, ribosomes, casein, hyalouranic acid, sodium hexametaphoshate etc., but not for lipids. Brock (1964) concluded from these differences that both streptomycim and dihydrostreptomycin act as cationic substances, since diguanidobutane, which has only the diguanidino group of the antibiotics, can also precipitate the lipids. The precipitation of polyanions by streptomycin was explained by the ability of the antibiotic to cross link between the adjacent polymers to make an insoluble lattice. That excess of polyanion can dissolve the precipitate supports this idea. Dihydrostreptomycin cannot precipitate the polyanions with the same efficiency as streptomycin and therefore one could look at streptomycin as a bivalent cation and dihydrostreptomycin as an univalent cation (Brock, 1964). The cationic guanidino groups of streptomycin could interact electrostastically with anionic groups. The secondary amine of the N-methylglucosamine moeity could serve as an additional cationic site and Brock further suggested that some interaction of the aldehydic group of streptose with the secondary amine must enhance the anion-attracting ability.

(B) Streptomycin and its action in vivo: The antibacterial action of streptomycin could be related to a variety of

resulting from the exposure of the living cells to the antibiotic. Early studies established that streptomycin in very high concentrations precipitates nucleic acids (Cohen, 1946) and inhibits the oxidation of several substrates (Oginsky, 1953). In addition, streptomycin, in different concentrations, is known to cause varying effects such as permeability changes, inhibition of protein synthesis, breakdown of RNA. Dubin et al., (1963) have made a careful study of changes that occur in bacteria growing in the presence of streptomycin and established the following sequence of changes:

1) There is an initial and almost instantaneous binding of streptomycin to the cell surface, which probable is an electrostatic interaction. The binding occurs almost equally on sensitive and resistant cells of E. coli, and on growing and non-growing cells. Futher, it is temperature independent and is inhibited by cations, orthophosphate and some organic acids (Brock, 1964).

2) Following the initial binding there is an increased rate of efflux of K^+, indicating an early effect on permeability. It was shown that this effect cannot be due to simple displacement of intracellular potassium, nor is it the result of a gross change in the cell surface-to-volume ratio; there is relative constancy of the ratio of the turbidity-to-cell number. This effect does not occur in streptomycin-resistant cells (Newton, 1965).

3) Coincident with the loss of K^+, there is a transient stimulation of net RNA synthesis, followed closely by an inhibition of protein synthesis. The stimulation of RNA is rather novel and little understood. The stimulation may be the result of the accumulation of RNA due to the decrease rate of breakdown shich ensues as the concentration of RNA is decreased. Dubin (1964) observed that a portion of the RNA stimulated by streptomycin has abnormally slow sedimenting properties, similar to those of the chloramphenicol particles reported by Normura and Watson, (1959). It was suggested that such stimulation of RNA synthesis may be the result of the inhibition of protein synthesis too slight to be detected. Inhibition of protein synthesis may be the direct effect of streptomycin on the protein synthesizing system or an indirect result associated with the effect of streptomycin on the membrane.

4) Later, there are marked changes in the permeability, leading to appearance of nucleotides in the growth medium. The release of nucleotides appears to be due to the breakdown of cellular RNA (Dubin and Davis, 1962). Such an effect follows an extensive uptake of streptomycin by the cells and also caused impairment of respiration, inhibition of RNA and DNA synthesis and breakdown of RNA and ribosomes.

Considering all the available evidence, it seems reasonable to conclude that streptomycin at certain concentration does two things to bacteria in vivo: (a) inhibition of

protein synthesis and (b) alteration of cell permeability. The question remains as to whether these two effects are independent or dependent upon one another. A change in permeability could cause inhibition of protein synthesis, and inhibition of protein synthesis could result in the impairment of the cell membrane. Although a unitary hypothesis based on a common substrate for streptomycin would be appealing, there is no evidence for this. In view of the wide variety of substances with which streptomycin can react, it is likely that both of these effects involve unlike targets of molecules for reaction with streptomycin.

Spotts and Stanier (1961) have drawn attention to the results of genetic analysis by transduction, indicating that streptomycin sensitivity resistance and dependence are due to alternate states of ribosomes. Recent investigations have provided considerable support for this view. Gorini and Kataja (1964) have shown that streptomycin causes phenotypic suppression of a variety of genes. This was explained on the basis of misreading the code in cell-free protein synthesis. Cox et al. (1964) have suggested that mutation at the classical streptomycin locus corresponds to structural changes in the 30s ribosomal sub-unit. The site of action of streptomycin in performing suppression is probably the ribosome (Gorini and Beckwith, 1966).

(C) Streptomycin and protein synthesis in vitro: Strepto-

mycin inhibits protein synthesis in vitro (Flaks et al., 1962a; Speyer et al., 1962). The formation of aminoacyl-tRNA is not affected by streptomycin at concentrations higher than those required for inhibition of protein synthesis in vivo or in vitro (Eaton and Caffery, 1961). Using all possible combinations of supernatant enzyme fractions and ribosomes from streptomycin-sensitive and resistant strains, several workers have independently localized the streptomycin semsitive site on ribosomes (Speyer et al., 1962; Flaks et al., 1962b. Davies (1964) and Cox et al., (1964) made hybrid ribosomes with combinations of 30s and 50s sub-units from streptomycin-sensitive and resistant strains and showed that the streptomycin semsitive site resided on the 30s sub-unit of the ribosome. Staehelin and Meselson (1966) and Traub et al. (1966) have further narrowed down the site of the streptomycin action by dissociating the 30s ribosomes into smaller fractions and reassociating them into native particles. They have shown that streptomycin sensitivity is determined by the 23s "core" ribonucleoprotein portion of 30s ribosomal particle, and not by the remaining "split" protein portion. Leon and Brock (1967) have shown recently streptomycin-C^{14} becomes attached to ribosomal protein and not to rRNA.

Streptomycin did not interfere with the binding of poly-U to ribosome (Davies, 1964); neither did it affect the C^{14}-phenyl-

alanine incorporation into protein in vitro (Cox et al., 1964). However, using cell-free extracts of streptomycin sensitive strains of E. coli, Davis et al. (1964) showed that low concentrations of streptomycin reduced poly-U-directed phenylalanine incorporated into protein and stimulated at the same time incorporation of isoleucine, leucine, and serine. This effect was not observed in a system containing ribosomes from streptomycin-resistant strains. Presence of streptomycin therefore stimulated mis-reading of the code in the cell-free system, and several groups (Schwartz, 1965; Old and Gorini, 1965) confirmed this finding by showing the miscoded amino acids appear to substitute for the proper amino acid in the polypeptide chain. An exhaustive study (Davies et al., 1966) on the effects of the aminoglycosidic antibiotics on polypeptide synthesis stimulated by polynucleotides of alternating nucleotide sequence resulted in the conclusion that coding changes produced by streptomycin are highly specific. For example, when poly-UC was used as template, which codes for serine (UCU) and leucine (CUC) streptomycin caused incorporation of phenylalanine (UUU, UUC) and proline (CCC, CCU, CCA, CCG). They concluded that streptomycin stimulated the misreading of the 5'-terminal or the internal position of the triplet codon, (only one base at a time) and of pyrimidine more frequently than purines. Misreading stimulated by streptomycin when homopolymers where used as templates

also give a similar result. In general, streptomycin causes misreading of U as C or A, and C as U or A, in both the 5'-terminal and internal positions of codon (Davies, 1966). Pestka (1965) and Kaji and Kaji (1965) have shown that, in addition to misrecognition of coding properties, the binding of aminoacyl-tRNA to ribosomes also was alternated by streptomycin. Since it has been established that streptomycin acts on the 30s sub-unit, the antibiotic could interfere with the process of codon recognition by reacting with the aminoacyl-tRNA site on the 30s sub-unit and therefore could interfere with the aminoacyl-tRNA-codon interaction.

Though there is sufficient evidence that streptomycin affects protein synthesis by causing substitution errors during polypeptide synthesis in vitro, there is very little evidence of misreading in whole cells, as in no case has an actual amino acid change been determined by whole cells. However, several bacterial mutants are known which are competent for streptomycin-activated suppression. At least in one of these mutants it was possible to demonstrate that streptomycin did not act directly upon the defective enzyme molecule (ornithine transcarbonylase) but interfered with its synthesis (Gorini, 1966). Further, it was shown that ribosomes isolated from both competent and noncompetent mutants differ in their response to the misreading action of streptomycin (Anderson et al., 1965). Bissell, (1965) has also demonstrated the formation of defective

B-galactosidase when the E. coli cells were grown in the presence of streptomycin. To account for these results it was proposed that streptomycin may alter the enzyme activity by interfering with the standard meaning of the genetic code at the translation level. The correlation of in vivo and in vitro results remains to be confirmed by direct evidence.

Chapter Two

RIBOSOMES AND PROTEIN SYNTHESIS

Polynucleotide-directed protein synthesis is demonstrated and characterized for S. marcescens. The basic requirements are the same as those described for E. coli (Nirenberg and Matthaei, 1961). The system provides a basis for studies on the nature of streptomycin action in the protein synthesizing system of S. marcescens.

I. Ribosome isolation and characterization:

Materials and Methods: S. marcescens wild-type strain Nima was grown on Bunting's medium as modified by Williams et al. (1956). This medium, referred to as complete medium (CM) from hereon, has the following composition:

Yeast extract 1.0 g, casein hydrolysate 2.0 g, glycerol 10.0 g, ammonium citrate dibasic 5.0 g, dipotassium phosphate 10.0 g, magnesium sulfate ($MgSO_4.7H_2O$) 0.5 g, sodium chloride 5.0 g, ferric ammonium citrate 0.05 g, and distilled water 1 liter.

The medium was adjusted to pH 7.1 ($\pm$0.1) with 1N NaOH and autoclaved for 15 min at 121 C. E. coli, when used, was grown in Pen-assay (Difco) broth medium.

For ribosome isolation, inoculum was produced from a loopful of stock culture inoculated into 50 ml of CM and grown overnight at 37 C on a rotary shaker. Two ml of the

above culture was added into 100 ml of CM and grown under similar conditions to build a 6 hr inoculum which was then added into 2 liters of CM contained in a 4 liter flask. Cells were grown at 37 C with constant aeration. After 3 hr growth, cells (approx. 4×10^8/ml) were harvested in the cold by centrifugation and washed once with 0.85% saline and twice with Tris-Mg buffer (Tris-HCl 0.01M, pH 7.8; magnesium acetate 0.01M). Cells were kept frozen until used. All subsequent procedures for isolation of ribosomes were conducted at 4 C.

Ribosomes were isolated according to the procedure described by Nirenberg and Matthaei (1961) with a few modifications (Cox et al., 1964). A weighed amount of cells were ground for 5-10 min in a mortar and pestle with alumina, approximately 2.5g/g of wet cell weight (Tissierres et al., 1959). To the paste from about 2 g of cells, 10 ml of TM buffer (0.01M Trus-Hcl, pH 7.8: 0.01M magnesium acetate: 0.06M KCl; 0.006M mercaptoethanol) and 5 ug/ml of DNAse were added. The mixture was centrifuged at 10,000 g for 20 min to remove the alumina, unbroken cells and cell debris. The supernatant was decanted and centrifuged at 20,000 g for 20 min respectively. The supernatant (S-20) was removed and centrifuged again at 105,000 g for 2 hr to separate the ribosomes. The supernatant fraction (S-100) was slowly pipetted out, discarding the lower 0.5 ml, and used for the amino acid incorporation

experiments. The ribosomal pellet was washed twice by re-suspending in TM buffer and centrifuging at 105,000 g for 2 hr each time. Ribosomes were finally suspended in 1.0 ml of TM buffer and dialyzed against 100 volumes of the same buffer with a desired concentration of Mg^{++} at 5C for 15 hr. Protein was separated from purified ribosomes by the method of Leboy et al., (1964) with the following modifications. To 0.5 ml of ribosomal suspensions in TM buffer, 0.5 ml of 4M LiCl containing 8M urea was added and the suspension was left overnight at 4 C. The precipitated RNA was removed by centrifugation at 20,000 g for 30 min. The supernatant protein was removed and dialyzed overnight against 4M urea and used for further analysis.

Electrophoresis of ribosomal protein on polyacrylamide gel was performed according to methods described by Reisfeld et al. (1962) with the modification by Hosokawa (personal communication). The details of proceduce and the composition of the ingredients are given in Annexure I. About 0.2 ml of sample containing 100-150 ug of ribosomal protein was applied to the gel to band the basic proteins at pH 4.5. The run was carried for 2.5-3 hr with a current of 3 ma/tube at 4 C. The staining was done with 1% Amido Schwarz in 7% acetic acid for 6 hr. The gels were destained with 7% acetic acid for 1-1.5 hr.

Protein was estimated by Lowry's method (1951), with serum bovine albumin as the standard, and RNA was determined

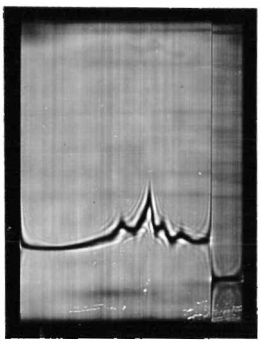
by the orcinol method as described by Dische (1955). RNA concentration was calculated by comparing the absorbances of test samples with a standard. A solution of 1 mg/ml in H_2O, has an absorbance at 260 mu of 24 (Von Ehrenstein and Lipmann, 1961). All O.D. measurements were made on Beckman, model DU and model DK-2, spectrophotometers.

Ribosomes were dissociated into 50s and 30s sub-units by dialyzing overnight the ribosomal extract against TM buffer containing 10^{-4}M of magnesium acetate. The ribosomal sub-units were separated by sucrose density gradient centrifugation. About 1.0 ml of a known concentration of purified ribosomes in 10^{-2}M or in 10^{-4} TM buffer was layered over 28 ml of 5-20% sucrose gradient. The centrifugation was done in a SW25 rotor for 5 hr for the separation of 70s ribosomes and for 10.5 hr for 50s and 30s ribosomal sub-units (Britten and Roberts, 1960). Fractions of 15 drops each, unless indicated otherwise, were collected separately from each tube by piercing through the bottom of the tube. For reading the O.D. at 260 mu, 0.1 ml from each tube was diluted to a proper concentration. Fractions containing ribosomes were pooled together and dialyzed against TM buffer with proper Mg^{++} concentration.

Studies on sedimentation velocities were performed on a Spinco model E ultra-centrifuge. Varying concentrations of crude extracts and purified ribosomal samples suspended in TM buffer with proper Mg^{++} concentrations were used for ultra-

Figure 1a. Schlieren diagram of crude extracts of ribosome from S. marcescens strain Nima

Figure 1b. Schlieren diagram of partially purified ribosomes from S. marcescens strain Nima



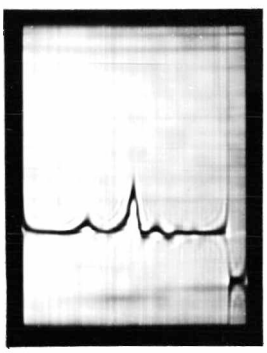
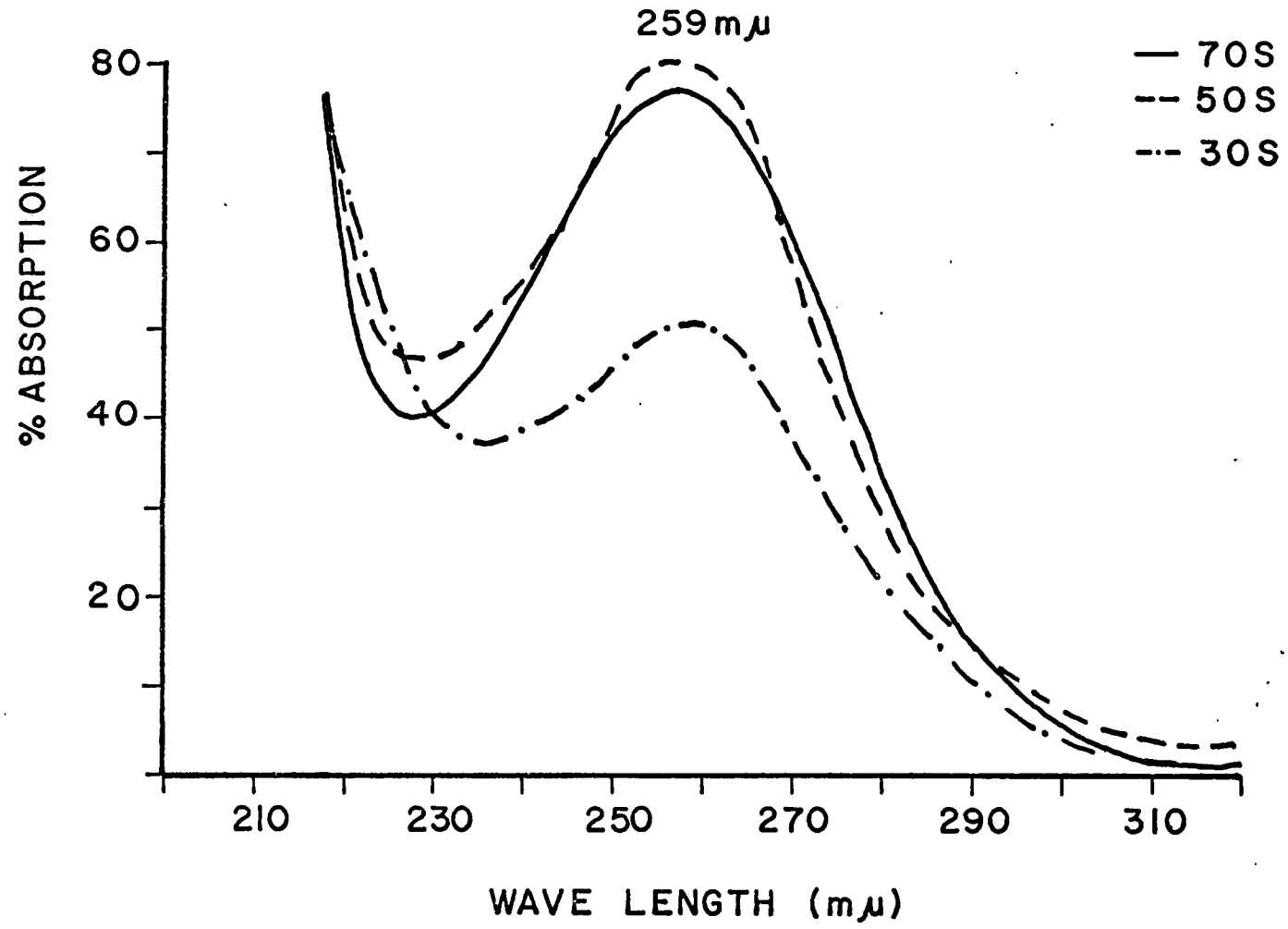


Figure 2. Absorption spectra of 70s, 50s, and 30s ribosomal units from S. marcescens strain Nima


259mμ
70S
50S
30S
% ABSORPTION
80
60
40
20
210
230
250
270
290
310
WAVE LENGTH (mμ)

centrifugation. Sedimentation boundaries were followed using a schlieren optics system.

