71-15,210

KULPA, Jr., Charles Frank, 1944-
CHARACTERIZATION OF THE ALTERATION IN RIBOSOMAL PROTEINS OF MESOPHILIC BACTERIA UPON CONVERSION TO PSYCHROPHILY.

The University of Michigan, Ph.D., 1970
Microbiology

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CHARACTERIZATION OF THE ALTERATION IN RIBOSOMAL PROTEINS OF MESOPHILIC BACTERIA UPON CONVERSION TO PSYCHROPHILY

by
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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology)
in The University of Michigan
1970

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ABSTRACT

CHARACTERIZATION OF THE ALTERATION
IN RIBOSOMAL PROTEINS OF MESOPHILIC BACTERIA
UPON CONVERSION TO PSYCHROPHILY

by Charles Frank Kulpa, Jr.

Chairman: Ronald H. Olsen

This investigation was undertaken to identify and characterize differences in the ribosomes of the mesophile, Pseudomonas aeruginosa, and several of its psychrophilic mutants and transductants. It was divided into three parts.

In the first part, certain physical characteristics of the ribosome were compared. Thermal denaturation points and stability in 8 M urea were used to estimate the relative degree of ribosomal RNA-protein interaction. These studies revealed that the psychrophilic mutants and transductants retained the characteristics of their mesophilic progenitors. Furthermore, when the ribosomal RNA base composition was analyzed by chromatography, psychrophilic mutants and transductants did not resemble wild type psychrophiles in their ribosomal RNA base composition, but were similar to wild type mesophiles.

In the second part, polyacrylamide gels were used to compare the 70S proteins of a psychrophilic transductant and its genetic donor and recipient. Also a psychrophilic

mutant and its wild type were compared as to their 70S, 50S and 30S ribosomal proteins. Comparison of the transductant and its progenitors indicated that there were at least 6 altered protein in the genetic recipient coincident with the transduction of psychrophily. In addition, comparison of the psychrophilic mutant ribosomal protein with its mesophilic progenitor showed that there were at least 10 altered proteins. Five of these protein differences were in the 50S subunits, and the other 5 were in the 30S subunits.

In the third part, the <u>in vitro</u> protein synthesis activity of wild type and psychrophilic mutant ribosomes was compared at 25 C and 3.5 C. These experiments revealed that the 50S subunit of the wild type was limiting to protein synthesis at 3.5 C. Furthermore, this limitation was overcome by the mutation to psychrophily.

In summary, comparison of certain physical properties of the ribosomes indicated that differences in these properties between wild type psychrophiles and mesophiles may be trivial to the determination of the growth temperature of these organisms. However, ribosomal protein differences between psychrophilic mutants and transductants, and their mesophilic progenitors indicated that ribosomal protein composition may be a factor in the determination of the minimum growth temperature of a bacterium.

Further experimentation revealed that the 50S ribosome of the psychrophilic mutant did not limit protein synthesis at 3.5 C as did the wild type 50S subunit. This implied a relationship between the ribosomal protein changes in the psychrophilic mutant and altered 50S ribosome activity coincident with a lowering of growth temperature range.

These results were discussed with regard to (a) the nature of the genetic change that had occurred to produce the psychrophilic mutants and transductants, and (b) the involvement of the ribosomal proteins and ribosomal subunits in the determination of the growth temperature range of a bacterium.

ACKNOWLEDGMENTS

I wish to thank:

Dr. Ronald H. Olsen for his time, patience, guidance and support of this research,

The members of my committee for their comments and discussions during the preparation of this dissertation,

The National Institutes of Health, General Medical Division, for financial assistance in the form of a predoctoral research fellowship,

My wife, Nancy, for encouragement, patience and sacrifices over the years of this study, also for her invaluable typing during the preparation of this dissertation.

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ABBREVIATIONS

O.D.	absorbancy of cells at 425 nm
RNA	ribonucleic acid
ATP	adenosine-5'-triphosphate
GTP	guanosine-5'-triphosphate
DNA	desoxyribonucleic acid
TCA	trichloroacetic acid
A_{260}	absorbancy at 260 nm
poly U	polyuridylic acid
RNase	ribonuclease
DNase	desoxyribonuclease
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
TGEB	tryptone glucose yeast extract broth
TSB	trypticase soy broth
G-C	guanine-cytosine
70S	ribosomal monomer characterized by 70S sedimentation coefficient
50S	ribosomal subunit characterized by 50S sedimentation coefficient
30S	ribosomal subunit characterized by 30S sedimentation coefficient

INTRODUCTION

Bacteria are capable of growing over a wide range of temperatures. However, individual species possess different maximum and minimum growth temperatures. Bacteria can be divided into three groups based on their maximum and minimum growth temperature. The first of these, the psychrophiles, will grow from as low as -7.5 C to 32 C (42). Ingraham (42) defines psychrophiles as being able to produce good growth at 0 C in 2 weeks. A second group, the mesophiles, grow from 8 C to 45 C. The mesophilic bacteria are the most intensively studied and characterized group of bacteria. The final group, the thermophiles, are capable of growing from 55 C to as high as 99 C under special conditions (9).

The mechanism(s) responsible for growth temperature range limits has been studied in recent years (15, 41, 42, 95). However, the nature of the factors responsible for the minimum and maximum temperatures of growth have not been clarified. Many enzymes from the various groups have been studied, particularly those of psychrophiles (95). The study of these enzymes has revealed that some enzymes are thermo-sensitive, but the majority are stable at temperatures far above the maximum growth temperature. Psychrophiles, when incubated at temperatures above their maximum for growth,

show leakage of cellular constituents (45). However, the thermo-sensitivity of the enzymes and cell permeability changes may be a result rather than the cause of the growth temperature limitation.

A step in protein synthesis, amino-acylation of tRNA, has been described as the molecular determinant of the maximum temperature of growth for the obligate psychrophile, Micrococcus cryophilus (53).

If the incubation temperature of bacteria is lowered below the minimum growth temperature gradually, to prevent "cold shock" (81), bacteria can survive without growth for long periods of time. In Escherichia coli the synthesis of protein, RNA and DNA eventually ceases when the cells are incubated at temperatures lower than 8 C (13). This cessation of protein synthesis at low temperature could be limiting the synthesis of other macromolecules (13). Therefore some aspect of protein synthesis could be responsible for the limitation of cell growth below the minimum temperature for growth.