Results: A schlieren diagram of crude extract from S. marcescens strain Nima was obtained from a run in the model E analytical ultra-centrifuge. The run was made at 20 C with a speed of 42,040 rpm and the picture was taken 12 min after the maximum speed was reached. Following the direction of sedimentation from right to left, the most prominent peak corresponds to 71s and heavier fractions have 97s and 133s values (Fig. 1a). The small peak following the 71s peak represents 54s particles and almost no trace of 30s fraction was observed. Ribosomes were partially purified by washing twice and dialyzing overnight against TM buffer. The schlieren diagram now showed peaks indicating a major component corresponding to 71.8s with slight peaks indicating particle of 50s and 30s sub-units (Fig. 1b). All sedimentation values were corrected for viscosity.

The absorption spectrum of purified ribosomes was taken from suspensions of ribosomes in TM buffer with 10^{-2}M Mg^{++}. The maximum absorption was at 259 mu. The absorption curves for 70s, 50s, and 30s fractions were identical (Fig. 2). The chemical composition of crude extract and purified samples of ribosomes show a slight excess of both RNA and protein in crude ribosomal sample (Table 1).

Table 1

CHEMICAL COMPOSITION AND ABSORPTION RATIO OF RIBOSOMES

Material	Protein mg/ml	RNA mg/ml	260/235
Crude extract Nima	7.5	8.9	1.88
Purified ribosome Nima	6.85	8.2	1.71
Crude extract D1	6.32	7.89	1.75
Purified ribosome D1	5.9	7.8	1.79

Figure 3. A sucrose gradient of non-dissociated ribosomes from S. marcescens. The 28 ml 5 to 20% sucrose gradient contained TM buffer with 10^{-2}M magnesium acetate. One ml of ribosomal suspension in TM buffer containing 80 ug of protein was applied to the grdient and centrifuged for 5 hr at 23,000 rpm. Fractions of 10 drops were collected and absortancy was measured at 260 mu.

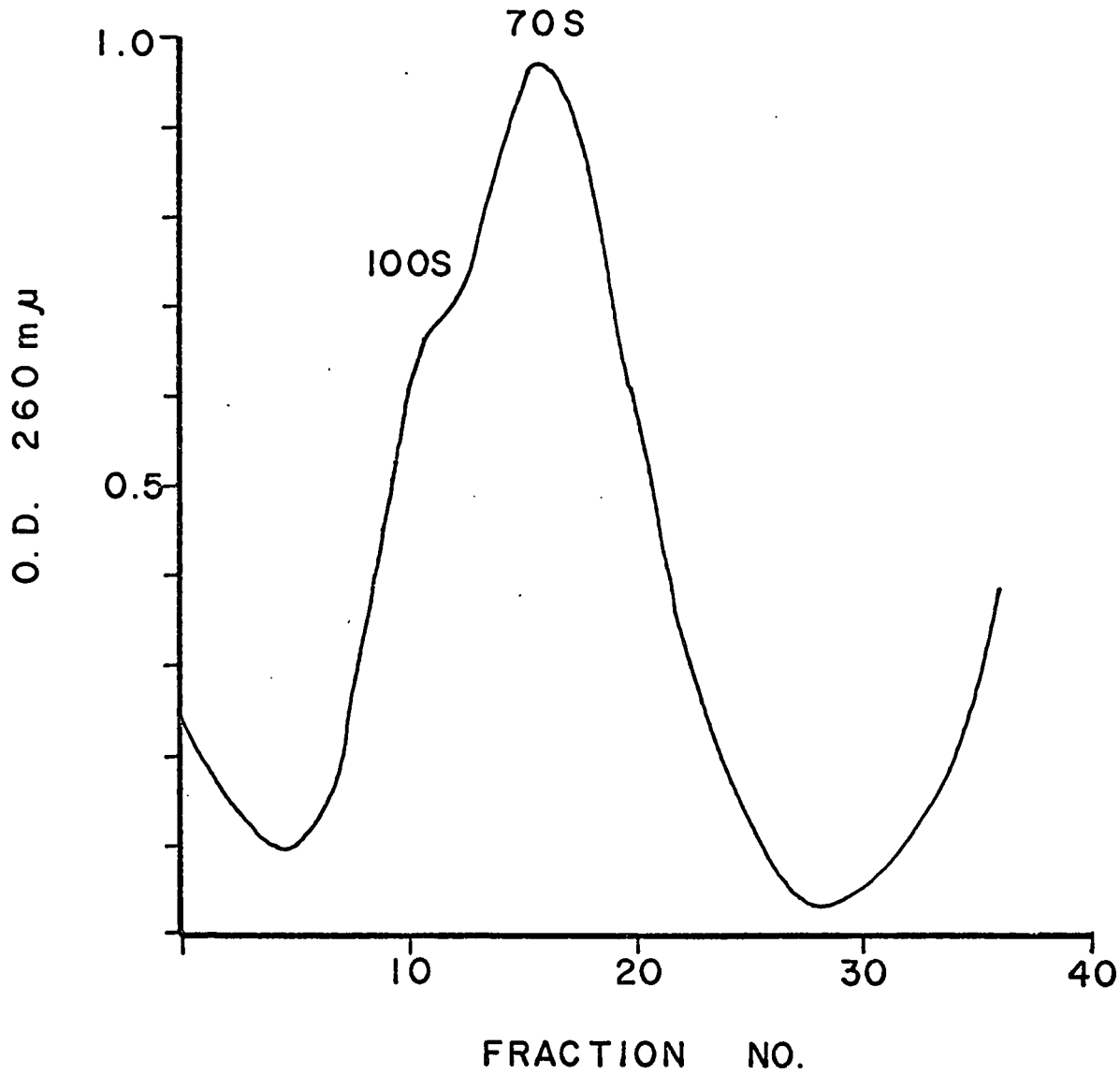

70S
100S
1.0
0.5
O.D. 260 mμ
10
20
30
40
FRACTION NO.

Figure 4. A sucrose gradient of dissociated ribosomes from S. marcescens strain Nima. The 28 ml of 5 to 20% sucrose gradient contained TM buffer with 10^{-4}M magnesium acetate. One ml of dissociated ribosomes layered on the gradient were centrifuged for 10 hr at 23,000 rpm. Fractions of 10 drops were collected and O.D. measured at 260 mu.

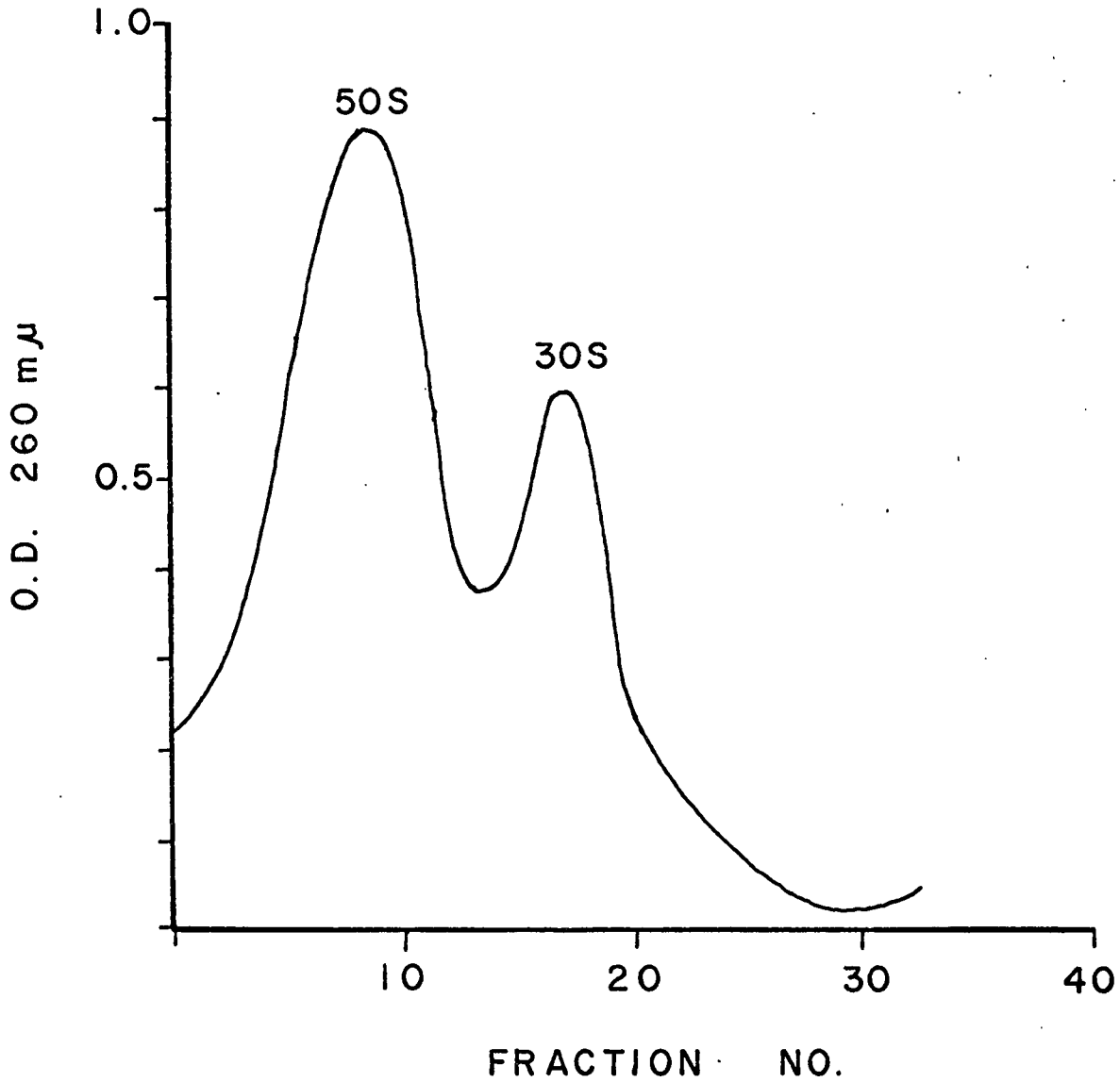

1.0
0.5
O.D. 260 mμ
50S
30S
10
20
30
40
FRACTION NO.

Figure 5. A sucrose gradient of reassociated 70s ribosomes from S. marcescens strain Nima. Centrifugation was carried out as in Figure 3.

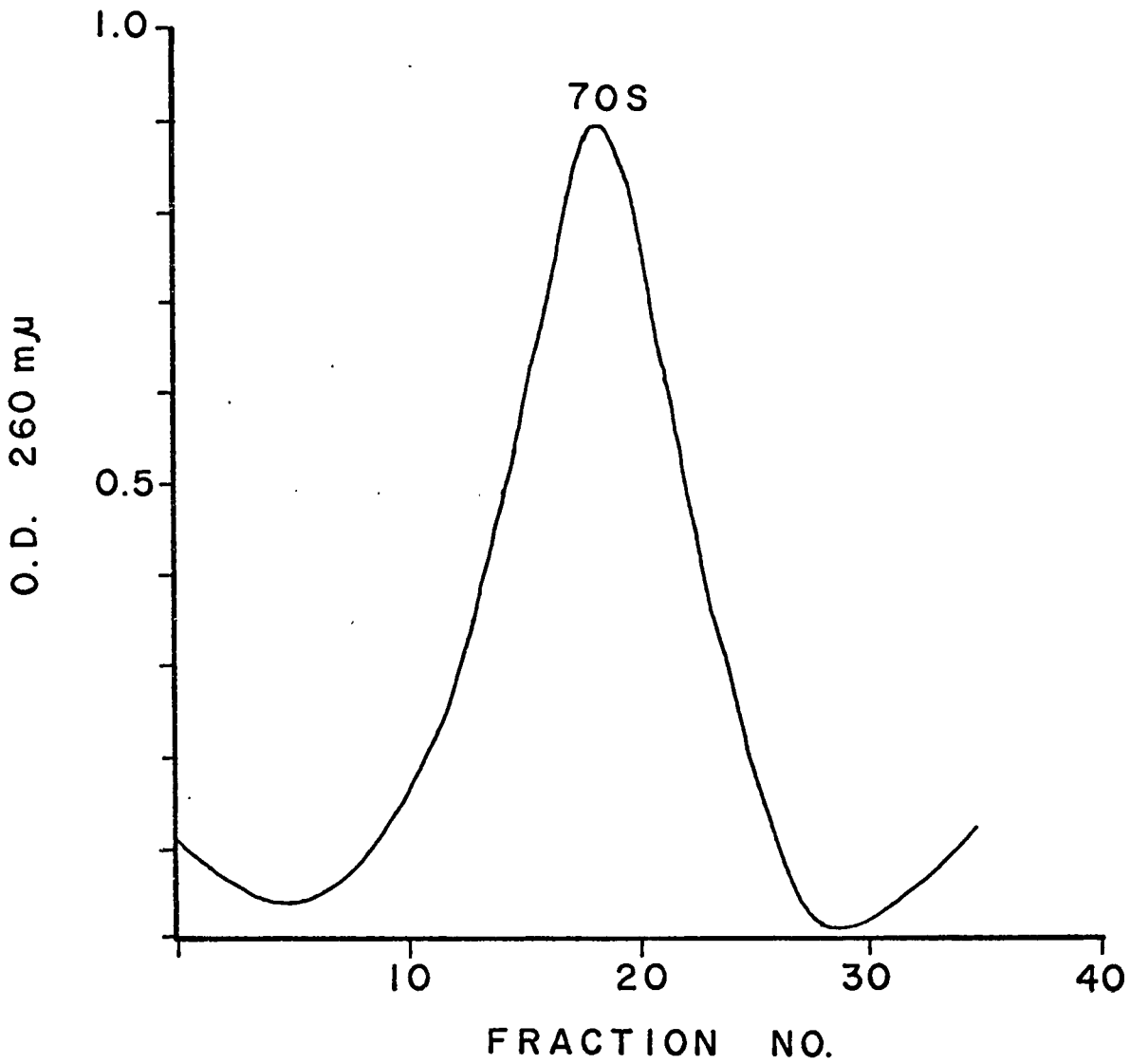
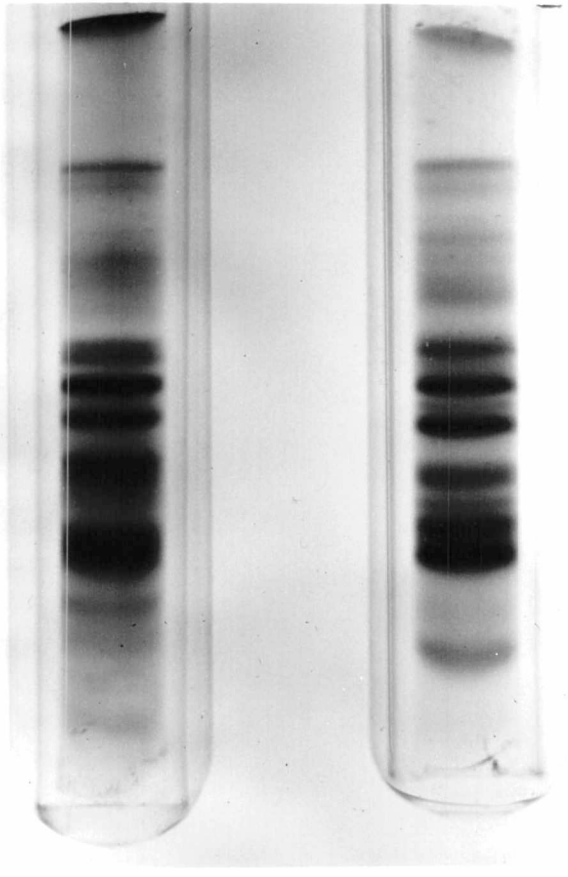

1.0
0.5
O.D. 260 mμ
70S
10
20
30
40
FRACTION NO.

Figure 6. Electrophoretic patterns of the basic ribosomal proteins from S. marcescens strain Nima and E. coli B. Runs were made on columns of polyacrylamide gel at pH 4.5.



E COLI NIMA

The sucrose density gradient profile of 1 ml of partially purified ribosomal sample containing about 80 ug of protein shows the major peak of 70s ribosomes and a small shoulder representing 100s fraction (Fig. 3). The ribosomes were dissociated by dialysis against TM buffer with lower Mg^{++} concentration and then applied to a density gradient for centrifugation. The gradient profile shows separate peaks but over lapping areas for the 50s and 30s ribosomal sub-units (Fig. 4): thus some tubes contained a mixture of both particles. For purification of the sub-units, these mixed fractions were discarded. The gradient pattern of the reassociated ribosomes indicated reassociation was almost complete (Fig. 5).

Ribosomal protein, freed of RNA, was banded by polyacrylamide gel electrophoresis. For comparison, ribosomal protein from E. coli was used as control. The comparison of basic proteins from S. marcescens and E. coli shows no noticeable difference in the pattern of banding (Fig. 6).

II. Protein synthesis, in vitro:

Materials and Methods: The isolation of ribosomes and the supernatant fraction (S-100) was done by the method described earlier in this chapter. The complete reaction

mixture for incorporation of amino acid was similar to that of Nirenberg and Matthaei (1961) with a few modifications. Unless otherwise specified the reaction mixture, 0.5 ml, consisted of Trus buffer 10^{-1}M (pH 7.8), magnesium acetate 10^{-2}M, KCl 6×10^{-2}M, mercaptoethanol 6×10^{-3}M, ATP 10^{-3}M, GTP 10^{-4}M, phosphoenolpyruvate 5×10^{-3}M, PEP-kinase 18.8 ug, sRNA 250 ug, C^{12} amino acids (total 18) 5×10^{-4}, C^{14}-proline or phenylalanine 5×10^{-4} (specific activity 165 mc/m mole), ribosomal protein 400 ug, supernatant protein 200 ug and poly-C or poly-U 50 ug. The incubation period was 40 min at 36 C, unless indicated otherwise. The ribosomes, supernatant were pre-incubated for 20 min along with ATP, GTP, and PEP (for removing the endogenous messenger activity). The reaction at the end of 40 min was stopped by adding 2.0 ml of 10% trichloroacetic acid. The precipitate was then heated for 30 min at 90 C and chilled at least for 30 min before it was filtered on a millipore filter paper. The precipitate was then washed with 15 ml of 5% trichloroacetic acid containing 1% caesamino acid. The activity for the C^{14} amino acid incorporation into the precipitated protein was counted for 10 min in a Packard scintillation spectrometer. Chemicals: C^{14}-proline and phenylalanine-C^{14} were purchased from New England Nuclear Corp.; some C^{14}-proline was also a gift from Dr. Weber of our department. Poly-C and poly-U were purchased from Miles Chemical Laboratory. Poly-U was also a gift from

Figure 7. In vitro protein synthesis. Poly-U-directed phenylalanine and poly-C-directed proline incorporation by cell-free extracts of S. marcescens strain Nima.

^{14}C PROLINE INCORPORATION/0.5ml
cpm X 10^2
30
20
10
PROLINE
Ø-ALANINE
12
8
4
^{14}C Ø-ALANINE INCORPORATION
cpm x 10^3
5
10
15
20
TIME (min)

Figure 8. Effect of temperature on poly-C-directed proline incorporation. Incubation conditions were same as in Figure 7.

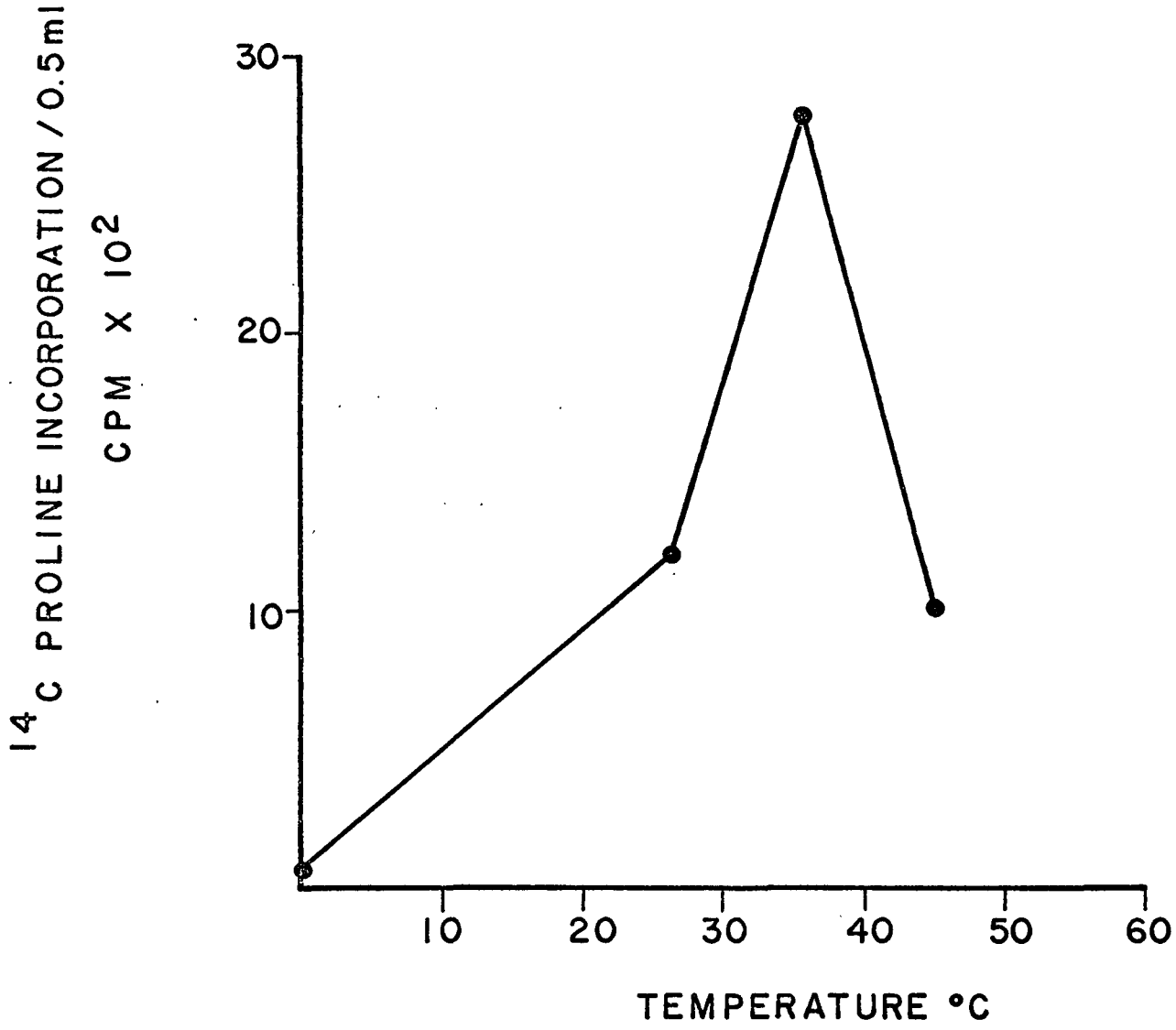
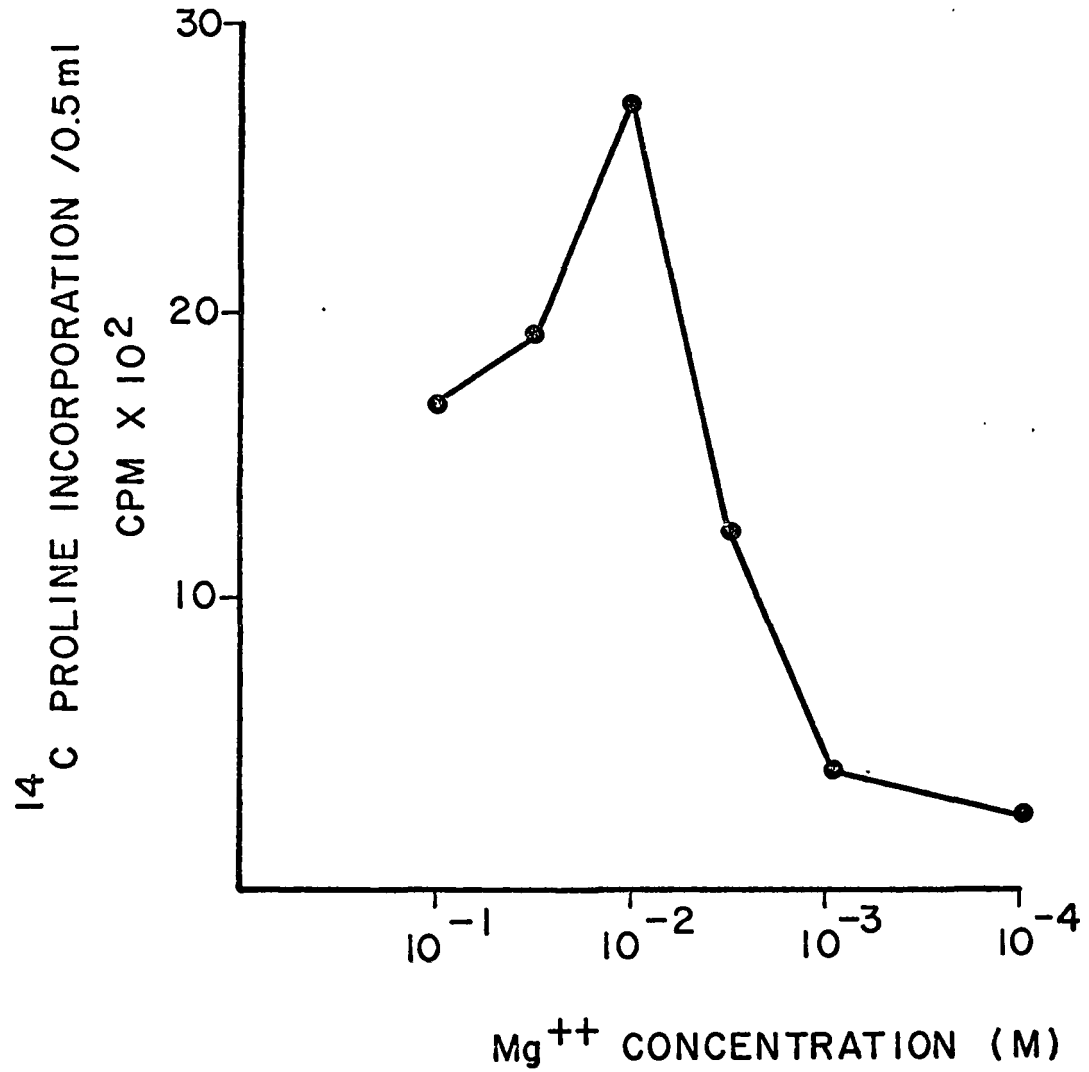

^{14}C PROLINE INCORPORATION / 0.5 ml
CPM X 10^2
30
20
10
10
20
30
40
50
60
TEMPERATURE °C

Figure 9. Effect of magnesium concentration on poly-C-directed proline incorporation. Incubation conditions same as in Figure 7.


30
20
10
^{14}C PROLINE INCORPORATION /0.5ml
CPM X 10^2
10^{-1}
10^{-2}
10^{-3}
10^{-4}
Mg^{++} CONCENTRATION (M)

Dr. Apirion of Washington University, St. Louis. PEP-kinase was purchased from Sigma Chemical Co., ATP, GTP, PEP, DNAse from Mann Research Laboratory.

Results: The time course for poly-C-directed C^{14}-proline and poly-U-directed phenylalanine incorporation were determined. Under the experimental conditions proline incorporation continued to increase sharply for about 5 min and then slowed down considerably to reach a plateau in about 10 min (Fig. 7). Phenylalanine incorporation followed a similar pattern with a somewhat prolonged linear increase in incorporation: the plateau was reached in about 15 min. Further, the maximum incorporation of phenylalanine was about 3-4 times more than that of proline. The optimal temperature for both proline and phenylalanine incorporation was 36 C. At 0 C the incorporation was almost negligible, and at 27 C and 45 C it was reduced to less than half the optimal amount (Fig. 8). The final concentration of the reaction mixture was brought to the required Mg^{++} concentration by adding the stock TM buffer containing high Mg^{++} concentration to bring the final volume to 0.5 ml. The optimum concentration for incorporation was 10^{-2}M (Fig. 9); there was a sharp reduction in the incorporation activity as Mg^{++} concentration was changed upward or downward.

As was shown for E. coli (Nirenberg and Matthaei, 1961) and Bacillus cereus (Kobayashi and Halvorson, 1966), the amino

Table 2

REQUIREMENTS FOR POLY-C-DIRECTED POLYPROLINE SYNTHESIS

Assay system	C^{14}-proline incorporation	
	cpm/tube	% of control
Complete system:	2,790	-
-Poly-C	920	33
-ribosomes	137	5
-supernatant	28	1.2
-ATP, GTP	485	17
-sRNA	381	13
$-C^{12}$-amino acids	1,360	50
+ribosomes (300 ug)	4,123	150
+sRNA (200 ug)	3,348	120

Conditions for incubation of the reaction were same as described in Materials and methods; all counts were corrected for 0 time incorporation.

Table 3

AMINO ACID INCORPORATION BY NATIVE AND REASSOCIATED RIBOSOMES

Assay system	Native CPM	Reassociated CPM
C^{14}-proline (Poly-C)	5,224	4,780
Phenylalanine-C^{14} (Poly-U)	14,880	12,570

Equal amounts of both native and reassociated were used; conditions of experiment were same as described in Materials and Methods.

Table 4

EFFECT OF STORAGE UPON AMINO ACID INCORPORATION BY RIBOSOMES AND SUPERNATANT

Fresh components		Stored components		C^{14}-proline incorporation
Ribosomes	Supernatant	Ribosome	Supernatant	CPM/TUBE
+	+	-	-	3,890
-	-	+	+	803
+	-	-	+	1,017
-	+	+	-	2,870

Reaction mixture same as described in Materials and Methods. Reaction mixture was preincubated and all counts were corrected for 0 reading. Fresh components ribosomes and supernatant were fresh after extraction; stored components = components were frozen at -10 C for one week.

acid incorporation was dependent upon ribosomes, supernantant and ATP. Poly-C stimulated proline incorporation by about 70% in the absence of preincubation and by about 90% when the components of the reaction mixture were preincubated for 20 min without the synthetic messenger. Ommission of sRNA and the C^{12}-amino acids from the mixture caused 85% and 50% reduction respectively. When ribosome and sRNA were added in excess, there was a significatn increase in the incorporation activity (Table 2).

Amino acid incorporation ability of native and reassociated ribosomes were compared. Equal amounts of ribosomes and supernatant fractions were used (concentrations were determined by absorbance at 260 mu) for both the incorporation experiments. Reassociated ribosomes seem to incorporate less amino acids into protein compared with native ribosomes (Table 3). Ribosomes used in this experiment were purified 70s particles separated by gradient centrifugation.

Finally, the effect of storing ribosomes and supernatant in the frozen state was studied. As shown in the Table 4, the supernatant fraction once frozen for a week shows very little incorporation (about 20% of the original); on the other hand storing the ribosomes for the same period at the same temperature causes about 28% reduction in the incorporation activity.

Conclusions: The characteristics of protein synthesis in cell-free extracts of S. marcescens are described. For this

purpose ribosomes were isolated, purified and their properties studied. The amino acid incorporation system from exponentially growing cells of S. marcescens is less stable than that described for E. coli. Maximum incorporation of C^{14}-proline occurred within 5-10 min of the incubation period, and phenylalanine-C^{14} was incorporated at a linear increases for about 15 min before reaching the plateau. Proline incorporation was maximum at 36 C and decreased rapidly both at 27 C and 45 C. The optimum concentration of Mg^{++} was 10^{-2} M, which is also the optimum level for maintaining the integrity of 70s ribosomes.

The native state of ribosomes seems to provide better conditions for in vitro protein synthesis than ribosomes reassociated from dissociated particles of 50s and 30s. The supernatant fraction loses its amino acid incorporating activity rapidly upon storage at freezing temperatures. Ribosomes, on the other hand, remained less susceptible to deterioration due to freezing.

Chapter Three

STREPTOMYCIN AND PROTEIN SYNTHESIS

Previous studies on streptomycin have revealed a multiplicity of effects of streptomycin on bacterial cultures. Inhibition of protein synthesis is considered a major action of the antibiotic.

Characteristics of streptomycin action on the protein synthesizing system for S. marcescens both in vivo and vitro are described in this chapter. Low concentrations of streptomycin (5 ug/ml) do not affect growth or protein synthesis. However, at higher concentrations (10 ug/ml) a close correlation was found between loss of viability and slowing of protein synthesis, as measured by the incorporation of C^{14}-proline into protein in vivo. The killing effect at this concentration is not the result of extensive membrane damage. Prolonged incubation of sensitive cells at sub-lethal concentrations of streptomycin (5 ug/ml) induces the formation of ribosomes different from those of streptomycin-free control cells. These ribosomes from streptomycin-treated cells show partial inhibition of amino acid incorporation in vitro with synthetic messengers in the absence of added streptomycin. Such an effect could be the result of an altered structure of ribosome synthesizes in the presence of streptomycin or due to the attachment of a small but significant amount of streptomycin to ribosome with forces that resist isolation process and dialysis. No gross structural differences, however, were found between

streptomycin-treated and control ribosomes.

Streptomycin inhibited both poly-C-directed and poly-U-directed proline and phenylalanine incorporation in cell-free systems containing ribosomes from streptomycin-sensitive cells, whereas extracts from resistant strains were relatively unaffected. The sensitivity to streptomycin resided on the 30s components of ribosomes. Marked differences between ribosomes of streptomycin-sensitive and resistant strains were noted upon electrophoretic separation of ribosomal proteins from these two strains of S. marcescens.

I. Streptomycin and protein synthesis, in vivo:

Materials and Methods: S. marcescens strain Nima and D1 were used in these studies. Nima is streptomycin-sensitive and D1 is resistant up to about 150-200 ug/ml of streptomycin. Inocula were grown in CM at 37 C on a rotary shaker in 250 ml Erlenmeyer flasks, and, after overnight growth, cells were harvested by centrifugation and suspended in half volume of 0.85% saline. One ml of this suspension was used as inoculum for 50 ml of CM. Streptomycin solution in H_2O, sterilized by filtering through a sintered glass filter, was added in desired concentrations to the autoclaved medium. Inoculated flasks were incubated on a rotary shaker at 37 C; when pigment was desired, the incubation temperature was lowered to 27 C.

Cell mass was estimated by measuring the turbidity in the Klett colorimeter, with a blue filter. Total cell counts

were done using a Petroff-Hauser counting chamber, and for viable counts, 0.1 ml of various dilution in 0.85% saline were plated on CM agar plates. Colonies were counted after overnight incubation at 37 C.

For protein determination measured aliquots of culture were harvested by centrifugation at 5,000 rpm for 10 min and washed once with 0.85% saline. Protein was precipitated with cold 10% trichloroacetic acid, and the precipitate was washed with 95% ethyl alcohol and dried with ether. The precipitate was then digested in 1N NaOH (2ml /2.5 ml of cell suspension) overnight at room temperature, and protein was determined by Lowry's method, with bovine serum albumin as standard.

Pigment was extracted for quantitation by a modification of the alkaline extraction procedure of Hubbard and Remington (1950). To each 20 ml aliquots of pigmented cells, 12.5 ml of 1N NaOH was added. The mixture was heated in a boiling water bath for 1 hr, and pigment was extracted by shaking with 95% ethyl alcohol petroleum ether mixture (1:2 v/v) for 15 min. The ether-ethanol layer was removed and evaporated to dryness at room temperature. The dried pigment was taken up in 6.0 ml of acid alcohol (1 part 1N HCl:9 parts ethyl alcohol), and the O.D. at 537 mu was reordered in a Beckman, model B, spectrophotometer.

C^{14}-proline incorporation into protein was determined

by adding 0.05 uc/ml of C^{14}-proline of specific activity 165 mc/m mole was added to 50 ml of mineral salt medium. This medium consists of all the mineral salts of CM with 1% glycerol and 0.5 mg% amino acid mixture. One ml of exponentially growing cells ($1x10^8$/ml) were added as inoculum. Streptomycin in desired concentrations were added in all flasks except controls. At intervals, 5 ml samples were removed and protein determined as before. The precipitate or a measured amount of it dissolved in 1N NaOH was used to assay radioactivity in the Geiger-Muller tube counter, model 181 A, Nuclear Corp., Chicago.

The effect of streptomycin on permeability changes was measured by following the releases of nucleotides into the medium. The absorbancy of the medium after proper dilution was recorded at 260 mu in a Beakman, model DU, spectrophotometer.

Results: To study the effects of streptomycin on growth in sensitive and resistant strains of S. marcescens, various concentrations of the antibiotic were added to 50 ml of CM inoculated with 1 ml of inoculum prepared as before. After 20 hr of incubation at 37 C on a rotary shaker, measured samples were taken out for the cell counts. The growth of sensitive strain Nima was unaffected up to 7-8 ug/ml of streptomycin, but concentrations above 10 ug/ml caused a sharp decline in the cell count. The growth of resistant strain D1 remained

Figure 10. Effect of various concentrations of streptomycin on growth of streptomycin-sensitive and resistant strains of S. marcescens.