The particular aspect of temperature effects on bacterial growth in which I am interested is the molecular determinant of the minimum temperature of growth. More specifically it is the characteristic in mesophiles that does not allow them to grow below 8-11 C. Das and Goldstein (13) proposed that at low temperatures the ribosomes of E. coli were not able to re-initiate protein synthesis upon release of the peptide chain at the end of the mRNA. More

recently (21) the size of the cellular pools of 50S and 30S ribosomal subunits *in vivo* was shown to increase when *E. coli* was incubated below 8 C. However, neither of these two studies showed specifically that the ribosome was the site of a block in protein synthesis at low temperature. Krajewska and Szer (47) and Greenwald and Szer (29) using an *E. coli* and psychrophilic *Pseudomonas* *in vitro* system to synthesize polyphenylalanine, showed that the supernatant enzymes and the *E. coli* 30S ribosomes functioned at 2 C, while the 50S ribosomes from *E. coli* were not functional. Therefore, the ribosome appears to be involved as a minimum growth temperature determinant in *E. coli*. No direct comparison of the difference between mesophilic and psychrophilic ribosomes was possible until the psychrophilic mutants and transductants of Olsen (71) became available. These organisms provided an opportunity to compare the ribosomes of a wild type mesophilic *Pseudomonas* with those from mutants and transductants possessing a lowered minimum and maximum growth temperature.

The objective of this investigation was, therefore, to study the ribosomes of wild type, mutant and transductant bacteria for structural or functional alterations which might be correlated with the different growth temperature ranges of the organisms.

The most significant results of this investigation were:

1. The electrophoretic mobilities of a number of 50S and 30S proteins from mutant ribosomes and 70S proteins from transductant ribosomes were found to be altered when compared with wild type ribosomal proteins.
2. In vitro studies showed that limitation of protein synthesis at low temperature, attributable to the 50S subunit, did not occur when 50S subunits of mutant cells were used.

HISTORICAL

Investigations of factors involved in the determination of the maximum and minimum growth temperatures of bacteria have been numerous and in many cases unrelated. Therefore, a number of different approaches to the study of growth temperature range contingencies for mesophiles and psychrophiles will be reviewed. Studies with thermophiles will not be described except when they are pertinent. Reviews by Gaughran (24), Koffler (46), Campbell (11) and Friedman (19) cover the topic of thermophiles. Though this investigation deals primarily with the minimum temperature of growth in mesophiles and psychrophiles, studies involving the maximum temperature of growth in psychrophiles will be included.

Early Studies

Forster (18) in 1887 reported the isolation of bacteria that would grow at 0 C. One year later, in 1888, Miquel (59) isolated bacteria that grew at high temperature. Schmidt-Nielson (84) in 1908 called bacteria that grew at 0 C psychrophiles. That same year Barber (5) studied the growth rate of E. coli at various temperatures. He discovered that there were finite maximum and minimum growth temperatures. Though growth at extreme temperatures by some

bacteria and a limited growth temperature range for mesophiles was known, little if any research was undertaken in the early years to analyze the cause of these growth temperature range differences. As Berry and Magoon (8) pointed out in 1933, the majority of the early investigations concerning psychrophiles were concerned with their isolation and taxonomic classification. Most of the psychrophilic bacteria that were isolated and identified were Pseudomonas species (42). It was not until the late 1950's that studies were undertaken to explain the difference in growth temperature range between mesophiles and psychrophiles.

Studies of Growth Characteristics and Enzymic Processes of Mesophiles and Psychrophiles

Brown (10) in 1957 studied the effect of temperature on the growth and utilization of glucose in psychrophilic and mesophilic pseudomonads. He found that there was no difference in growth requirements but that the psychrophiles exhibited greater oxidative activity at low temperatures. He suggested, therefore, that a study of the enzymes of these organisms might elucidate differences between mesophiles and psychrophiles. Ingraham (39) in 1958 studied the growth of psychrophiles, applying the Arrhenius equation and μ as the temperature characteristic of growth. He stated that the minimum and maximum temperatures of growth may not be related. Ingraham and Bailey (40) in 1959 compared the effect of temperature on the metabolism of

mesophiles and psychrophiles. They felt, after a study of intact and ruptured cells, that the differences between mesophiles and psychrophiles were dependent on structural integrity of cellular components rather than on enzymatic differences. Sultzer (98) in 1961 compared the oxidative activity of mesophiles and psychrophiles on saturated fatty acids. He concluded that there was no relationship between maximum growth temperature and oxidative capabilities. However, the psychrophilic enzymes did appear to be less sensitive to lower temperatures than the mesophiles. Ng, Ingraham and Marr (64) in 1962 studied the effect of a shift to lower incubation temperature on the growth of E. coli. They found cellular damage and depression of enzymes. However, it appears that they were observing "cold shock", since the lowest temperature they studied was 10 C, and this would still allow the growth of the organism.

Hagen and Rose (31) in 1962 studied the biochemical basis for the low maximum temperature of growth in a psychrophilic yeast. They found that the cells could not produce adequate amounts of amino acids at supramaximal temperatures. Their studies indicated an impairment of Krebs cycle enzymes.

Baxter and Gibbons (6) in 1962 studied the minimum growth temperature determinant in yeasts. They concluded that even though the enzymes for glucose oxidation in mesophiles would not function at low temperature, the

minimum temperature determinant in yeasts may be in the sugar transport mechanism. This limitation could come about through physical changes in the lipids of the mesophile at low temperature.

Olsen and Jezeski (70) in 1963 studied the effect of growth medium on growth rate at various temperatures with Pseudomonas fluorescens. They used temperature coefficients (Q_{10} values) to show that the effect of temperature on growth rate was influenced by carbon source and aeration.

In 1964, Marr, Ingraham, and Squires (57) studied the effect of low temperature on the synthesis of beta-galactosidase in E. coli. They found a progressive decline in the rate of synthesis with temperature. This was attributed to two things, either an increased catabolite repression at low temperature or a temperature effect on the rate of transcription.

Rose and Evison (79) in 1965 analyzed the minimum temperature of growth using psychrophilic and mesophilic Corynebacterium species. They found that a defect in sugar transport may be partially responsible for the minimum temperature of growth. However, their studies did not rule out the possibility of a block in the synthesis of some essential compound at low temperature. Shaw and Ingraham (88) studied the relationship of minimum growth temperature to the fatty acid composition of E. coli. They found no direct relationship between them. Studies have been carried

out with marine psychrophiles, as well as those with terrestrial bacteria considered here. These are detailed in a review by Morita (60).

Shaw (85) in 1967 studied the temperature characteristic (μ) of psychrophilic and mesophilic yeasts. He found no difference between their μ values. This did not agree with the early studies by Ingraham (39) utilizing psychrophilic and mesophilic bacteria. In 1968, Hanus and Morita (32) using marine bacteria also found no differences between mesophiles and psychrophiles with regards to their temperature characteristics.

Stokes in 1967 (95) summarized the theories concerning enzymes and cell membranes and their relationship to the ability of psychrophiles to grow at low temperature. First, enzymes and enzyme-forming systems are able to function at low temperature and are perhaps simultaneously susceptible to heat inactivation. Second, the psychrophile's cell membrane allows the transport of substrates at low temperature. He stated that the enzyme theory was most plausible. Purchit and Stokes (74) in 1967 added more evidence to the involvement of enzymes as a temperature determinant. They showed that at temperatures above the maximum the oxidative and fermentative enzymes of a psychrophilic bacterium were destroyed. They found this to be true in both intact and ruptured cells. This was in contradiction to the work of Ingraham and Bailey (40) in 1959. Therefore, possibly it was not only structural

integrity of the total cell but also the enzyme components that were limiting growth.