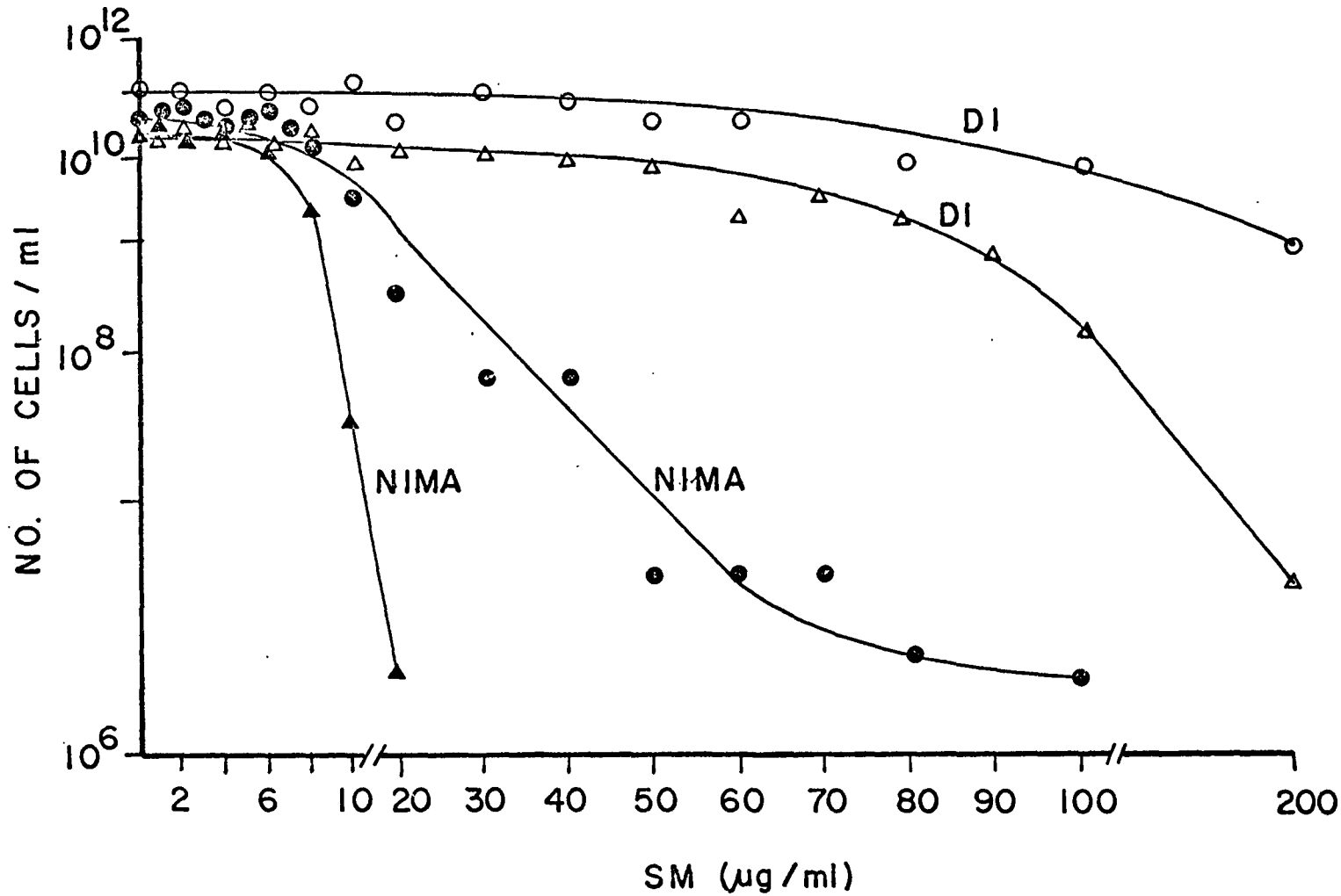

● ○ TOTAL CELL COUNT
△ ▲ VIABLE CELL COUNT
10^{12}
10^{10}
10^{8}
10^{6}
NO. OF CELLS / ml
DI
DI
NIMA
NIMA
2
6
10
20
30
40
50
60
70
80
90
100
200
SM (µg / ml)

Figure 11. Effect of streptomycin on growth pattern of sensitive strain Nima of S. marcescens.

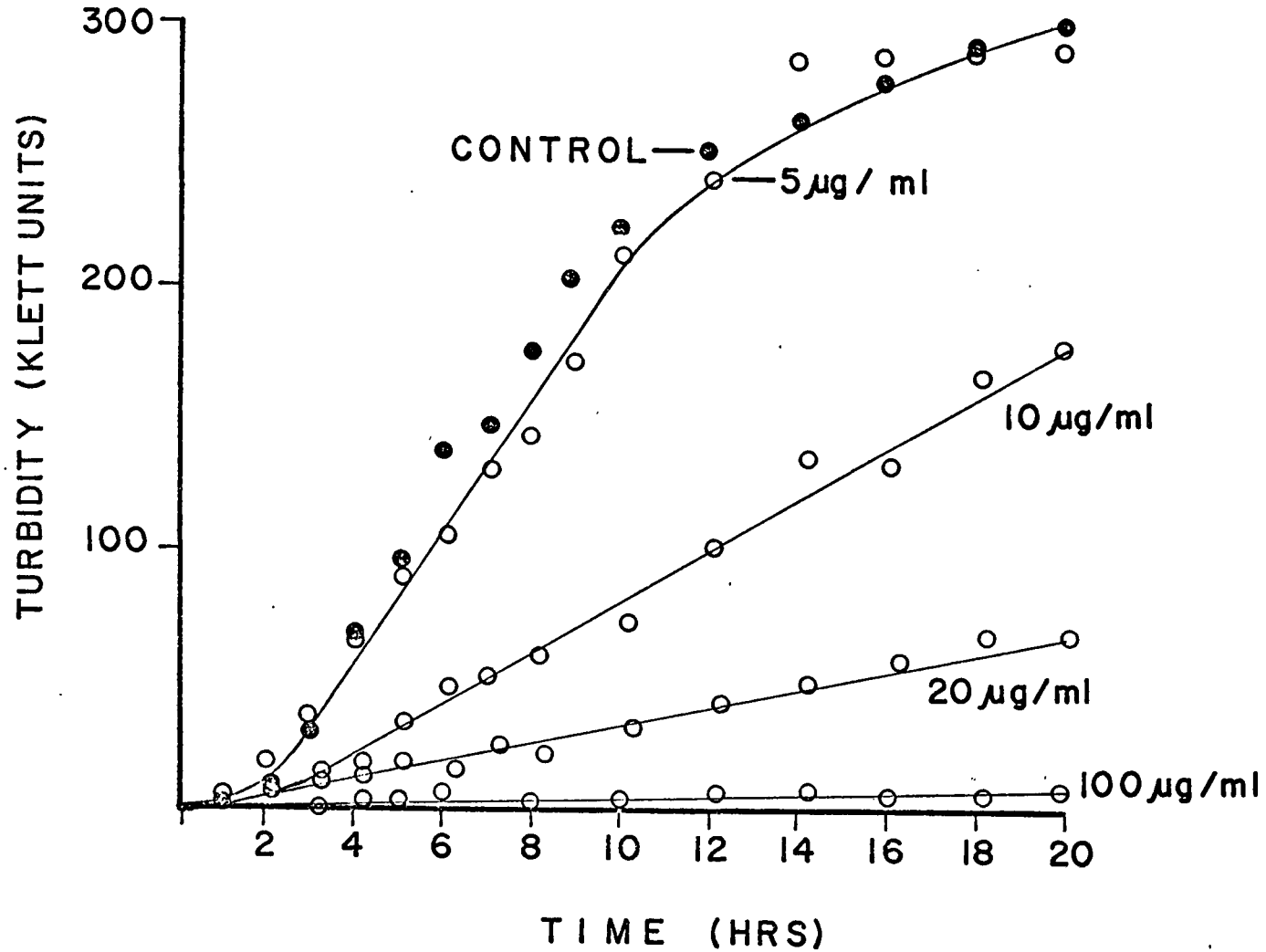
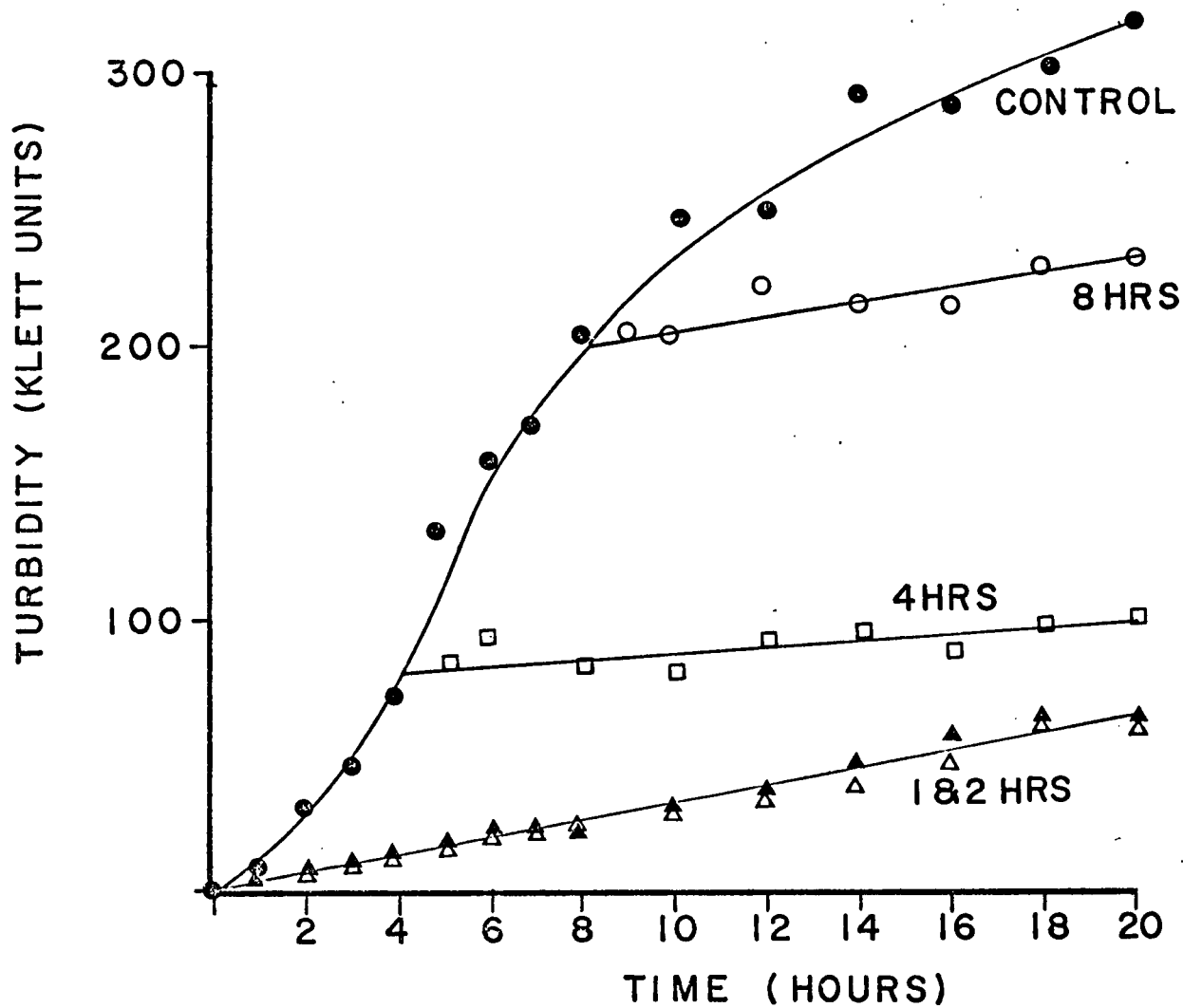

300
200
100
TURBIDITY (KLETT UNITS)
CONTROL
5 µg / ml
10 µg/ml
20 µg/ml
100 µg/ml
2
4
6
8
10
12
14
16
18
20
TIME (HRS)

Figure 12. Effect of streptomycin added at intervals during growth of S. marcescens strain Nima.


TURBIDITY (KLETT UNITS)
300
200
100
CONTROL
8 HRS
4HRS
1&2 HRS
2
4
6
8
10
12
14
16
18
20
TIME (HOURS)

unaffected up to about 150 ug/ml of streptomycin (Figure 10). The entire growth pattern of strain Nima, in the presence of 5, 10, 20 and 100 ug/ml of streptomycin, was followed at hourly intervals for 20 hours. Streptomycin at the level of 5 ug/ml did not affect the growth, except that a slightly prolonged lag period was noticeable. However, both 10 and 20 ug/ml of streptomycin caused significant reduction in the cell mass as shown by measurement of turbidity (Figure 11). To measure whether the streptomycin killing at 20 ug/ml was similar during different phases of growth, the antibiotic was added to the growing culture at intervals. The addition of the drug at 0 time (along with the inoculum) and after 1 and 2 hr of growth appears to have produced similar effects. When streptomycin was added late, after the cells are well into exponential phase (4 Hr), and also after 8 hr of growth, there was almost immediate cessation of growth (Figure 12). Inhibition of growth at this concentration (20 ug/ml) could be the result of one of several effects known for streptomycin. One of them is an extensive damage to the cell membrane, causing the appearance of large molecules, such as nucleotides, in the growth medium. Various concentrations of streptomycin were used to measure the effects of such permeability changes in relation to the loss of viability of the cells. The interesting result is that sensitive cells are killed at low concentrations of streptomycin well before a measurable effect appears on the

Figure 13. Effect of various concentrations of streptomycin on viable cell count and permeability of S. marcescens strains Nima and D1.

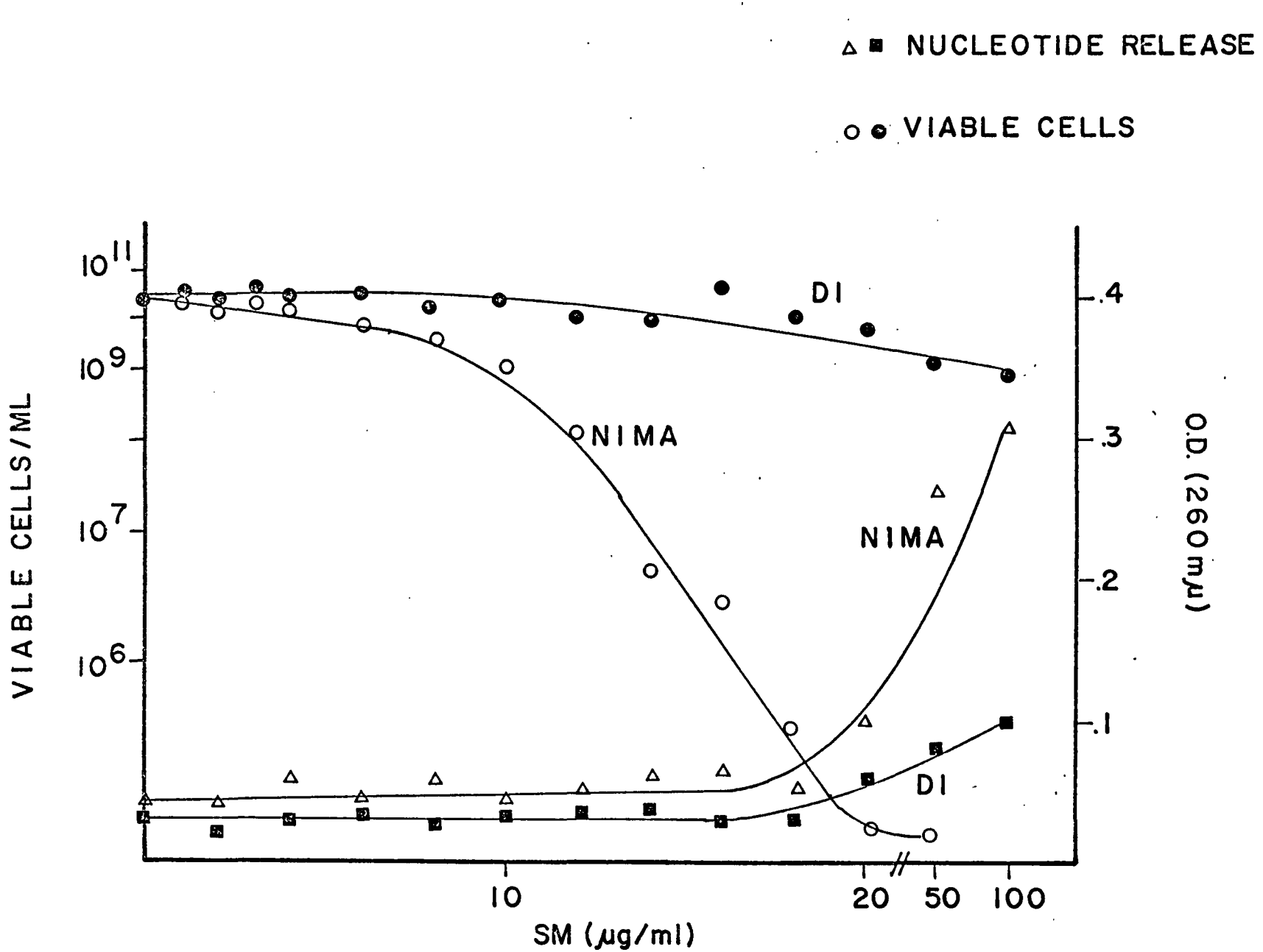
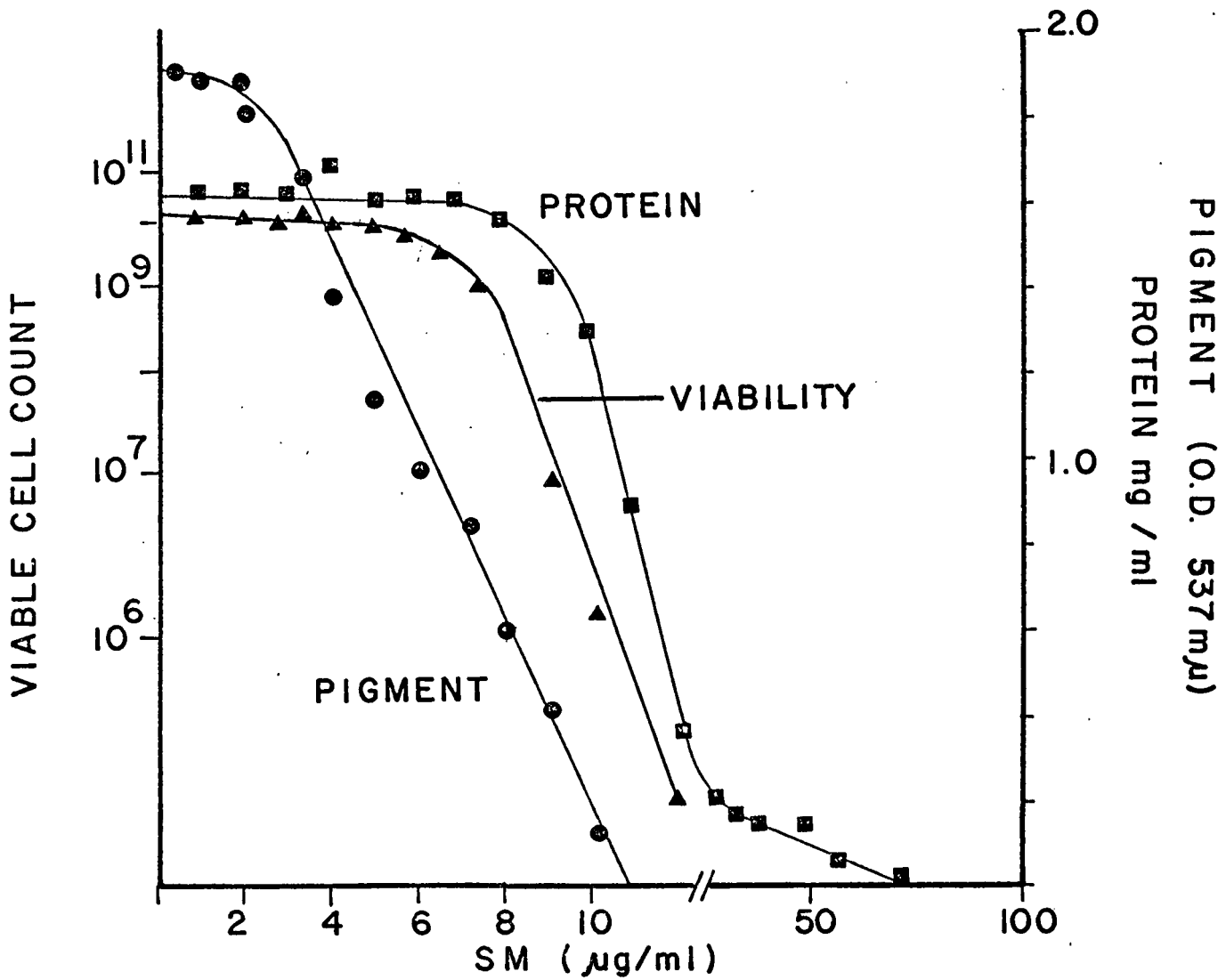