In 1968, Harder and Veldkamp (34) studied the physiology of a psychrophilic Pseudomonas. They eliminated respiratory enzymes as the maximum temperature determinant. They showed the existence of a defect in protein and RNA synthesis at supramaximal temperatures. Stokes and Larkin (96) in 1968 compared the oxidative capabilities of whole and disrupted psychrophilic and mesophilic Bacillus species at various temperatures. They found that the psychrophile functioned better at low temperature than the mesophile. They also found no difference between cell free extracts and intact cells. Farrell and Rose (16) in 1968 compared the effect of cold shock on a mesophilic and a psychrophilic pseudomonad. The mesophile was rapidly killed under certain ionic conditions whereas the psychrophile was not. This seemed to indicate some effect upon the membrane of the mesophile causing loss of viability. Kenis and Morita (45) showed in 1968 that increases in temperature caused leakage of cellular constituents and a loss of viability with a psychrophilic marine bacterium. They also suggested the possibility that there may be temperature-sensitive synthetic mechanisms.

Quist and Stokes (75) in 1969 observed differences in the induction of formic hydrogenlyase when comparing mesophiles and psychrophiles. Differences in the temperatures for optimal enzyme activity and optimal enzyme synthesis were

also observed. Their results indicated that the temperature characteristics of enzyme synthesis and activity were important determinants for psychrophilic and mesophilic growth.

Therefore, the comparison of metabolic processes of psychrophiles and mesophiles leads to the theory that the synthesis and activity of enzymes within the cell are directly involved in the determination of the growth temperature range of a bacterium. Membrane permeability changes and transport limitations do not appear to be the cause of growth temperature limits.

Mutations for the Study of Growth Temperature Range

Temperature-sensitive (ts) mutations which affect the maximum temperature of growth have been known for a number of years. However, since these have dealt primarily with the maximum temperature of growth in mesophiles, they will not be dealt with here. We will consider these mutations that involve the minimum temperature of growth in mesophiles and psychrophiles. The first mutation affecting the minimum temperature of growth in mesophiles was reported in 1962 by Azuma, Newton and Witter (4). They reported lowering the minimum temperature of growth of *Pseudomonas aeruginosa* to that of psychrophiles. However, they did not say if they also lowered the maximum growth temperature, and no further studies have been conducted with these mutants.

O'Donovan and Ingraham (68) in 1965 reported mutants of E. coli with elevated minimum temperatures of growth. They suggested that these be called "cold-sensitive" (cs). In a subsequent report, O'Donovan, Kearney and Ingraham (69) described three classes of cold-sensitive mutants. These mutants were nutritional mutants requiring a specific nutrient to grow at low temperature. They appeared to have a block in regulation of enzyme function at low temperature.

In 1967, Condon and Ingraham (12) reported a cold-sensitive mutation in Pseudomonas putida that affected the synthesis of an enzyme necessary for the utilization of mandelate as a carbon source. They proposed that the minimum temperature of growth may be determined by factors that regulate the synthesis and activity of enzymes at normal temperatures.

Ingraham and Maaløe (41) in 1967 discussed the relationship of cold-sensitive mutants to the study of the minimum temperature of growth. They noted that mutants of E. coli had not been isolated with a lowered minimum growth temperature and that possibly they could not be. They stated that the cause of the limitation of growth at low temperature may be a metabolic block that cannot be overcome by providing small molecules in the medium. This meant that the wild type mesophiles may cease to grow at low temperature because of the excessive sensitivity of one or more control mechanisms (primarily enzyme control) which

cannot be overcome by supplementing the media with effectors.

Olsen and Metcalf (71) in 1968 reported production of mutants and transductants of P. aeruginosa which had their minimum growth temperature changed from 11 C to 0 C and their maximum growth temperature lowered from 44 C to 32 C. The mutants were produced by ultraviolet irradiation at 99% kill with a frequency of 1×10^{-8}. The transductants were produced using a Pseudomonas generalized transducing phage. Psychrophily cotransduced 50% of the time with tryptophan independence. The mutants and transductants no longer retained the phage sensitivity of their progenitor in most cases. They were, in most cases, pigmented differently than their progenitors.

Squires and Ingraham (92) in 1969 reported cs mutants of E. coli which required lactose for growth at low temperature. They stated that this was due to the production of a heat-sensitive galactokinase that was non-functional at permissive temperatures and that the cold-sensitivity appeared to involve the repression of enzyme synthesis by the buildup of toxic product. Tai, Kessler and Ingraham (101) also reported a mutation in Salmonella typhimurium that resulted in cold-sensitivity of the synthesis of ribosomes. This mutation resulted in the accumulation of RNA and altered ribosomal profiles at low temperature. The mutation also co-transduced with streptomycin resistance. They felt that a high proportion of mutants exhibiting

the cs phenotype may be mutations affecting the synthesis or regulation of ribosomes. Guthrie, Nashimoto and Nomura (30) also reported a similar mutation in E. coli. Their mutants accumulated ribosomal precursors at low temperature indicating a block in the assembly of ribosomes.

Another mutant was isolated separately by Tai and Jackson (100) and by Malcolm (55). They used the obligate psychrophile, Micrococcus cryophilus. They were able to raise the maximum growth temperature of this psychrophile by mutagenesis. This mutation allows a definitive study of maximum growth temperature, since the limitation of growth at high temperatures in the wild type was characterized previously (54).

Finally, this year, 1970, Hoffmann and Ingraham (37) have reported a mutant of S. typhimurium that requires tryptophan for growth at 20 C. There appears to be a reversible inactivation of an enzyme essential in trytophan biosynthesis at low temperature.

Relationship of Macromolecular Synthesis to Growth Temperature Range

The study of macromolecular synthesis as a possible factor in the determination of growth temperature range appeared to be necessary after the enzyme and mutant studies. It seemed that the synthesis and regulation of enzymes was a determining factor in growth temperature range. The study of macromolecular synthesis has involved primarily in vivo analysis of DNA, RNA and protein. However, there

have been a few studies concerning the structure and function of ribosomes, and their relationship to the growth temperature range of bacteria.

Strange and Shon (97) in 1964 studied the effect of thermal stress on the ribonucleic acid composition of Aerobacter aerogenes. Their studies suggested a relationship between rate of death and RNA degradation.

Goldstein, Goldstein and Lowney in 1964 (28) studied in vivo the protein-synthesizing capabilities of E. coli at 0 C. Protein synthesis ceased after a few hours at 0 C. Their results implied that the formation of polyribosomes on messenger RNA (mRNA) was defective at 0 C. They suggested that the ribosome may be involved in the determination of the minimum temperature of growth. Possibly there was a defect in ribosomal activity(s) at low temperature which could then limit the synthesis of enzymes at low temperature.

In 1966, Saunders and Campbell (82) compared ribosomes of E. coli and Bacillus stearothermophilus. They found that the ribosomes of the thermophile had a higher melting temperature than the mesophile, but there were no differences in the amino acid composition of the ribosomal proteins. Therefore, this difference may have been in the organization of ribosomal proteins as influenced by rRNA.

Pace and Campbell (73) in 1967 correlated thermal stability of ribosomes and the maximum growth temperature of a number of psychrophiles, mesophiles and thermophiles.

They proposed that the thermal stability of ribosomes was due to the interaction of ribosomal protein and rRNA. They also found a correlation between increasing guanine-cytosine (G-C) content of rRNA and the maximum growth temperature. Stenesh and Yang (94) and Stenesh and Holazo (93) found essentially the same results when comparing the rRNA and ribosomes from mesophilic and thermophilic Bacillus species. Friedman, Axel and Weinstein (20) compared the capabilities for poly U-directed protein synthesis after heating of E. coli and B. stearothermophilus ribosomes. They found that there was much less inactivation of thermophile ribosomes by heating at 65 C than there was for mesophile ribosomes.

Harder and Veldkamp (33) studied a psychrophilic marine Pseudomonas. They found that rRNA synthesis appeared to be temperature dependent.

Shaw and Ingraham (88) in 1967 studied the synthesis of macromolecules in E. coli after a shift from 37 C to 10 C. They found an inhibition of macromolecular synthesis but respiration and permeability were not affected. After 4-8 hours macromolecular synthesis began again. There did not appear to be any relationship between the lag and the minimum growth temperature determinant. However, it must be noted that 10 C was still above the minimum temperature of growth of 8 C for E. coli.

Krajewska and Szer (47) in 1967 compared at various temperatures the in vitro protein synthesis capabilities of

psychrophilic *Pseudomonas* ribosomes with those from *E. coli*. They found that at 25 C there was no apparent difference in the capabilities of both species of ribosomes and that all components were interchangeable. However, at 2 C the *E. coli* system would not function even though the soluble fraction was shown to be functional at low temperature. They found that the psychrophilic ribosomes when combined with the mesophilic soluble fraction were completely functional at 2 C. Therefore it appeared that the block in protein synthesis observed by Goldstein *et al* (28) did involve the ribosomes of the mesophile.

Das and Goldstein (13) in 1968 studied further the cessation of protein synthesis at 0 C in *E. coli*. Protein synthesis ceased after about 4 hours at 0 C. However, it resumed immediately upon shifting to 37 C. There appeared to be a dispersion of polysomes at 0 C. They also found that *E. coli* ribosomes could be activated for *in vitro* protein synthesis at low temperature by previous incubation at 37 C. Their data suggested that the ribosomes were reversibly blocked in the initiation of new protein synthesis at 0 C. Shaw (86) in 1968 studied DNA, RNA, protein synthesis and cell division in *E. coli* at 6 C. He found that all macromolecular synthesis eventually ceased at 6 C, but that the cells could not divide before cessation of synthesis. Therefore, he concluded that shifting the cells to temperatures below their minimum either directly or indirectly disrupted the mechanism controlling cell division. Malcolm (52)

in 1968 studied the synthesis of protein and RNA in the psychrophile, M. cryophilus, at normal and restrictive temperatures. He found that, as protein synthesis ceased, the cells lost viability. He has since characterized the defect in protein synthesis at supramaximal temperatures to be a temperature-sensitive step in the attachment of amino acids to transfer RNA (53, 54, 55).

Friedman, Lu and Rich (21) in 1969 showed that the in vivo ribosomal subunit pools of E. coli increased with a corresponding decrease in polyribosomes when the cells were held at temperatures below the minimum for growth. This further substantiated the results of Goldstein et al (28) and Das and Goldstein (13) that the ribosomes did not appear to be able to reinitiate protein synthesis at temperatures below the minimum for growth. Greenwald and Szer (29) in 1969 reported that the defect in in vitro protein synthesis in E. coli at low temperature resided in the 50S ribosome. They showed that if they added an E. coli 30S ribosome and a psychrophile 50S ribosome, they functioned well in in vitro protein synthesis at low temperature, whereas the mesophilic 50S inhibited the reaction at low temperature. Nash, Grant and Sinclair (62) in 1969 studied the thermolability of protein synthesis in an obligately psychrophilic yeast. They found temperature-sensitive aminoacyl-tRNA synthetases and a possible sensitivity of peptide elongation factors on the ribosomes. In a later report, Nash and Grant (63) compared the ribosomes of mesophilic and psychrophilic yeasts. They found greater

thermal stability for the mesophilic ribosomes than for the psychrophilic ribosomes.

Ansley, Campbell and Sypherd (1) in 1969 found no unusual chemical differences between ribosomal proteins of E. coli and B. stearothermophilus that could account for the increased heat stability of the thermophilic ribosomes. They concluded, therefore, that the structural arrangements within the ribosomes were the important factors.

Using tissues from higher organisms, Haschemeyer (36) studied the low temperature acclimation of protein synthesis in toad fish livers. When toad fish liver was exposed to low temperature for an interval of time an increased rate of protein synthesis resulted. Haschemeyer eliminated the possibility that stimulation of RNA synthesis and the formation of mRNA-ribosome complexes was a cause of this phenomenon. However, she stated that a step in the peptide elongation reaction on the ribosome may be rate limiting and that the rate of this step was increased after exposure to low temperatures. Therefore, it appears that the ribosomes may be involved as one of the factors in determining the minimum and maximum growth temperatures of not only bacteria, but also higher organisms.

Polyacrylamide Gel Analysis of Ribosomal Proteins

The efficacy of polyacrylamide gel electrophoresis for the analysis of basic proteins was demonstrated in 1962 by Reisfeld, Lewis and Williams (78). Though polyacrylamide

had been in use for a number of years (25), the technique had not been applied to the analysis of basic proteins prior to this. In 1961, Waller and Harris (104) analyzed the ribosomes of E. coli on starch gel, and their procedure for isolating the proteins was used for many years with acrylamide gels. In 1964, Leboy, Cox and Flaks (50) described the use of LiCl-urea to extract ribosomal proteins and the technique for running two samples for comparison on one gel. This was a significant advance since protein samples do not always give the same pattern on single gels. In 1968, Fogel and Sypherd (17) reported a new technique for the preparation of ribosomal protein using 2-chloroethanol. This technique was advantageous since small amounts of ribosomes could be used; however it was time consuming. In 1969 Hardy, Kurland, Voynow and Mora (35) reported an adaptation of the procedure of Waller and Harris (104) which gave very low levels of contaminating rRNA and was especially good for the isolation of proteins from 30S ribosomal subunits. The gel technique has been retained with very few modifications since it was reported by Reisfeld et al (78) for basic proteins. Gel electrophoresis has been used to analyze ribosomal proteins from a variety of sources such as plants (43), bacteria (17) and yeasts (83).