△ ■ NUCLEOTIDE RELEASE
○ ● VIABLE CELLS
VIABLE CELLS/ML
10^{11}
10^{9}
10^{7}
10^{6}
O.D. (260 mμ)
.4
.3
.2
.1
DI
NIMA
NIMA
DI
10
20
50
100
SM (μg/ml)

Figure 14. Effect of streptomycin on protein and pigment syntheses and viable cell count in S. marcescens strain Nima.


VIABLE CELL COUNT
10^{11}
10^{9}
10^{7}
10^{6}
PROTEIN
VIABILITY
PIGMENT
2
4
6
8
10
50
100
SM (µg/ml)
2.0
1.0
PIGMENT (O.D. 537mµ)
PROTEIN mg / ml

permeability of nucleotides. No significant rise in the 260-mu-absorbing compounds was found in the medium with streptomycin concentrations below 20 ug/ml, whereas a concentration as low as 10 ug/ml reduces the number of viable cells approximately by a half (Figure 13). Thus, the inhibition of growth and protein synthesis in sensitive cells appears not to be the direct results of extensive membrane damage, though it is possible that leakage of smaller molecules or ions, such as the loss of K^+ (Dubin et al., 1963), may cause inhibition of growth. The killing of resistant bacteria seems to accompany the concomitant lysis of the cells due to high concentrations of the antibiotic.

Next, the effect of streptomycin on total protein synthesis was studied by adding various concentrations of the antibiotic to the medium inoculated with Nima or D1. After 20 hr of growth, protein content was determined for measured samples of cell suspensions by Lowry's method. A comparison of the inhibition of protein synthesis by streptomycin with the loss of viable cell count suggests that the effect on protein synthesis is related to cell death (Figure 14). White and Flaks (1962) have reported cessation of protein synthesis before the onset of killing; on the other hand Hurwitz et al. (1962), using lower concentrations, have shown that the static effect coincides with the inhibition of protein synthesis.

In an attempt to resolve this point, kinetics of strepto-

Figure 15. Effect of 5 and 10 ug/ml of streptomycin on protein synthesis and viable cell count in growing culture of S. marcescens strain Nima.

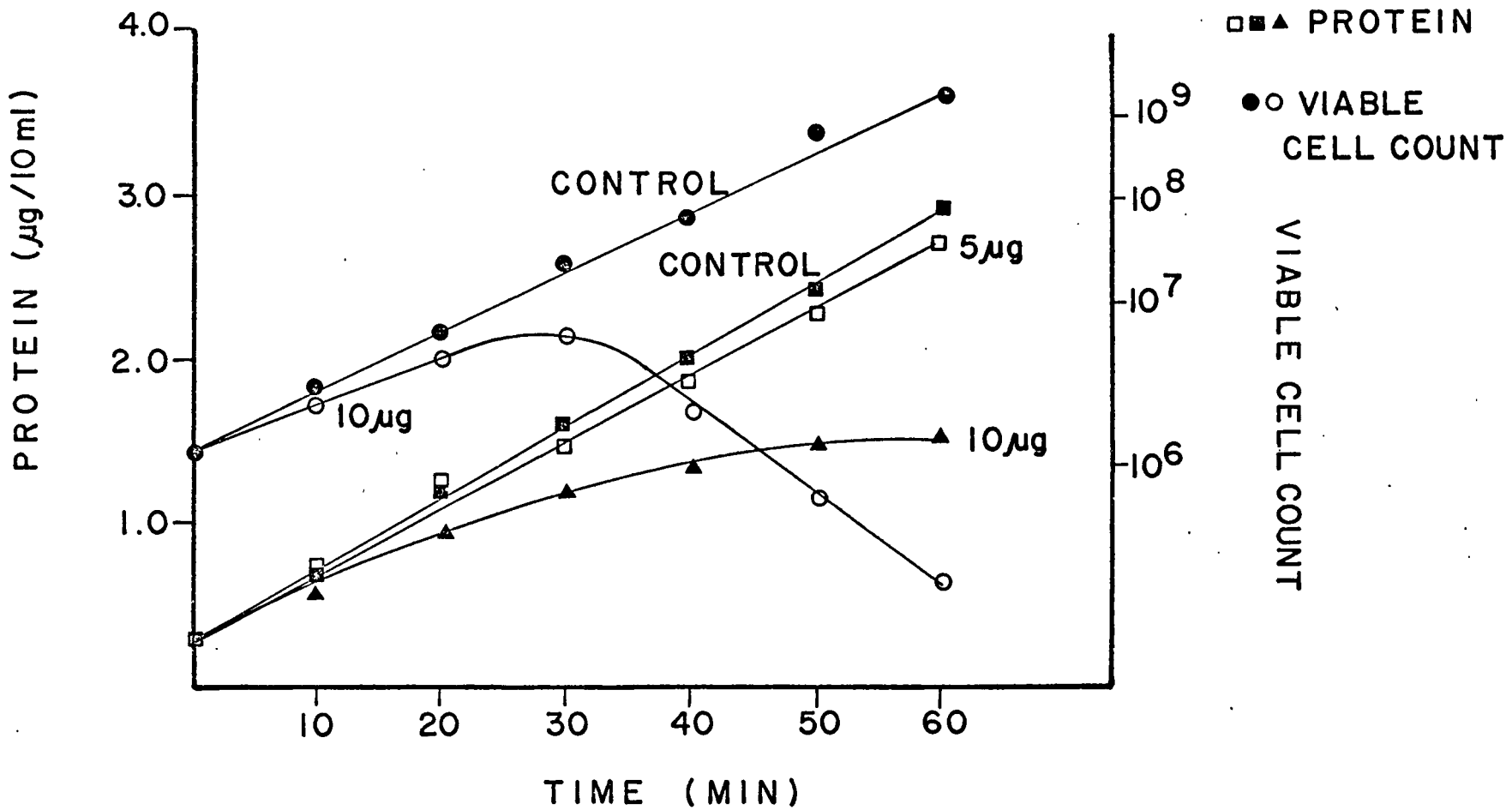
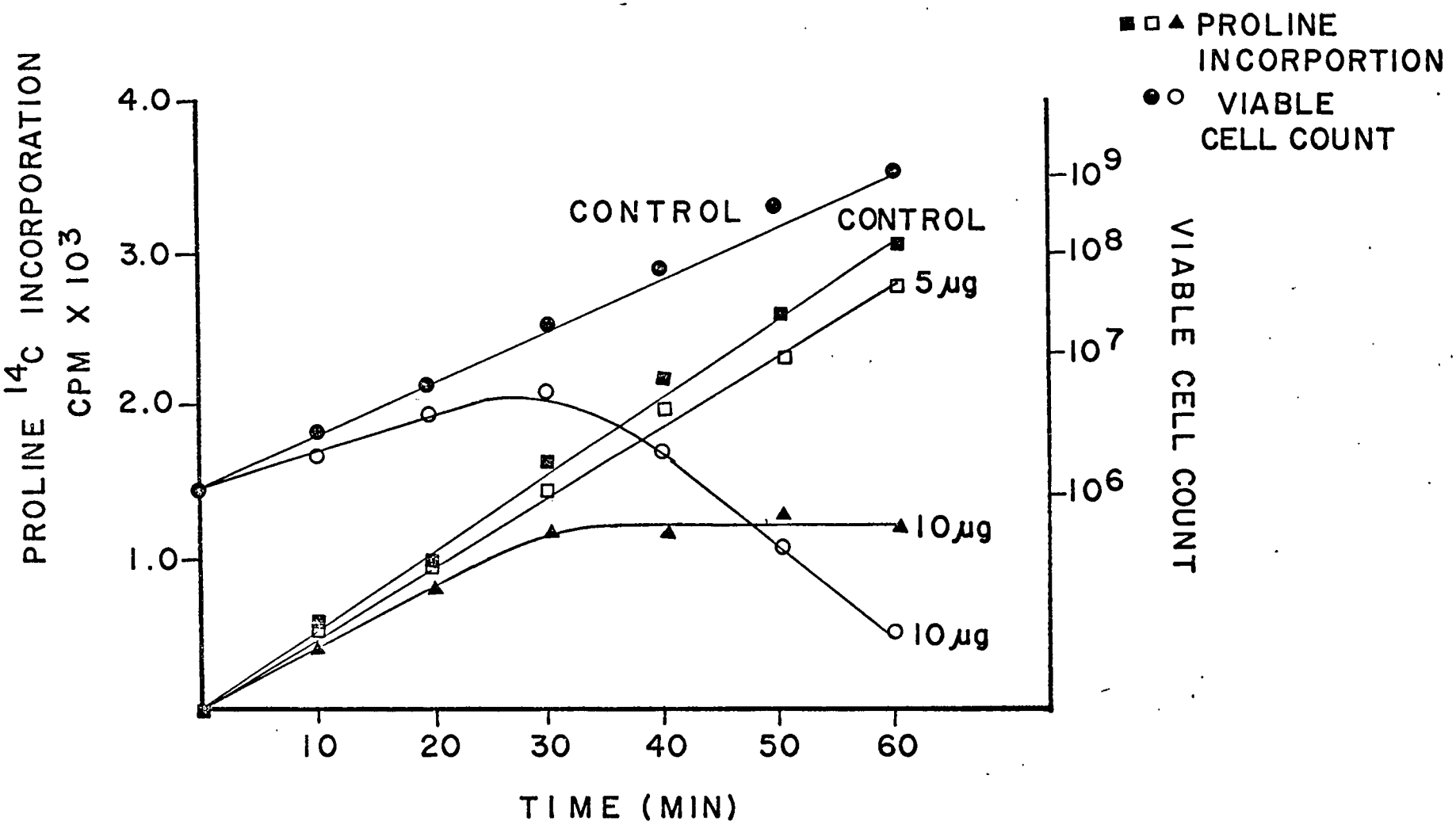

4.0
3.0
2.0
1.0
PROTEIN (µg/10ml)
CONTROL
CONTROL
5µg
10µg
10µg
10
20
30
40
50
60
TIME (MIN)
10^9
10^8
10^7
10^6
VIABLE CELL COUNT
PROTEIN
VIABLE CELL COUNT

Figure 16. Effect of 5 and 10 ug/ml of streptomycin on C^{14}-proline incorporation into protein and and on viable cell counts in S. marcescens strain Nima.


PROLINE ^{14}C INCORPORATION CPM X 10^3
4.0
3.0
2.0
1.0
CONTROL
CONTROL
5 µg
10 µg
10 µg
10
20
30
40
50
60
TIME (MIN)
10^9
10^8
10^7
10^6
VIABLE CELL COUNT
PROLINE INCORPORTION
VIABLE CELL COUNT

mycin action was studied with 5 and 10 ug/ml of streptomycin in a system for incorporation of C^{14}-proline into protein in vivo. Streptomycin was added to a 2-3 hr old culture in CM, and samples were taken at the intervals of every 10 min during the next 1 hr period of incubation at 37 C. Each sample was analyzed for protein content by Lowry's method, for viable cell count as before, and for incorporation of labeled proline into protein as described in the materials and methods. There was a sharp killing effect after 20-30 min of exposure of sensitive cells to streptomycin at 10 ug/ml (Figure 15). A close correlation was found (Figure 16) between loss of viability and slowing of protein synthesis measured by proline incorporation. The effect with lower concentrations of streptomycin (5ug/ml) was negligible. These studies suggest that killing of cells by streptomycin is related to an abrupt reduction of protein synthesis, and this occurs before an extensive damage to cell membrane takes place as measured by appearance of nucleotides in the growth medium.

Sub-lethal concentrations (5ug/ml) of the antibiotic, however, do not produce any measurable effect on protein content, and in agreement with this, the effect on proline incorporation was also negligible (Figure 16). However, streptomycin at these low levels may cause other effects, and one of them is the inhibition of the pigment prodigiosin synthesis. There was a sharp reduction in pigment formation by concentra-

tions which showed no inhibition of protein synthesis or growth (Figure 14). Whether this effect could be related to any of the known effects of streptomycin is not known.

II. Streptomycin and protein synthesis, in vitro:

Materials and Methods: Ribosomes and supernatant fractions were prepared from log-phase cells as described in section I of this chapter. Growth conditions for both S. marcescens and E. coli were also similar to the previous experiments.

For isolation of 30s and 50s ribosomal sub-units, 70s ribosomes were dissociated by overnight dialysis against TM buffer containing 10^{-4} M Mg^{++}. One ml of the resulting ribosomal solution containing about 30 mg of ribosomes was layered on 28 ml of a 5-20% sucrose density gradient and centrifuged for 10 hr at 23,000 rpm in a Spinco SW 25 rotor as before. One ml fractions from the peak concentration of 30s and 50s sub-units were separated and used with or without further purification or dialyzed again to remove sucrose. The concentrations of ribosomal fractions were determined by estimating the protein content by Lowry's method or by recording the O. D. at 260 mu (60 ug/ml of ribosomes give an O. D. at 260 mu of 1).

Poly-C-directed proline incorporation and poly-U-directed phenylalanine incorporation were carried out in a tital volume of 0.5 ml by the methods of Nirenberg and Matthaei (1961) with the minor modifications described in section I of this chapter.

The incubation conditions and the concentration of components were also similar. The specific activities of C^{14}-phenylalanine and C^{14}-proline were 1.04 and 1.02 mc/mg respecticely. The reaction mixtures were preincubated in the absence of the polynucleotides. All values were corrected for 0 time incorporation. Streptomycin in desired concentration was added along with the polynucleotide addition.

The incorporation of amino acids by hybrid ribosomes was carried out under conditions similar to those for the measurements on normal ribosomes. The 30s and 50s sub-units were first preincubated at 0 C for 30 min in the absence of poly-U or poly-C to allow the 70s hybrid formation. The reaction mixture was then preincubated at 36 C with all the components except the polynucleotide and then further incubated in the presence of all the components of the incorporation experiment. For each 400 ug of total ribosomes 250 ug of 50s and 150 ug of 30s ribosomal particles were used.

Seperation of 70s, 50s and 30s particles was done by zonal centrifugation in a linear sucrose density gradient. Measured amounts of ribosomes were layered on a 5-20% gradient and centrifuged at 23,000 rpm for 5 hr in a SW 25 rotor to separate 70s particles; 50s and 30s fractions were separated by centrifuging for 10.5 hr under similar conditions, or with a SW 39 rotor using a 4.4 ml gradient and centrifuging at 37,000 rpm for 190 min. Fractions of equal volume were collected and those

from the peak concentration were pooled together and dialyzed to remove sucrose, if necessary.

For experiments on ribosomes from cells grown in the presence of streptomycin, S. marcescens strain Nima was grown overnight with 5 ug/ml of streptomycin to produce the inoculum which was then added to 2.5 liters of CM containing the same concertration of the antibiotic. Cells were harvested by centrifugation after 3-3.5 hr growth at 37 C. Ribosomes were isolated as before and one sample of ribosomes was dialyzed against 1,000 volume of streptomycin-free TM buffer for 15 hr at 4 C. Amino acid incorporation experiments with ribosomes from a control treatment, from streptomycin-treated cells, and from streptomycin-treated and dialyzed cells were all carried out by methods described in Section I of this chapter.

The binding capacity of these ribosomal samples to streptomycin was observed by adding 2 mg of C^{14}-streptomycin (specific activity 0.054 ug/mg) to 1 ml of solution containing about 10 mg of ribosomes. After incubation of 4 C for 30 min the ribosomes were centrifuged at 23,000 rpm for 5 hr in a Spinco SW 25 rotor. Fractions of 10 drops each were collected and the activity of 5-drop samples counted in a Packard scintillation spectrometer.

The ribosomes from normal cells, from cells grown in the presence of streptomycin and from those of the streptomycin-resistant mutant strain D1 were compared by banding the proteins

Figure 17. Effect of streptomycin on C^{14}-proline incorporation into protein by cell-free extracts of S. marcescens strains Nima and D1.