In Vitro Protein Synthesis with Pseudomonas

Since Nirenberg (65) detailed his cell-free, protein-synthesizing system directed by either natural mRNA or synthetic messengers in 1963, many studies have been carried out to elucidate the function of the ribosome in protein synthesis. Spirin and Gavrilova (91) detail in depth the results of the analysis of the function of the ribosome in protein synthesis. However, the majority, if not all, of the studies were done with E. coli. Krajewska and Szer in 1966 (47) were the first to characterize an in vitro protein-synthesizing system for a Pseudomonas species. They described in detail the requirements for in vitro protein synthesis at various temperatures by components from a psychrophilic Pseudomonas species. Migita and Doi (58) in in 1970 reported the use of Pseudomonas extracts to make formylmethionyl-tRNA; however they use an unfractionated, cell-free supernatant for their system.

MATERIALS AND METHODS

Reagents

All inorganic chemicals were either Baker Analyzed Reagent grade or Mallinckrodt Analyzed Reagent grade.

Tryptone, yeast extract and Bacto-agar were obtained from Difco Laboratories, Detroit, Michigan. Glucose (anhydrous) was Baker Analyzed Reagent grade. Trypticase soy broth was obtained from Baltimore Biological Laboratories, Baltimore, Maryland.

Absolute isopropanol and toluene were Baker Analyzed Reagent grade.

Tris-(hydroxymethyl)-aminomethane (Sigma 7-9) was obtained from Sigma Chemical Company, St. Louis, Missouri.

Sucrose was Mallinckrodt Analytical Reagent grade.

Acrylamide, 2-mercaptoethanol, beta-alanine, N, N', methylene-bisacrylamide and 2-chloroethanol were the highest purity attainable from Eastman Organic Chemicals, Rochester, New York.

N, N, N', and N'', -tetramethylethylenediamine was obtained from Matheson, Coleman and Bell, Cincinnati, Ohio.

Folin-Ciocalteau Reagent for Lowry protein determination was from E. H. Sargent and Company, Chicago, Illinois.

3,5-dihydroxytoluene (orcinol) for RNA determinations and desoxyribonuclease (crystalline) were

obtained from Nutritional Biochemicals Company, Cleveland, Ohio.

The disodium tetrahydrate of creatine phosphate, creatine kinase, disodium trihydrate of adenosine-5'-triphosphate, trilithium tetrahydrate of guanosine-5'-triphosphate, potassium salt of polyuridylic acid and L-phenylalanine were obtained as A grade from Calbiochem, Los Angles, California.

L-^{14}C-phenylalanine, specific activity 455 mc/mmole, was obtained from Schwartz Bioresearch Inc., Orangeburg, New York.

Buffalo Black NBR was obtained from Allied Chemical, Morristown, New Jersey.

Bovine albumin powder, Fraction V, and yeast ribose nucleic acid, reagent grade were obtained from Nutritional Biochemicals Company, Cleveland, Ohio.

2,5-diphenyloxazole (PPO) and 1,4-bis-(2-(5-phenyloxazoyl))-benzene (POPOP) were obtained in Scintillation Grade from Packard Instrument Company, Inc., Downers Grove, Illinois.

Media

The media used for the growth of Pseudomonas species and Escherichia coli was 0.5% tryptone, 0.25% yeast extract and 0.1% glucose (TGEB). When TGEB was used in fermenters, $NaNO_3$ was added to 0.4%. The Micrococcus species were grown on trypticase boy broth (TSB) prepared as directed by the

manufacturer. Whenever solid media was used, Bacto-agar was added to 1.5%.

Buffers

The buffers described here were used in the preparation of ribosomes. The pH was determined using a Beckman pH meter with expanded scale. After preparation the buffers were stored in the cold to prevent contamination.

1. Phosphate Magnesium (PM)

   0.01 M phosphate pH 7.0
   0.01 M Mg-Acetate

2. Tris Magnesium Ammonium Chloride 2-mercaptoethanol (TMNB)

   0.01 M Tris pH 7.8
   0.01 M $MgCl_2$
   0.06 M NH_4Cl
   0.006 M 2-mercaptoethanol

3. Tris Ammonium Chloride 2-mercaptoethanol (TNB)

   0.01 M Tris pH 7.8
   0.0001 M $MgCl_2$
   0.06 M NH_4Cl
   0.006 M 2-mercaptoethanol

Organisms

The following organisms were obtained from the University of Michigan culture collection, *Micrococcus cryophilus* and *Micrococcus lysodeikticus*. Dr. Ronald Olsen, Department of Microbiology, University of Michigan, provided *Pseudomonas aeruginosa* strains PT0629 and PAT38, *Pseudomonas fluorescens* strains PF012 and PF014, *Pseudomonas geniculata* PG050, *Pseudomonas putida* A.3.12.

PTOPM22 and PTOPM23 were psychrophilic U.V. mutants of PT0629 (71). PFT293 and PFT300 were psychrophilic transductants of PAT38 prepared using Pseudomonas phage PX4 with PF014 as genetic donor (71).

The mutants and transductants used in this study were selected on the basis of growth rate at 3.5 C. A number of mutants and transductants were streaked on TGEB agar plates (TGEA) and incubated at 3.5 C for 4-7 days and earliest visible growth was scored. Wild type psychrophiles were streaked as a measure of normal growth at 3.5 C. The mutants, PTOPM22 and PTOPM23, that were selected showed visible growth about the same time as the wild type psychrophiles, 4 days. PFT293 and PFT300 were selected from the group of transductants. Though they showed the earliest growth among the transductants they were 1-2 days slower than the mutants.

Micrococcus freudenreichii and Micrococcus sodonensis were gifts from Dr. I. J. McDonald, Division of Bioscience, Natural Resources Council, Ottawa, Canada, and Dr. J. J. Perry, North Carolina State University, Raleigh, North Carolina, respectively. Escherichia coli D_{10} was a gift of Dr. Theophil Staehelin, Department of Zoology, University of Michigan.

Analytical Methods

The protein concentrations were determined by the method of Lowry (51) using bovine albumin powder (Fraction

V) as standard. RNA was determined chemically by orcinol (61) using yeast ribose nucleic acid as standard. The absorbance at 260 and 280 nm was determined using a Beckman DU spectrophotometer. Cell absorbancies (O.D.) were determined at 425 nm using a Spectronic 20.

Growth of Organisms

Cultures were grown in two ways, either in 1 liter batches in Fernbach flasks or 7 and 15 liter fermenters. The 1 liter batches were grown 16-18 hours at 25 C with aeration in a New Brunswick Psychrotherm to an absorbance of 0.6-2.0 depending on the concentration or age of cells desired. Growth in fermenters was done only for PT0629, PTOM22 and E. coli D_{10}. The growth in fermenters was at room temperature (23-26 C) for 18-20 hours with forced aeration by bubbling air through sterile glass wool into the fermenter. Agitation was provided by vibrating baffles.

Harvest of Cells and Preparation of Ribosomes

Cells were harvested and ribosomes were prepared in two ways depending on their use. Ribosomes to be used in urea studies, thermal denaturation, ribosomal RNA preparation and 70S ribosomal protein analysis were prepared by the first method. This was similar to that of Tissieres (103). The ribosomes used for in vitro protein synthesis and ribosomal subunit protein analysis were prepared by the second method. The following flow sheets describe the two methods of ribosome preparation.

A. Preparation of Ribosomes with PM Buffer

Cells (O.D. 0.6-2.0) (all steps done at 4-5 C.)

pour over crushed ice 40 g/200 ml cells

centrifuge 8,000 x g for 20 minutes

Cells

cells resuspended in 1/3 vol. PM and recentrifuged

Washed cells

resuspended in 20 ml PM

sonicate in Raytheon 10 kc sonicator at maximum output for 5-20 minutes depending on the cells used (Sonication chamber cooled by water at 5-7 C)

Lysed cells

centrifuge at 10,000 x g for 10 minutes at 5 C

discard pellet

centrifuge at 30,000 x g for 10 minutes at 5 C

discard pellet

S-30

centrifuge at 105,000 x g for 2 hours in 30 rotor in Beckman Model L preparative ultracentrifuge at 5 C

discard supernate

P-105 (ribosomal pellet)

resuspend pellet in 30 ml PM and recentrifuge at 105,000 x g for 2 hours at 5 C

discard supernate

P-105

Ribosomes are resuspended at 10-100A_{260} units/ml in PM. This constitutes the working ribosomal solution. An A_{260} unit is the quantity of ribosomes that has an absorbance of 1 at 260 nm.

Ribosomes prepared in this manner have a 260/280 nm absorbancy ratio of 1.75-1.90. The RNA/protein ratio was 1.40-1.60. Sucrose density gradient analysis of these ribosomes in 5-20% sucrose gradients in SW40 Ti rotor at 30,000 rpm for 2 hours in a Beckman L2-65B ultracentrifuge showed a single peak in the 70S region.

B. Preparation of Ribosomes for In Vitro Protein Synthesis and Subunit Protein Analysis.

Cells (O.D. 0.5-1.0)

centrifuge at 8,000 x g for 20 minutes at room temperature

Pellet

resuspend in 100-200 ml 0.15M NaCl

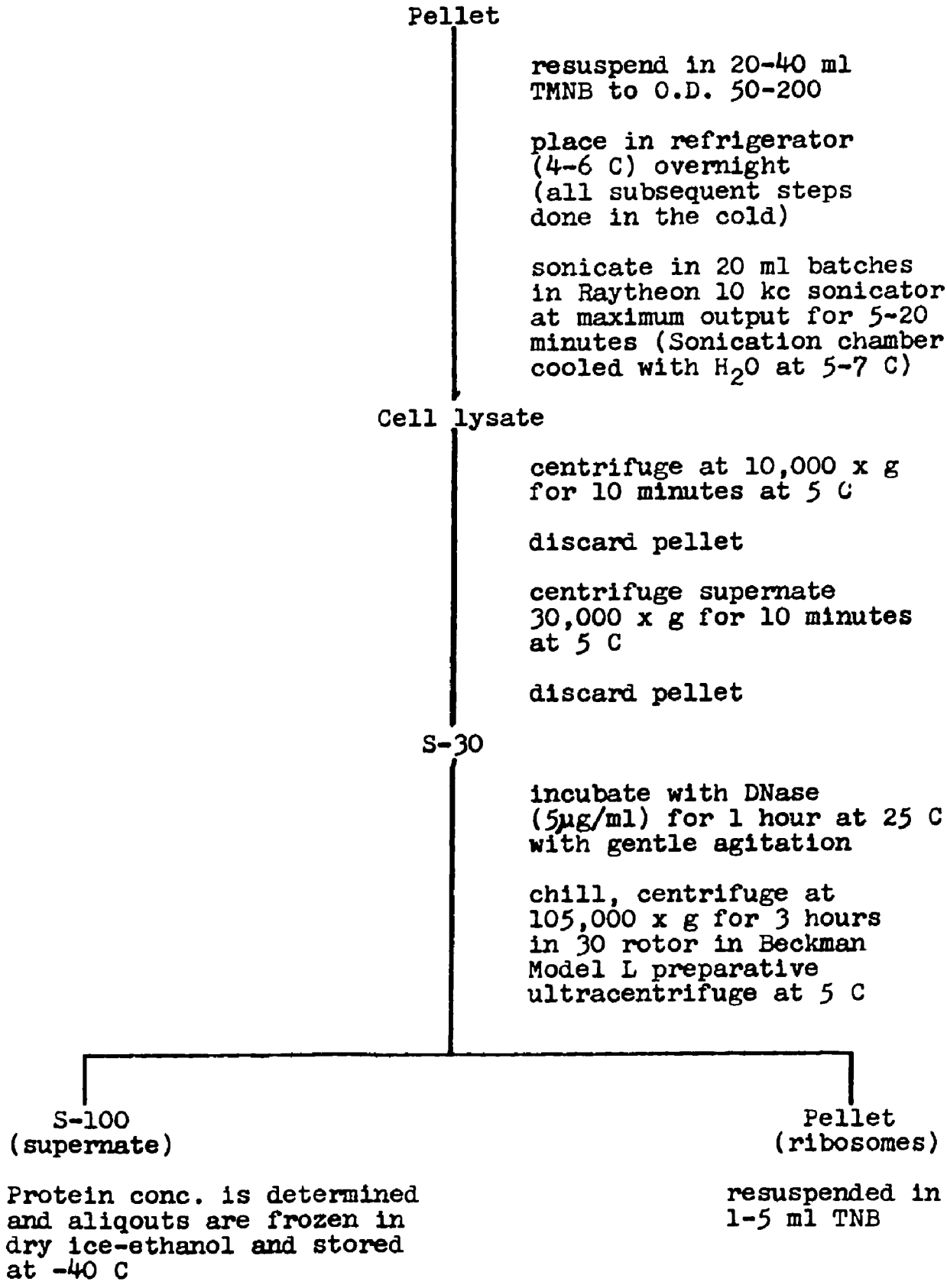
centrifuge 8,000 x g for 20 minutes at room temperature

Pellet

resuspend in 100 ml TMNB

centrifuge 8,000 x g for 20 minutes at room temperature

Pellet


Pellet
resuspend in 20-40 ml TMNB to O.D. 50-200
place in refrigerator (4-6 C) overnight (all subsequent steps done in the cold)
sonicate in 20 ml batches in Raytheon 10 kc sonicator at maximum output for 5-20 minutes (Sonication chamber cooled with H_2O at 5-7 C)
Cell lysate
centrifuge at 10,000 x g for 10 minutes at 5 C
discard pellet
centrifuge supernate 30,000 x g for 10 minutes at 5 C
discard pellet
S-30
incubate with DNase (5µg/ml) for 1 hour at 25 C with gentle agitation
chill, centrifuge at 105,000 x g for 3 hours in 30 rotor in Beckman Model L preparative ultracentrifuge at 5 C
S-100 (supernate)
Protein conc. is determined and aliqouts are frozen in dry ice-ethanol and stored at -40 C
Pellet (ribosomes)
resuspended in 1-5 ml TNB

The ribosomes prepared in this way have a 260/280 nm absorbancy ratio of 1.80-1.90. When the ribosomal pellet was suspended in TNB, there was an immediate dissociation into subunits. The RNA/protein ratio was 1.2-1.4 for PT0629 and 1.0-1.3 for PTOPM22. These ribosomes are then used for *in vitro* protein synthesis or for preparation of ribosomal subunits which will then be used for analysis of proteins on polyacrylamide gels. These same ribosomal subunits will also be used in *in vitro* protein synthesis.