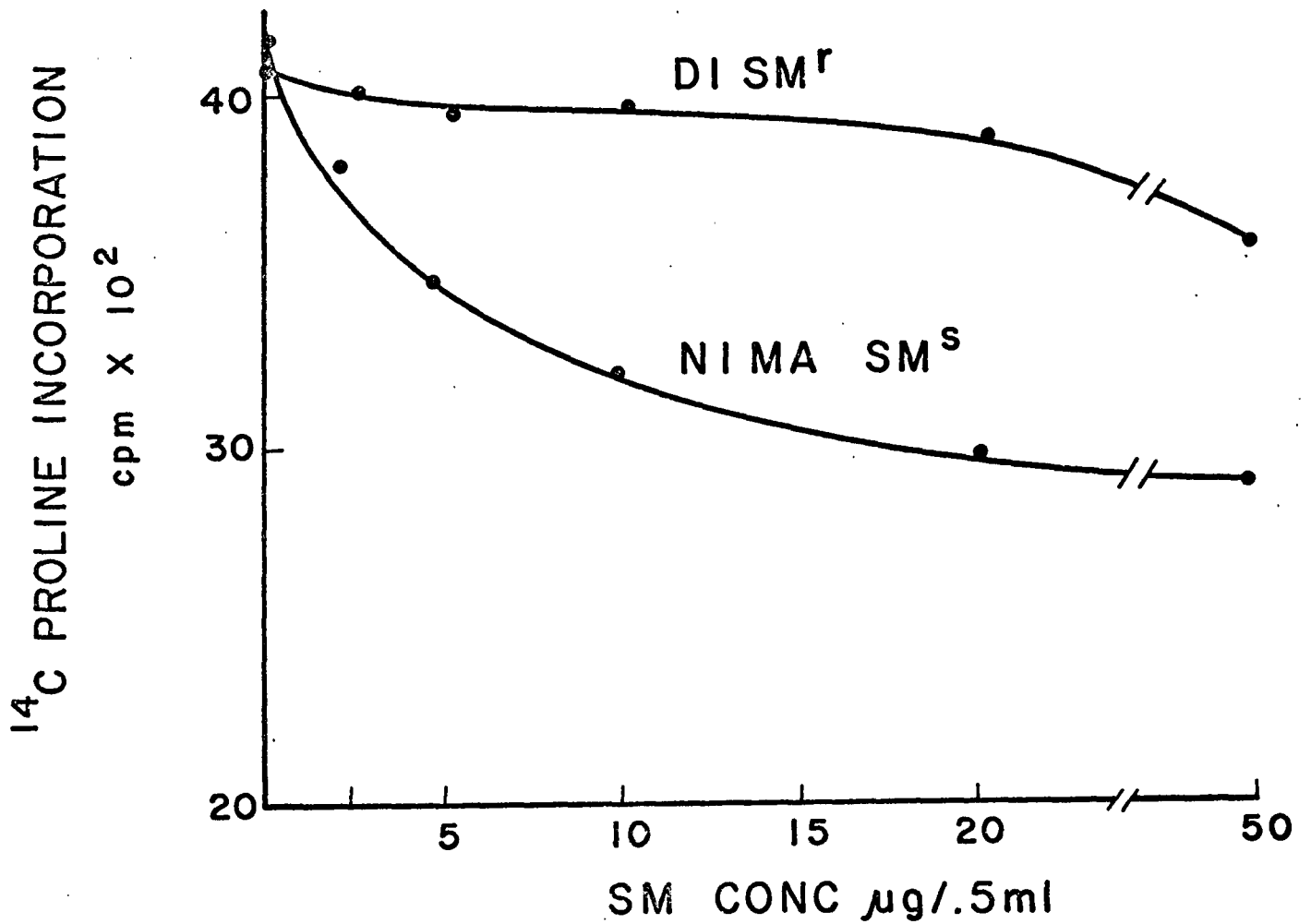

DI SM^r
NIMA SM^s
40
30
20
5
10
15
20
50
SM CONC µg/.5ml
14C PROLINE INCORPORATION
cpm X 10^2

Figure 18. Time course of C^{14}-proline incorporation and effect of streptomycin on in vitro protein synthesis in S. marcescens strain Lima.

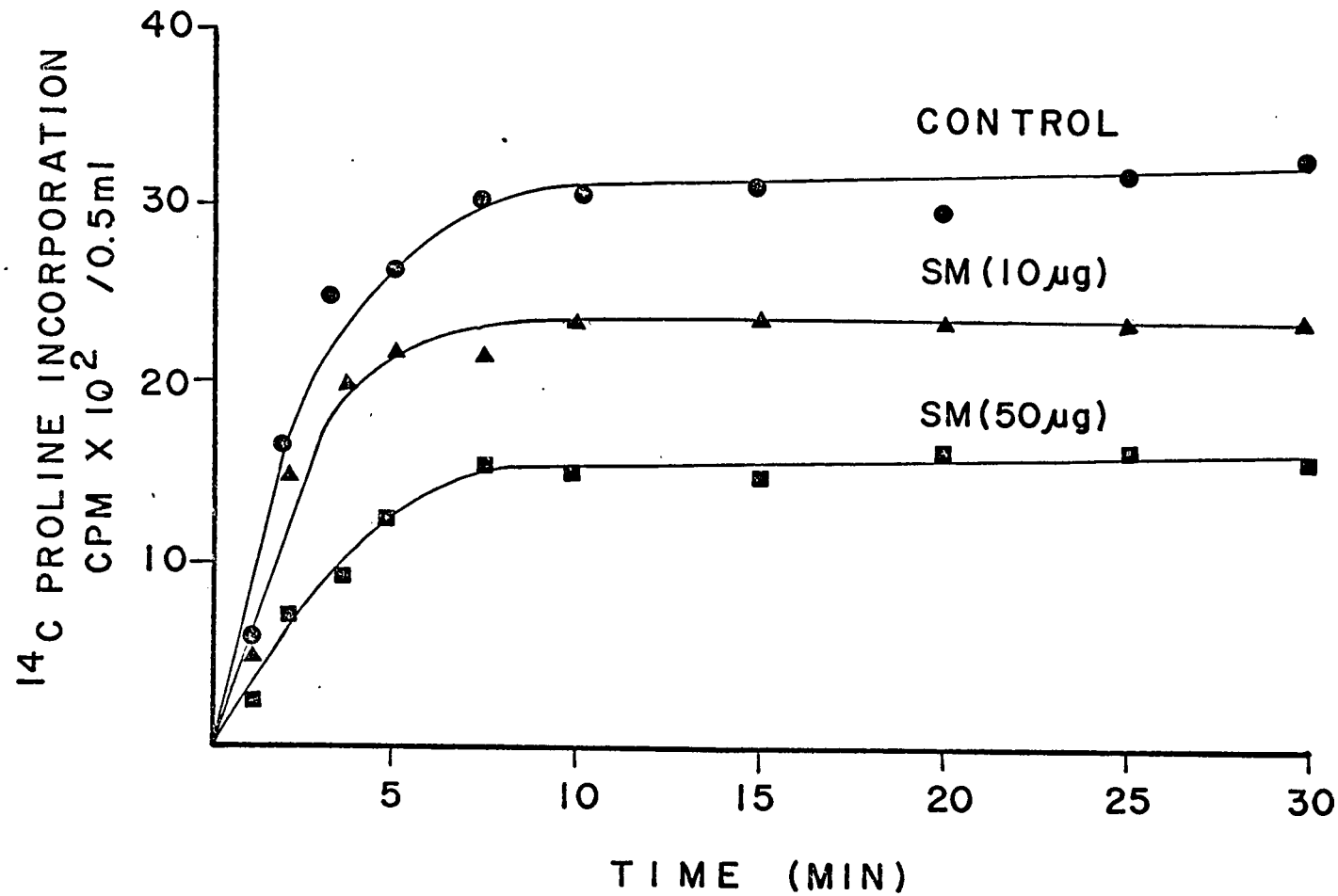

40
30
20
10
^{14}C PROLINE INCORPORATION
CPM X 10^2 /0.5ml
CONTROL
SM (10μg)
SM (50μg)
5
10
15
20
25
30
TIME (MIN)

Table 5

STREPTOMYCIN INHIBITION OF C^{14}-PROLINE AND C^{14} PHENYLALANINE INCORPORATION

C^{14} Amino Acid	Polynucleotide	Incorporation (cpm/.5ml)	
		-SM	+SM (50 ug)
Proline	Poly-C	3,880	2,083
Proline	-	887	738
Phenylalanine	Poly-U	15,780	7,021
Phenylalanine	-	923	698

The components of the reaction mixture and conditions for incubation same as described in Materials and Methods; all counts were corrected for 0 time.

on polyacrylamide gel electrophoresis. The method is given in Annexure I.

Results: Streptomycin inhibited the protein synthesis-by cell-free extracts from S. marcescens strain Nima, whereas the system from strain D1 remained relatively unaffected (Figure 17). Poly-C-directed proline incorporation was reduced sharply at low concentrations of streptomycin; 5 ug/ml of the antibiotic caused about half the inhibition obtained with 50 ug/ml of streptomycin. In these early experiments the maximum inhibition was of the order of only 30%. Addition of streptomycin after the polynucleotide template was added reduces the inhibitory activity of the drug to some extent. In later experiments streptomycin was added before or with the template. With very low concentrations (1 ug) slight stumulation of proline incorporation was used. The relation between the time course and streptomycin inhibition indicated that the poly-C-directed incorporation of proline is sensitive throughout the incubation period (Figure 18). Streptomycin in concentrations of 50 ug/ml caused about 40% inhibition of the incorporation of proline, and the inhibition reached the plateau within 10 min of the incubation period. With identical conditions for the incorporation experiments, poly-U-directed phenylalanine incorporation appears more sensitive than poly-C-directed proline incorporation (Table 5). In the absence of synthetic template, inhibition of endogenous mRNA-dependent

Table 6

EFFECT OF STREPTOMYCIN ON AMINO ACID INCORPORATION

NATIVE AND REASSOCIATED RIBOSOMES

Assay system	Native		Reassociated	
	-SM	+SM	-SM	+SM
C^{14}-proline (Poly-C)	5,224	3,619	4,780	3,280
C^{14}-phenylalanine (Poly-U)	14,880	8,081	12,570	4,323

Experimental conditions were same as described in Materials and Methods; all counts are given as CPM and corrected for 0 time; SM = streptomycin (50 ug when used)

Table 7

STREPTOMYCIN INHIBITION OF C^{14}-PROLINE INCORPORATION BY RIBOSOMES OF SENSITIVE STRAIN NIMA OF S. MARCESCENS

Ribosome	Supernatant	Incorporation (CPM/TUBE)	
		-SM	+SM
sensitive	sensitive	4,020	2,573
sensitive	resistant	3,581	2,011
resistant	sensitive	3,901	3,590
resistant	resistant	3,870	3,501

Experimental conditions same as described in Materials and Methods: sensitive = extracts from streptomycin-sensitive Nima; resistant = extracts from resistant strain D1; SM = streptomycin (50 ug when used); all counts were corrected for 0 time.

protein synthesis by streptomycin was insignificant.

When ribosomes are dissociated by lowering the Mg^{++} concentration and then reassociated for incorporation experiments in the presence of 10^{-2}M Mg^{++}, the activity of C^{14} amino acid incorporation in vitro is reduced to some extent (Table 6). However, the relative efficiency of streptomycin action on such ribosomes is stimulated. This is true particularly for poly-U-directed phenylalanine incorporation, which showed an inhibition of about 65% incorporation as compared to 30% for poly-C-directed proline incorporation.

Incorporation experiments, in the system prepared by interchanging the components, ribosomes and supernatant obtained from sensitive and resistant cells were done in an attempt to localize the cellular fraction sensitive to the action of streptomycin. There was no inhibition when the system contained ribosomes of streptomycin resistant S. marcescens, regardless of the source of the supernatant fraction. The results were consistent in both poly-C and poly-U-directed systems (Table 7), and showed that streptomycin interferes with ribosomal function in S. marcescens, as was reported for extracts from E. coli (Spreyer et al., 1962).

In an attempt to localize further the site of action of streptomycin on sensitive ribosomes, an experiment was done on poly-C-directed polyproline synthesis with hybrid ribosomes, reconstituted with sub-units derived from resistant and sensi-

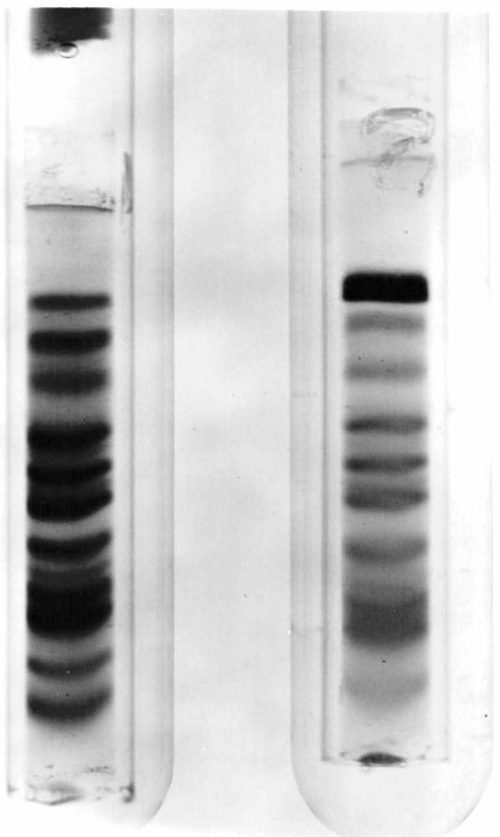
Table 8

EFFECT OF STREPTOMYCIN ON C^{14}-PROLINE INCORPORATION BY HYBRID RIBOSOMES

Ribosomes		Incorporation (CPM/TUBE)	
30s	50s	-SM	+SM
sensitive	sensitive	5,082	3,683
sensitive	resistant	4,633	3,001
resistant	sensitive	4,972	4,601
sensitive	-	214	201
-	sensitive	501	333

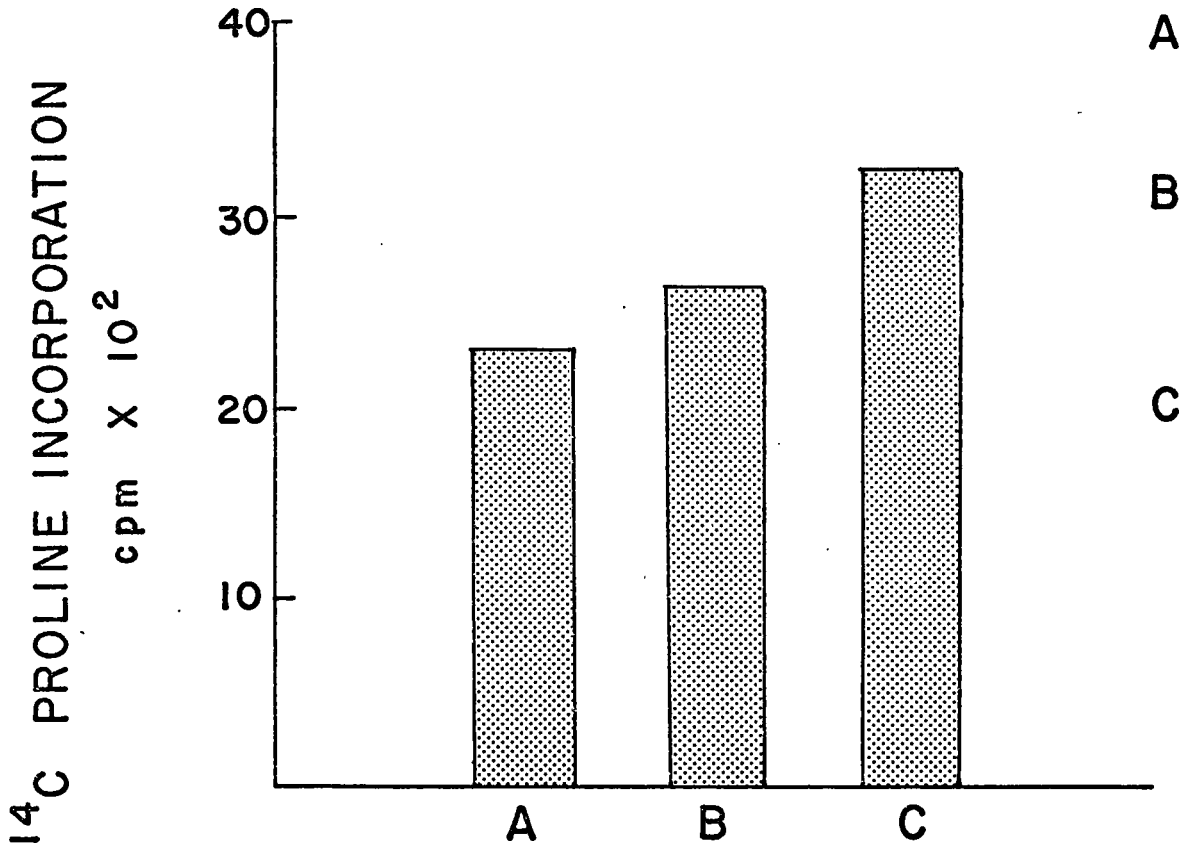
Experimental conditions same as described in Materials and Methods; all counts were corrected for 0 time; SM = streptomycin (50 ug when used); sensitive = extracts from strain Nima; resistant = extracts from strain D1.

Figure 19. Electrophoretic separation of ribosomal protein from S. marcescens strains Nima and D1 on polyacrylamide gel at pH 4.5. The column on the right represents proteins from streptomycin-resistant strain D1 and on the left are proteins from sensitive strain Nima.



NIMA D1

Figure 20. C^{14}-proline incorporation by ribosomes from streptomycin-grown cells of S. marcescens strain Nima



A RIBOSOMES FROM CELLS GROWN IN SM.

B RIBOSOMES FROM "A" DIALYZED AGAINST SM-FREE TRIS BUFFER.

C CONTROL

tive strains. The hybrid ribosomes are sensitive to streptomycin inhibition only when they contain a 30s sub-unit derived from a streptomycin-sensitive strain (Table 8). The origin of the 50s sub-unit was of no significante. No protein synthesis occurs in the absence of eitherof the two sub-units of ribosomes.

The separation of ribosomal protein from streptomycin sensitive strain Nima and resistant strain D1 of S. marcescens showed at least one significant difference in their pattern of banding. The characteristic, thick, top band from D1 ribosomes were markedly less from strain Nima. The significance of this is discussed later (Figure 19) [illegible].

Ribosomes isolated from cells grown in the presence of 5 ug/ml of streptomycin show partial inhibition of amino acid incorporated with polynucleotide in the absence of added streptomycin. Such reduced activity of ribosomes from streptomycin treated cells could be due to streptomycin that attached to ribosomes and survived the isolation process. To test this hypothesis the ribosomes from streptomycin grown cells were dialyzed extensively against streptomycin-free TM buffer. The source of supernatant was common for all three samples and came from the control streptomycin-free cells. Ribosomes grown in the presence of streptomycin showed reduced activity of incorporation of proline, even after 15 hr dialysis (Figure 20). The fact that reduced incorporation ability was associated with the ribosome and not with the supernatant faction was demonstrated

Table 9. C^{14}-proline incorporation by cell-free extracts from streptomycin-grown and streptomycin-free cells of S. marcescens strain Nima.