The analytical sucrose density gradient patterns of the ribosomal subunits will be described in the results section.

Preparation of Ribosomal Subunits

The subunits were separated on 5-20% sucrose gradients (34 ml) in nitrocellulose tubes in an SW25.1 rotor at 17,000 rpm for 16 hours at 5 C. The stock sucrose solution was 2 M in TNB, and autoclaved to inactivate any RNase activity. The gradients were prepared using a Beckman Density Gradient Former. The heavy solution was 26.5% sucrose in TNB buffer and the light solution was 1% sucrose in TNB. The gradients were made at room temperature and cooled for 1 hour in the cold and then layered with 1 ml suspension of ribosomes containing 8-15 mg ribosomal protein/ml. The gradients were collected through the bottom of the tube in 9 drop fractions. If the absorbancy

was read at 260 nm, every 5th drop of each fraction was collected separately and diluted with 3 ml distilled water and the absorbance measured. Figure 1 shows a graph of the separation of subunits by this method. The dotted line indicates the fractions that were pooled containing either the 50S or 30S ribosomal subunits. After these were pooled, 1 M $MgCl_2$ solution was added to 0.01 M and they were centrifuged at 105,000 x g for 16 hours in a 30 rotor at 5 C. This eliminated the use of dialysis to remove the sucrose. It was found that dialysis lowered the efficiency of the ribosomes for in vitro protein synthesis. The pellets of the subunits were rinsed with 10 ml of TMNB and then suspended in 0.5-1.0 ml of TMNB. These subunits were then used in in vitro protein synthesis or in the preparation of ribosomal proteins for analysis on polyacrylamide gels. The purity of the subunits was checked by centrifuging 0.400-0.600 A_{260} units in 0.1 ml in 5-20% gradients (5 ml) in polyallomer tubes in a SW50.1 rotor for 100 minutes at 39,000 rpm at 5 C. These gradients were prepared using a Beckman Density Gradient Former with the same sucrose solutions described above. The gradients were collected in 6 drop fractions from the bottom of the tube, diluted with 3 ml distilled water and the absorbance read at 260 nm.

Preparation of Ribosomal RNA

The ribosomal RNA was prepared in a manner similar to the phenol method of Kurland (49). The ribosomes in PM,

Figure 1. Separation of ribosomal subunits on sucrose density gradients.

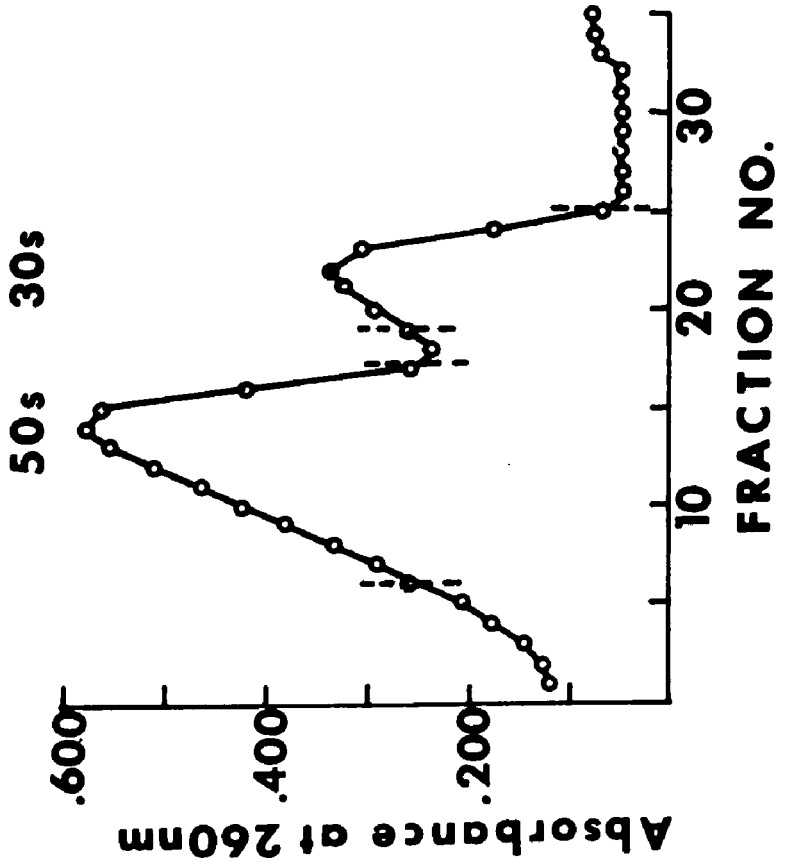


Figure 1

usually between 100-500 A_{260} units, were mixed with sodium lauryl sulphate to 0.2% (w/v) and incubated at room temperature for 5 minutes. This suspension was then diluted to 20 ml with 0.01 M Tris pH 7.6, 0.02 M Mg-Acetate (TM). To the dilute ribosomal suspension was added an equal volume of TM-equilibrated redistilled phenol. The phenol-ribosome mixture was mixed in a 50 ml screw cap tube slowly by a rotating shaker platform for 15 minutes. This shaker platform was placed perpendicular to the floor so that the mixing was by slowly inverting the tube. The suspension was then centrifuged at low speed for 10 minutes and the aqueous phase was aspirated off and the organic phase discarded. The phenol step and centrifugation were repeated twice with 1/2 volume of phenol. The aqueous phase has two volumes of absolute ethanol added to precipitate the RNA. The RNA precipitate was collected by centrifugation at 5 C and redissolved in TM. The ethanol precipitation was repeated twice. The RNA was then placed in a vacuum dessicator, which was then evacuated and placed at 37 C until the RNA was dry. The RNA was then weighed and was ready for analysis. The recovery of ribosomal RNA was 0.03-0.07 mg/A_{260} unit of ribosomes.

Analysis of Ribosomal RNA Composition

The hydrolysis and chromatography of the ribosomal RNA was done by the procedure of Bendich (7). The dried ribosomal RNA, usually 5-10 mg, was dissolved in 70% $HClO_4$.