ASSAY SYSTEM	RIBOSOME	SUPERNATANT	CPM
POLY U (ø ALA)	C	C	12,618
	SM	C	10,952
	C	SM	12,013
POLY C (PROLINE)	C	C	3,355
	SM	C	2,569
	C	SM	3,405

SM CELLS GROWN IN SM C CONTROL

Figure 21. Streptomycin-C^{14} binding to ribosomes of S. marcescens strain Nima

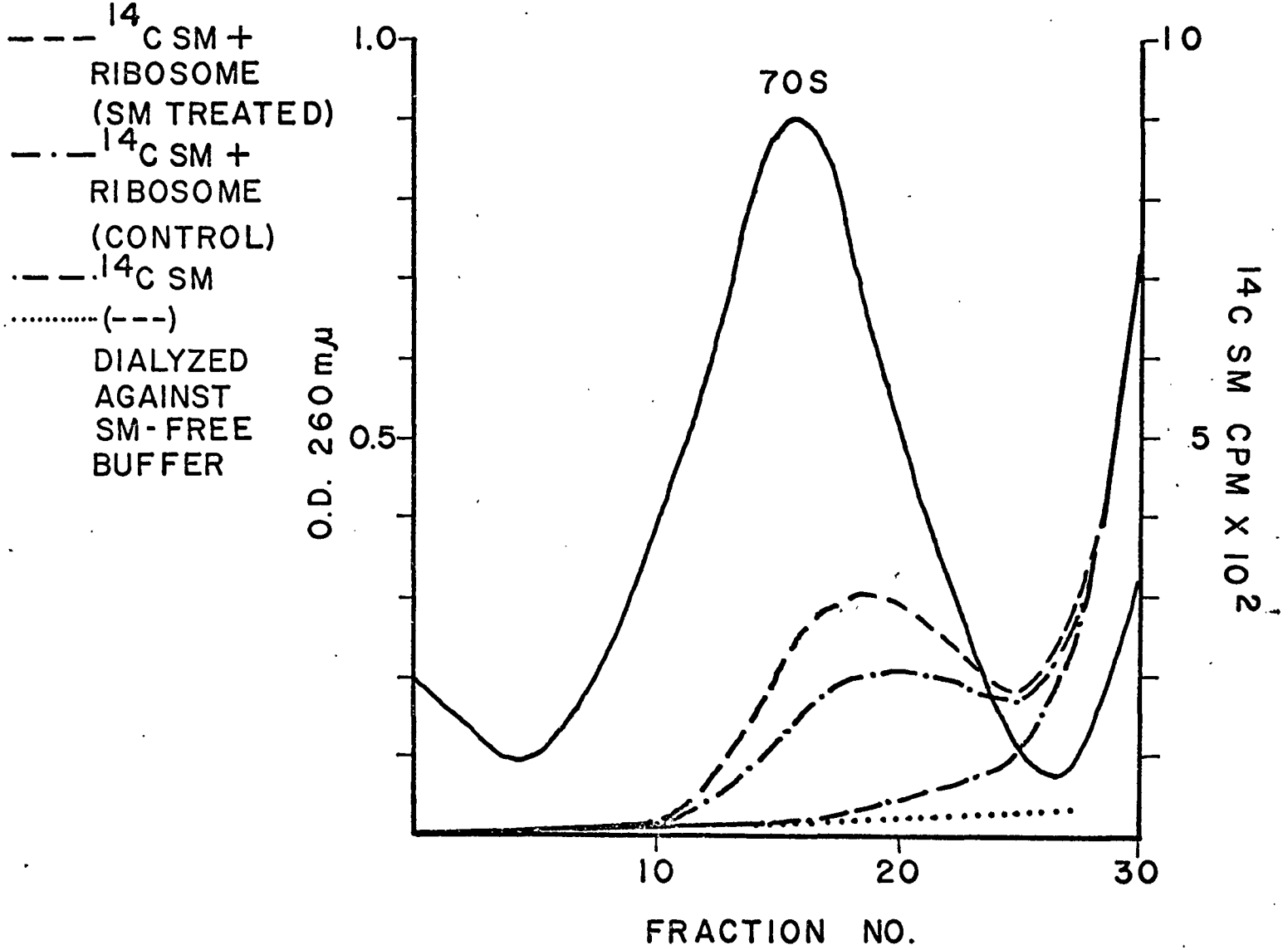

14C SM + RIBOSOME (SM TREATED)
14C SM + RIBOSOME (CONTROL)
14C SM
(---) DIALYZED AGAINST SM-FREE BUFFER
1.0
0.5
O.D. 260 mμ
70S
10
5
14C SM CPM X 10²
10
20
30
FRACTION NO.

Figure 22. Sucrose gradient profile of ribosomal extracts from cells grown in the presence of streptomycin, cells grown in streptomycin and dialyzed, and streptomycin-free control cells of *S. marcescens* strain Nima.

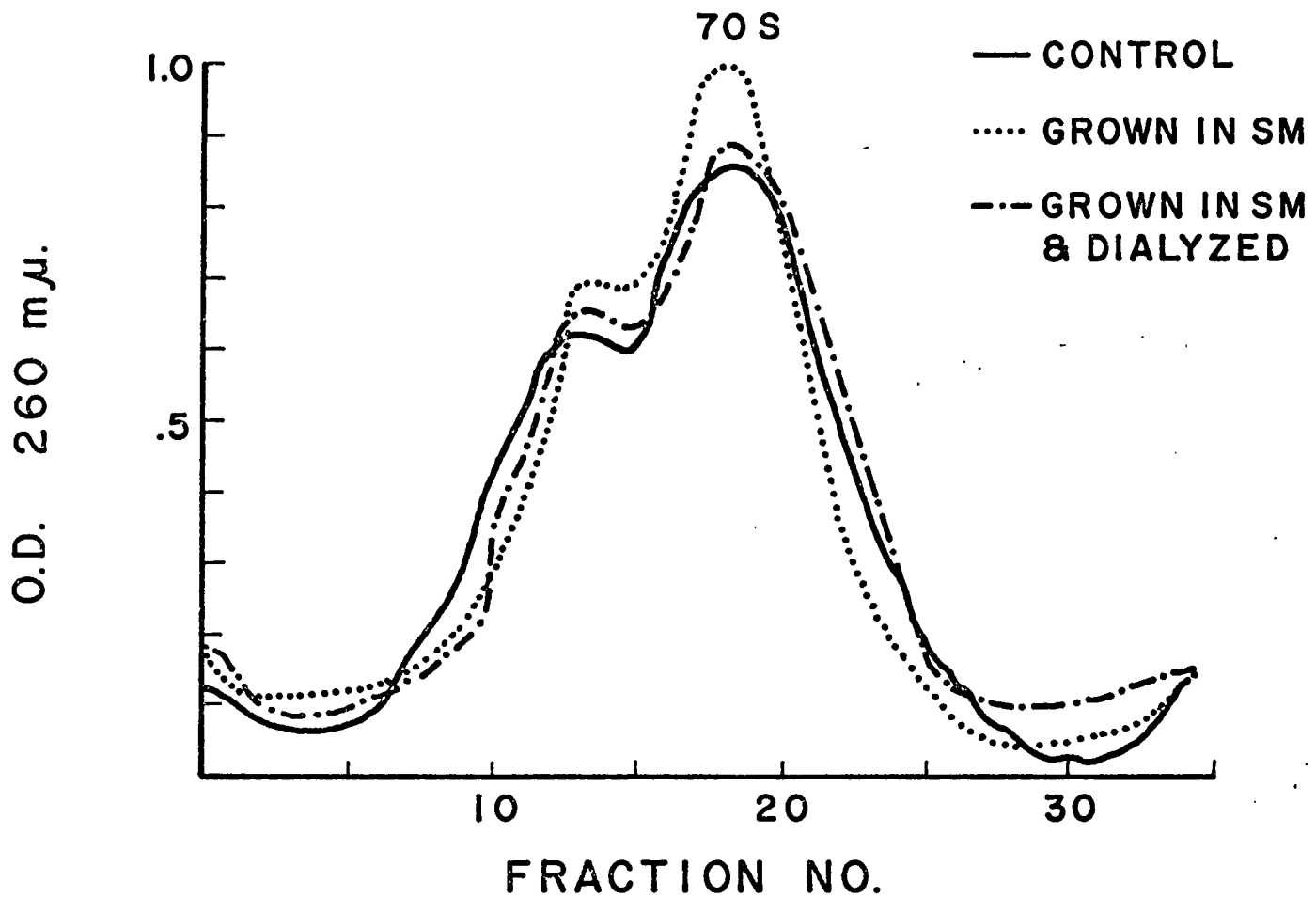

70 S
1.0
.5
O.D. 260 mμ.
10
20
30
FRACTION NO.
CONTROL
GROWN IN SM
GROWN IN SM
& DIALYZED

Figure 23. Sucrose gradient profiles of dissociated ribosomes from streptomycin-grown cells and streptomycin-free control cells of S. marcescens strain Nima.

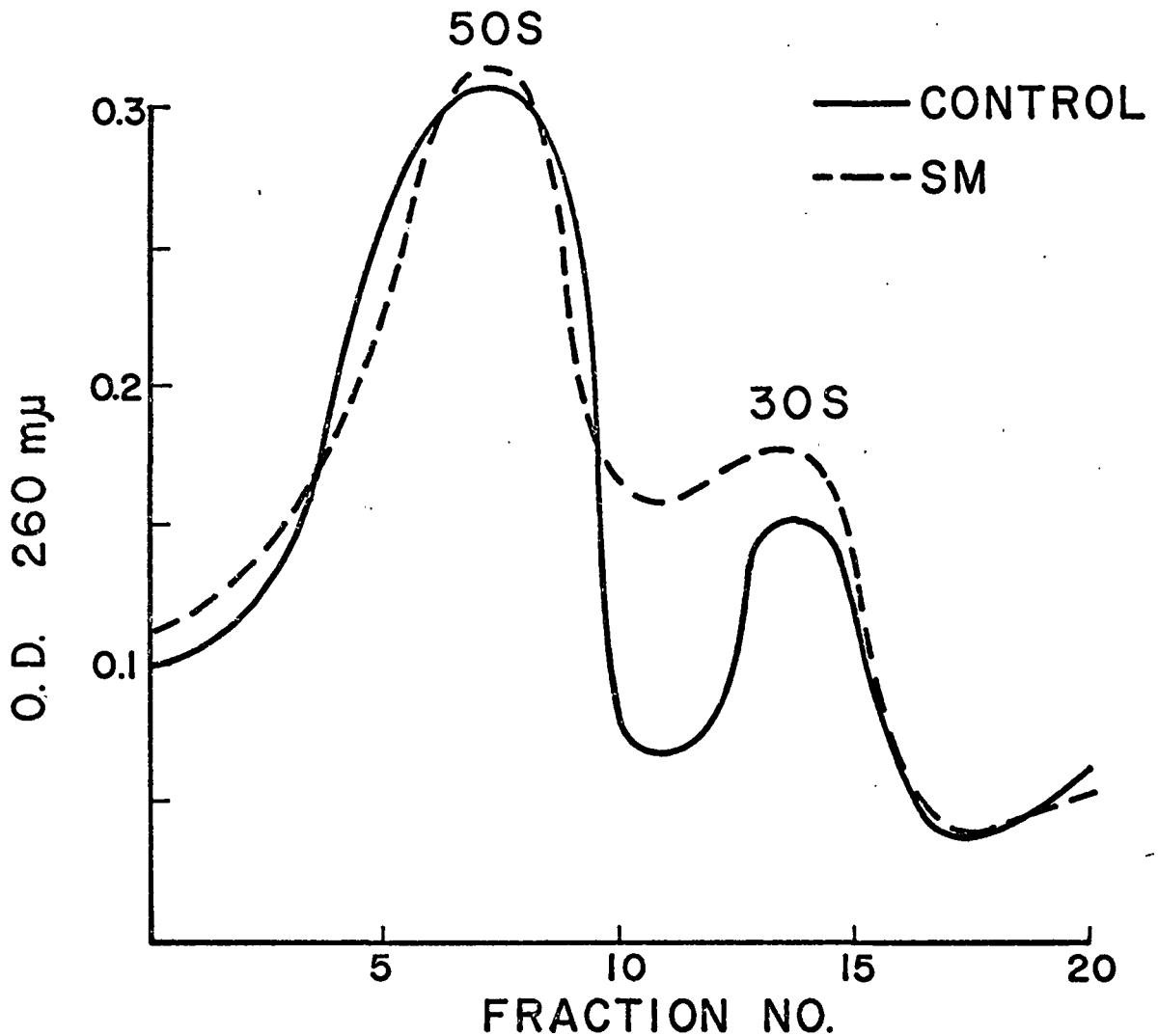

50S
30S
CONTROL
SM
0.3
0.2
0.1
O.D. 260 mμ
5
10
15
20
FRACTION NO.

Table 10

CHEMICAL COMPOSITION AND ABSORPTION RATIO OF RIBOSOMES FROM STREPTOMYCIN-GROWN AND CONTROL CELLS OF *S. MARCESCENS*

Ribosome source	Protein mg/ml	RNA mg/ml	260/235
Control	7.38	8.81	1.79
SM-treated	7.4	8.9	1.809

RNA and protein were estimated as described in Chapter Two. RNA was also estimated by assuming that 1 mg/ml in H_2O has a absorbance of 24 at 260 mu.

by interchanging the components, ribosomes and supernatant, from streptomycin treated cells and streptomycin-free control cells. Both ribosomes and supernatant were dialyzed against streptomycin-free TM buffer. The partial inhibition of incorporation of proline and phenylalanine was due to the ribosomal source; the origin of supernatant was of no consequence (Table 9). Whether streptomycin remains bound to ribosomes even after dialysis could not be demonstrated with experiments performed by adding streptomycin-C^{14} to ribosomal suspension. After dialysis for 15 hr no significant activity remained associated with ribosomes (Figure 21).

The possibility that streptomycin acted upon ribosomal synthesis itself to induce gross conformational or particle weight changes was tested by applying a measured amount of ribosomes from the above sources to gradient analysis. No measurable difference was observed in the profiles for both 70s and 30s and 50s particles (Figure 22, 23). Further, an analysis on chemical composition of these ribosomes did not reveal any significant differences (Table 10).

One interesting property of the ribosomes from cells grown in streptomycin was the capacity to bind more streptomycin than control ribosomes. Two mg of C^{14}-streptomycin was added to about 10 mg of ribosomes and the mixture was incubated for 30 min at 4 C. A sample of ribosome from normal cells was treated as control. Both of these samples were layered on a

sucrose gradient for separation of the 70s particles. Fractions of equal volume were collected and radioactivity of a representative sample from each fraction was counted. A significantly high activity was associated with the ribosomes from streptomycin-treated cells in the region of 70s particles. Most of the activity, however, remained at the top of the tube. The significance of these results is discussed in chapter 4. (see Figure 21).

Conclusions: The characteristics of streptomycin action on protein synthesis in S. marcescens are described for both in vivo and in vitro systems. Streptomycin at 5 ug/ml does not decrease either the total amount of protein synthesized or the growth of sensitive strain Nima. However, ribosomes isolated from cells grown in the presence of sub-lethal concentrations of streptomycin show partial inhibition of amino acid incorporation in vitro in the absence of added streptomycin. No gross structural differences were observed in the sucrose density profiles of these ribosomes compared with those of control cells. Also experiments with C^{14}-streptomycin did not confirm that streptomycin is bound to ribosomes with sufficient strength to resist dialysis. It is possible at the same time that an extremely small amount of the streptomycin-C^{14}, approximately 1-2 molecules per ribosome, remained attached to ribosomes but could not be detected. Whether the reduced ability of these ribosomes to synthesize proteins

is the result of irreversible binding of low amount of streptomycin or due to a change in the ribosomal conformation during ribosomal synthesis is not evident from these data.

At higher concentrations of streptomycin (10 ug/ml), a close correlation exists between loss of viable cells and slowing down of protein synthesis as measured by the incorporation of C^{14}-proline into protein in vivo. The killing effect at this concentration is apparently not the result of extensive damage to the cell membrane. In resistant cells of strain D1 loss of viability occurs with concomitant damage to the membrane.

Streptomycin inhibits both poly-C-directed proline incorporation and poly-U-directed phenylalanine incorporation by cell-free extracts from streptomycin-sensitive strain Nima; whereas extracts containing ribosomes from resistant strain were relatively unaffected. The sensitivity to streptomycin resides on the 30s component of the ribosomes. A marked difference between ribosomes of sensitive and resistant strains was noted upon electrophoresis of ribosomal proteins on polyacrylamide gel columns.

Chapter Four

DISCUSSION

Streptomycin is known to produce numerous effects before the ultimate destruction of bacterial cells sensitive to the antibiotic. In E. coli the sequence of effects occurring while the cells are growing in the presence of streptomycin starts with the acceleration of potassium efflux, immediately followed by an inhibition of protein synthesis and loss of viability. Later, there are gross changes in cellular metabolism leading to impairment of respiration, breakdown of RNA, extensive damage to the cell membrane, and lysis of cells.

It is well established that streptomycin inhibits protein synthesis in several bacterial species (Erdos and Ullmann, 1960; Anand and Davis, 1960; Spotts and Stanier, 1961). The mechanism of action on protein synthesis was mainly elucidated by experiments with cell-free bacterial extracts. The site of action of the antibiotic was found to be the 30s ribosomal sub-unit (Cox et al., 1964). This interaction of streptomycin with sensitive ribosomes in vitro brings about misreading in the translation of the amino acid code (Davies et al., 1964). The purpose of this thesis was to examine the effects of sub-lethal concentrations of streptomycin on intact cells and on protein synthesis, especially on ribosomal function, in vitro and vivo in streptomycin-sensitive and resistant strains of S. marcescens. The study necessitated demonstrating and

characterizing in this species, protein synthesis in vitro and its stimulation by synthetic polynucleotides.

The characteristics of in vitro protein synthesis in S. marcescens are very similar to those observed for E. coli by Matthaei and Nirenberg, (1961), though the cellular extracts from S. marcescens sustain protein synthesis for a shorter time than in the system from E. coli. The linear increase in the amino acid incorporation during the early incubation period reaches a plateau within 5-10 min., compared to 40-60 min on E. coli and B. cereus (Kobayashi and Halvorson, 1966). In another report (Ismande and Caston, 1966), B. cereus extracts showed incorporation kinetics similar to those for S. marcescens. The decreased rate of incorporation after the initial linear increase in S. marcescens does not seem to be due to any non-specific breakdown of the incorporation system, as preincubation does not affect the initial rate of synthesis. It is unclear whether mRNA breakdown, slow release of polypeptide, or reduced polypeptide chain initiation became the limiting factors for protein synthesis in vitro.

The general properties of protein synthesis in vitro in S. marcescens were examined under a variety of conditions. The energy requirements and the dependence of the system upon ribosomes and supernatant indicate that this system is similar to those found in other bacterial systems. The maximum incorporation occurred in the presence of 10^{-2}M Mg^{++},

which is also required for the integrity of the ribosomal 70s unit. The optimal temperature for incorporation is about 36 C; lower and higher temperatures affected the system significantly. The native state of the ribosomes is also of significance for the system. When ribosomes were dissociated into sub-units and reassociated, the incorporation of amino acids was reduced. The fact that reassociated 70s ribosomes are recovered with good efficiency from the dissociated particles suggests that the effect was not the result of incomplete reassociation of 30s and 50s particles into 70s ribosomes. Ismande and Caston (1966) reported that repeated washings of ribosomes also caused decreased amino acid incorporation. The change in ribosomal activity upon dissociation and reassociation or upon washing could be due to the removal of ribosome-bound enzymes or due to a general disruption or distortion of the structure in the ribosome dependent upon weak interaction and ionic bonding.

The kinetics of the inhibition of protein synthesis caused by the presence of streptomycin in the growth medium suggest that this effect is related to cell death. Total protein synthesis in sensitive strains of S. marcescens not affected up to about 8-10 ug/ml of streptomycin (Figure 14). As the concentration was raised past 10 ug/ml, there was a sharp decrease in protein synthesis with a simultaneous and rapid reduction in viability of the cells. White and Flaks (1962)

have reported that cessation of protein synthesis occurs before the onset of killing in sensitive bacteria: on the other hand, in lower concentrations of streptomycin (Hurwitz et al., 1962) the killing effect of streptomycin coincides with the initial inhibition of protein synthesis. In the present studies, reduction in the amount of total protein started slightly after the start of the decline in cell count. However, upon precise measuring of the slowing of protein synthesis, by C^{14}-proline incorporation, there was shown a close correlation between the loss of viability and the decrease in protein synthesis. The close association of these two effects suggests that the inhibition of protein synthesis may be the principal cause of the bactericidal action of this antibiotic.

The study of the killing effect of streptomycin becomes more complicated when the streptomycin action on the permeability of the cell membrane is also considered. The results (Figure 13) indicate that damage to the cell membrane, measured in terms of nucleotide excretion into the medium starts at a concentration of about 20 ug/ml of streptomycin, a concentration higher than that which causes the onset of protein inhibition and loss of viability. In *E. coli* it was shown clearly (Dubin et al., 1963) that marked changes in the permeability occur, which are associated with the extensive uptake of streptomycin. This altered permea-

bility may result in the death of the cell. However, much before such abnormal channels develop in the membrane, there is an initial escape of K^+ ions into the medium. This occurs with consentrations significantly lower than required for lethal action of the drug and constitutes one of the earliest effects in the sequence of changes brought about by streptomycin (Dubin et al., 1963). It was suggested (Dubin et al., 1963) that streptomycin absorbed to the surface of the cell causes local distortion in the growing membrane and allows K^+ efflux; extensive uptake of streptomycin, these authors suggested further, may therefore be an indirect result of the depletion of K^+, which appears to be accompanied by Mg^{++}. This hypothesis supports the notion that the membrane becomes permeable to streptomycin; in fact, the effect on protein synthesis, which starts much earlier than measurable damage to the membrane, readily explains the early penetration of at least a small amount of the streptomycin. It seems reasonable to conclude that streptomycin does at lease two things to the bacterial cell: (1) inhibits protein synthesis, and (2) affects permeability and membrane stability. It is not clear whether these are independent or dependent effects.

The question about what causes the lethal effect of streptomycin still remains undefined. The results from these studies on S. marcescens and those on E. coli (Hurwitz et al., 1962) provide leads to postulate that streptomycin ex-

erts its killing effect in sensitive bacteria by inhibiting protein synthesis at concentrations which do not cause gross breakdown of RNA, damage to membranes, or lysis of the cells. The onset of the killing effect in S. marcescens starts at external concentrations of approximately 10 ug/ml in the presence of about 10^{11} cell/ml, which is equivalent to about 10^5 molecules of streptomycin bound to each cell.

One interpretation of the above results is that inhibition of protein synthesis occurs when the concentration of streptomycin reaches approximately 10 molecules per ribosome, assuming there are 10^4 ribosomes per cell. With data from a cell-free system for protein synthesis, Flake et al. (1962) have estimated that 1-2 molecules of streptomycin per ribosome are sufficient to inhibit protein synthesis in sensitive cells of E. coli, ie, about 50% reduction in the incorporation. This means that, at an external concentration of 10^5 molecules of streptomycin per cell, it is relatively easy to saturate ribosomes with streptomycin.

The effect of streptomycin on protein synthesis in cell-free systems was shown by a variety of workers. With all possible combinations of supernatant, ribosomes, and ribosomal sub-units in E. coli, the streptomycin-sensitive site was shown to reside on the 30s component of sensitive ribosomes (Davies, 1964; Cox et al., 1964). The data presented in this thesis demonstrates that, as in E. coli, the sensitivity to strepto-

mycin in S. marcescens resides on the 30s component of ribosomes from sensitive cells (Table 8). Streptomycin did not affect poly-U-directed phenylalanine and poly-C-directed proline incorporation into protein when the system utilized ribosomes from resistant strains. The kinetics of streptomycin inhibition of protein synthesis in vitro reveals that a concentration as low as 5 ug per 0.5 ml of reaction mixture, containing approximately 400 ug of ribosomes, inhibits amino acid incorporation significantly. A slight stimulation was observed in poly-C-directed proline incorporation when the concentration was about 1 ug or lower. No attempts were made to study the inhibition at concentrations below 1 ug per reaction mixture. Such a stimulation of proline incorporation is not understood; results of Davies et al., (1965) also support this finding. Addition of streptomycin after adding the polynucleotide template reduces the inhibitory activity of streptomycin. The low inhibition suggests that, once the ribosomes-mRNA complex is formed, it becomes less susceptible to streptomycin action. The level of phenylalanine incorporation directed by poly-U is four to five times higher than that of poly-C-directed proline incorporation, and a difference is also observed in the levels of inhibition by streptomycin on these two systems.

The negative effect on protein synthesis of dissociating and reassociating ribosomes appears to be accentuated by

streptomycin. Phenylalanine incorporation by reassociated ribosomes was inhibited by streptomycin to the extent of about 65%, whereas inhibition in the presence of native ribosomes was never higher than 50%. It can be noted that a similar relationship exists in the absence of streptomycin, where reassociated ribosomes showed reduced incorporation of both amino acids. Repeated washings and prolonged storage exerted a similar effect of reducing ribosomal function.

Although streptomycin was shown to influence protein synthesis in cell-free extracts, the specific reaction that is affected is rather uncertain. Several workers have shown that the recognition process between codon and anticodon is affected by streptomycin (Pestka et al., 1965; Kaji and Kaji, 1965; Davies et al., 1964). Misrecognition leads, for example, to phenylalanine being substituted by isoleucine, leucine, or serine. Old and Gorini (1965) presented evidence that streptomycin-induced misreading results in the substitution of an amino acid in the place of the proper amino acid in the polypeptide chain. However, the data do not show strict stoichiometry between the losses and the gains in amino acid residues; there are fewer gains than losses. There is ample evidence that misreading by streptomycin also results in suppression of nonsense codons (Gorini and Beckwith, 1966). Direct evidence for suppression is scanty; however, there is indirect evidence suggesting an amino acid substitution caused by streptomycin.

The streptomycin-stimulated misreading appears to be dependent upon the concentration of the antibiotic. Pestka (1967) estimated that a concentration of approximately one molecule per ribosome is sufficient to produce misreading in vitro, and, in fact, it is maximum at a concentration of about 1-2 molecules.

At higher concentrations there is net inhibition of protein synthesis. It appears therefore that streptomycin action on protein synthesis is twofold: misreading and inhibition. These two actions of streptomycin are quite distinct and highly dependent upon concentration of the drug, even though they are demonstrable in the same extracts with the same template RNA and at the same Mg^{++} concentration (Schwartz, 1966). The precise way, in chemical terms, in which streptomycin induces the misreading or causes inhibition is what remains uncertain

Evidence presented in chapter 3 indicates cells grown in sub-lethal concentrations of streptomycin contain defective ribosomes that remain defective after isolation. These ribosomes, even after washing and dialysis, show consistently less incorporation of amino acid in vitro, in the absence of added streptomycin, than shown by native ribosomes. The fact that the reduced incorporation activity was associated with ribosomes and not supernatant was demonstrated by interchanging the ribosomes and supernatant fractions from streptomycin-grown and control cells. Two hypotheses can explain these

results: (1) the presence of streptomycin in the growth medium, in sub-lethal concentrations, induces defective synthesis of ribosomes and (2) a small but significant amount of streptomycin remains attached to ribosomes with forces that resist washing and dialysis.

The first hypothesis was tested by analyzing ribosomes for protein and RNA content, and by examining for the formation of abnormally-sedimenting particles. No gross difference in RNA and protein content was noted. The sedimentation pattern on a sucrose density gradient did not reveal any grossly abnormal ribosomal particle weight or density. With higher concentrations (25 ug/ml) Dubin (1964) reported slow-sedimenting ribosomes in E. coli. Such high concentrations also affected growth of the bacterium. However, in experiments in S. marcescens, 5 ug/ml of streptomycin did not affect growth or protein synthesis, neither did it stimulate RNA in measurable amounts. Therefore, formation of a gross structural change is ruled out by these results, however, a slight change, non-detectable by conventional methods of gradient analysis, is not excluded.

The second hypothesis could only be tested with labeled streptomycin. The non-availability of C^{14}-streptomycin of high specific activity was a serious limitation in testing this hypothesis. About 2 mg of labeled streptomycin was added to approximately 10 mg of ribosomes (approximately 100 molecules of streptomycin per ribosome). When the ribosomes were centrifuged on

a gradient to separate the 70s particles, a significant amount of activity remained associated with ribosomes, however, upon dialysis the activity was very close to background. Calculation, based on the particle weight of 70s ribosome as 2.6×10^6 indicates that before dialysis, the ratio of streptomycin molecules to ribosomes was 20:1. A recent report of Leon and Brock (1967) indicates that a low but significant activity (total counts 334/min) remain associated with the ribosomes even after there washings. Based on this, they calculated that at least one molecule remains firmly attached to ribosomes. If this were true, the reduced capacity of ribosomes to synthesize proteins can be explained on the basis that one molecule per ribosome can cause misreading. However, this reasoning fails to agree with the data, particularly when dialyzed ribosomal samples too had reduced incorporation activity. Also, the fact that streptomycin-grown ribosomes exhibit increased affinity for the drug itself (Figure 21) suggests that ribosomes have undergone some conformational changes which results in providing more sensitive sites for the attachment of streptomycin than on normal ribosomes.

The conclusion most consistent with this discussion and with the data on the thesis is that the presence of low concentrations of streptomycin induces the formation of structurally abnormal ribosomes and results in streptomycin binding to

ribosomes (reversibly, at least in part). These dual effects reduce the ability of the treated ribosomes to function in protein synthesis in vitro, in the absence of disolved streptomycin.

Chapter Five

SUMMARY

1) Polynucleotide-directed protein synthesis is demonstrated and characterized for S. marcescens. The basic requirements are the same as those described for E. coli. C^{14}-proline and C^{14}-phenylalanine were incorporated with a linear increase for the first 10 min before reaching the plateau. Proline incorporation was maximum at 35 C and decreased rapidly both at 27 C and 45 C. The optimum concentration of Mg^{++} was 10^{-2}M, which is also the optimum level for maintaining the integrity of 70s ribosomes. The native state of ribosomes seems to provide better conditions for in vitro protein synthesis than ribosomes reassociated from the dissociated 50s and 30s particles. The supernatant fraction loses its amino acid incorporating activity rapidly upon storage at freezing temperatures. Ribosomes, on the other hand, remained less susceptible to deterioration due to freezing.

2) Streptomycin inhibits both poly-U-directed phenylalanine and poly-C-directed proline incorporation into protein by cell-free extractions of streptomycin-sensitive cells, whereas extracts from resistant strain are relatively unaffected. The sensitivity to ribosomes resides on the 30s component of the ribosomes. Upon electrophoretic separation of ribosomal protein a marked difference was noted between streptomycin-sensitive and resistant ribosomal proteins.

3) The kinetics of streptomycin action on protein

synthesis was also studied in vivo. Streptomycin at 5 μg/ml does not inhibit the total protein synthesis or the growth of sensitive strain Nima. However, ribosomes isolated from cells grown in the presence of sub-lethal concentrations of streptomycin show partial inhibition of amino acid incorporation in vitro in the absence of added streptomycin. No gross structural differences are present in the sucrose density profiles of the ribosomes compared with those of streptomycin-free control cells. Also, experiments with C^{14}-streptomycin do not confirm that streptomycin is bound to ribosomes with sufficient strength to resist dialysis. It is possible at the same time that an extremely small amount of the antibiotic, approximately 1-2 molecules per ribosome, remains attached to ribosomes but could not be detected. At higher concentrations of streptomycin (10 μg/ml), a close correlation exists between loss of viable cells and slowing down of protein synthesis as measured by the incorporation of C^{14}-proline into protein in vivo. The killing effect at this concentration is not the result of extensive damage to the cell membrane.

Annexure I

GEL SYSTEMS FOR RIBOSOMAL PROTEINS

Electrophoresis at pH 4.5[a] for ribosomal proteins			Electrophoresis at pH 8.7[b]		
1. Stock Solutions[c]					
A. KOH, 1N,	24	ml	A. Tris[d]	6.05	g
Acetic acid,	8.6	ml	HCl, 1N,	8	ml
TEMED[d],	2.0	ml	TEMED,	0.09	ml
Urea,	24	g	Urea,	24	g
Water, to 50 ml			water, to 50 ml		
B. KOH, 1N,	24	ml	B. Tris,	1.50	g
Acetic acid,	1.44	ml	HCl, 1N,	12	ml
TEMED,	0.23	ml	Urea,	24	g
Urea,	24	g	water, to 50 ml		
Water, to 50 ml					
			C. Acrylamide,	7.5	g
C. Acrylamide (Eastman),	6.65	g	MBA,	0.2	g
MBA[d]	0.1	g	$K_3Fe(CN)_6$,	3.75	mg
Urea,	24	g	Urea,	24	g
Water, to 50 ml			Water, to 50 ml		
D. Acrylamide,	2.5	g	D. Acrylamide,	3.33	g
MBA,	0.625	g	MBA,	0.83	g
Urea,	24	g	Urea,	24	g
Water, to 50 ml			Water, to 50 ml		
E. Riboflavin,	1.0	mg	E. Riboflavin,	2.0	mg
Urea, 8M,	50	ml	Urea, 8M	50	ml

II. Other Reagents

1. Ammonium persulfate, made up fresh daily, 1.12% in 8M Urea	1. Ammonium persulfate, made up fresh daily, 0.56 % in 8M Urea
2. Buffer; stock soln., store at 3°	2. Buffer; stock soln., store at 3
β-Alanine 31.2 g	Tris, 6.0 g
Acetic acid 8.0 ml	Glycine, 28.8 g
Water, to 1 liter;	Water, to 1 liter;
adjust to pH 4.5	adjust to pH 8.2 - 8.4
3. Urea, 8M	3. DMAPN[d,e], 10% in water
	4. Urea, 8M

III. Gel Preparations

1. Lower Gel:	1. Lower Gel:
Mix: 1 part A	Mix: 3 part A
6 part C	4 part C
1 part Ammonium persulfate	1 part Ammonium persulfate
2. Upper Gels (spacer and sample):	2. Upper Gels (spacer and sample):
Mix: 1 part B	Mix: 2 parts B
4 part D	3 parts D
1 part E	1 part E
2 part Urea, 8M	2 parts Urea, 8M
(Add 1 ul DMAPN per ml of gel) Not needed	Add 1 ul DMAPN per ml of gel

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[a]Modified from R. A. Reisfeld, U. J. Lewis and D. E. Williams, Nature 195, 281 (1962

[b]Modified from B. J. Davis, reprint obtained from Distillation Products Ind., 1961

[c]Store in amber plastic bottles at 3°. Usually include a magnetic stirring bar in those stock solutions (C and D) which tend to crystallize out at 3°.

[d]Trus - trishydroxymethylaminomethane, (Fisher Reagent)
TEMED N,N,N',N' - tetramethylethylenediamine, (Eastman, Reagent grade)
MBA - N,N' -methylenebiscarylamide, (Eastman, Crystallized Reagent)
DMAPN - 3-Dimethylaminopropionitrile, (Eastman, Reagent grade)

[e]The DMAPN is added to facilitate polymerization of the upper gels, which is otherwise particularly difficult in the case of the pH 8.7 upper gel, perhaps because of omission of Temed from B.

[f]The % polymere in the gels as formulated are: lower gel, pH 4.5-10%; upper gel, pH 4.5-2.5%: lower gel, pH 8.7-7.5%; upper gel, pH 8.7-2.5%

PROCEDURES

The procedures, with some modification follow those of Reisfeld, et al., Nature 195, 281 (1962). The runs are made in glass tubes, 3.25 inches long and with an I. D. of 5 mm. The volumes of gel solutions for the various layers are: 1.0 ml for the lower gel layer, 0.1 ml for the spacer gel layer and 0.15 ml for the sample gel layer. Each gel layer is overlaid with water to hasten polymerization.

We generally run from 50 to 100 ug of ribosomal protein, generally in a volume of 10 to 20 ug. Considerably more protein can be run, although some of the closely running bands begin to run together and cannot be distinguished. Up to about 50 ul of soln. can be tolerated before the presence of LiCl begins to interfere with formation of the Kohlrausch boundary and resolution is lost.

The electrophoretic runs are carried out at 3° for optimum resolution. For the run at pH 4.5 the stock buffer is diluted with 4 parts of cold water, the run is carried out for 90 to 120 mins. with the cathode at the bottom and a current of 3 ma/tube. A trace of a 0.1% solution of pyronine red (National Aniline; Allied Chemical) is applied to the top of the sample gel after it has been polymerized to provide a tracking dye indicating the solvent front. A 90 to 120 min. run will move the dye band to the base of the tube. Shorter runs can be achieved with lower dilutions of the buffer but it is

not advisable to increase the current as heating will occur and lower the resolution. For the run at pH 8.7 the stock buffer is diluted with 1 part of cold water; the run is carried out as above with the anode at the bottom and bromphenol blue used as the tracking dye.

At the conclusion of the electrophoretic run the gel columsn are removed from the glass tubes by first rimming with water and are then stained for at least 1 hour by immersion in a 1% solution of Amido Schwarz in 7.5% acetic acid. Destaining is carried out over night at room temperature, at a current of 4 to 5 ma/tube with the anode at the bottom and 7.5% acetic acid in the buffer trays.

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