The suspension was rapidly mixed for 5 minutes on a vortex mixer and then centrifuged at approximately 1,000 x g for 10 minutes at room temperature. The supernatant (hydrolyzed RNA solution) was used for analysis by paper chromatography. The descending chromatography was performed at room temperature. The chromatographic solvent used was a mixture of 65 ml of peroxide-free absolute isopropanol, 16.7 ml concentrated HCl and 18.3 ml distilled water. The absolute isopropanol was tested for contamination by peroxide as described by Bendich (7). The chromatography was on Whatman No. 1 chromatography paper, cut into 15 x 60 cm sheets. Three positions were marked 5 cm from the upper end of the paper. One was a blank, the next two were used for a control of a known amount of bases, 10 µl, and rRNA hydrolysate, 10-30 µl. The chromatography was run for 16-20 hours at room temperature in which time the solvent front moved approximately 40 cm. The spots were located with a Wood's light and cut out using a template for uniform size. A blank spot was cut out 1/2 way between the solvent front and starting point in the blank column. This was used as the blank in the spectrophotometer and gave the same results as blanks cut out parallel with each spot. These spots were then cut into thin strips and placed in screw-capped tubes, and 5 ml of 0.1 N HCl was added. These were shaken at 37 C for 2 hours to elute the bases. The HCl was decanted and the concentrations were then determined from a spectral analysis of the control and rRNA eluates.

Hyperchromicity Studies of Ribosomes in 8 M Urea

A solution of 8.9 M urea was prepared in 0.01 M phosphate pH 7.0. Ribosomes, usually 3.0-4.0 A_{260} units, were diluted 1:10 into the urea solution giving a final concentration of 8 M urea. These were then shaken at 20 C for 20 hours. All samples were done in triplicate. The absorbancy at 260 nm was read and the results were expressed as % hyperchromicity change.

Thermal Denaturation of Ribosomes

The thermal denaturation data were obtained by measuring A_{260} with a Beckman DU spectrophotometer and temperature with a temperature probe in a cuvette in the sample chamber. The ribosomes were diluted into 0.01 M Tris, pH 7.6, 0.01 M Mg-Acetate for the thermal denaturation. The initial absorbancy of the ribosomes was 0.300-0.400. The temperature increase was maintained as close as possible to 1.0 C/5 minutes.

Preparation of Ribosomal Protein

The ribosomal protein from 70S and 50S ribosomes was prepared according to the method of Fogel and Sypherd (17). The ribosomes, 3-5 ml, constituting 5-15 mg protein had 5 volumes of cold 2-chloroethanol and HCl to 0.06 N added. The suspension was then held on ice for 2-3 hours with occasional mixing and centrifuged at 5,000 x g at 5 C for 20 minutes to remove the rRNA precipitate. The pellet was discarded and the supernate dialysed in the cold against

distilled water with at least two changes and a total dilution not less than 1:10,000. The dialyzed solution was lyophilized, and the protein was dissolved in either 4 M urea in distilled water or 6 M urea in 0.48 N KOH, 0.28 N acetic acid resulting in a final pH of 5.9. The protein was weighed and dissolved at 5-6 mg/ml. The recovery of ribosomal protein prepared in this way was 50-70% of the initial protein depending on the source of the ribosomes.

The 2-chloroethanol procedure was found inadequate for the preparation of 30S ribosomal protein for polyacrylamide gel electrophoresis. The method of Hardy (35), an adaptation of Waller and Harris (104), gave the best preparation of 30S protein. 30S ribosomal subunits, 2-4 mg of protein in 1 ml TMNB, were mixed with 0.1 ml of 1 M $MgCl_2$ and 2 ml glacial acetic acid. This was then held in an ice water bath for 45 minutes with gentle mixing every 5 minutes on a vortex mixer. The suspension was then centrifuged at 20,000 x g for 10 minutes at 5 C to sediment the precipitated rRNA. The supernatant was decanted and saved. The pellet was washed once in 1 ml of 1 vol. TMNB, 0.1 M $MgCl_2$ and 2 vol. glacial acetic acid. The two supernates were combined and constituted the protein solution. It was not necessary to dialyze the acetic acid away since the solution could be used directly for electrophoresis on polyacrylamide gels. The amount of protein recovered by this method, while not determined directly, was sufficient

to give distinct protein band patterns with small sample volumes on polyacrylamide gels.

Preparation of Polyacrylamide Gels and Electrophoresis of Ribosomal Protein

The preparation of the polyacrylamide gels was essentially the method described by Gesteland and Staehelin (26). This method employed a final separating acrylamide gel concentration of 10% in 8 M urea at pH 4.5. The 7.5% gel concentration of Reisfeld (78) was tried but gave poorer resolution than the 10% concentration. Table 1 details the various solutions used in the preparation of the polyacrylamide gels. The riboflavin solution was twice as concentrated as that used by Gesteland and Staehelin (26). The ammonium persulfate solution was made fresh. The other solutions were stable for several weeks in brown glass bottles stored in the cold.

The various solutions were mixed to provide two kinds of gels. First, the separating gel solution was made. In this the actual protein separation by charge and molecular weight occurs. Secondly, the spacer and sample gel solution were constituted. The presence of a spacer gel was found to increase the resolution of the proteins. The sample gel was used to prevent any convection currents from disturbing the sample of protein on top of the gel.

The separating gel was prepared by mixing 1 part of solution I, 6 parts of solution III and 1 part of Solution VI.

TABLE 1

PREPARATIVE SOLUTIONS FOR POLYACRYLAMIDE GELS

Solution[1]	Acrylamide	Methylene-bis-acrylamide	1N KOH	glacial acetic acid	TEMED[2]
I	-	-	24 ml	8.6 ml	2 ml
II	-	-	24 ml	1.44 ml	0.23 ml
III	6.65 g	0.1 g	-	-	-
IV	2.5 g	0.625 g	-	-	-
V	4 mg riboflavin/100 ml 8 M urea				
VI	1.12% ammonium persulfate in 8 M urea				

[1]After all components were added, 24 g of urea was added and the solution was brought to 50 ml with distilled water.

[2]TEMED,- N, N, N', N'', -tetramethylethylenediamine

To this was added 0.5 mg $K_3Fe(CN)_6$/8ml gel solution from a 2.5 mg/ml solution in 8 M urea. This was added to slow down the polymerization rate and provided a more uniform gel. Glass tubes, 9 cm x 6 mm (I.D.), which had been coated with a 1:50 dilution of Kodak Photo-Flo solution, were placed in the small end of a serological cap to hold them upright and stopper one end before the gel solution was added. A portion (1.7 ml) of separating gel solution was placed in the tubes. Approximately 0.1 ml of distilled water was layered over the gel to prevent the formation of a meniscus. The gel polymerization was a chemical reaction and did not need direct light. The polymerization took 1-1.5 hours. After the separating gel had been polymerized, the water above was decanted and 0.3 ml spacer gel was placed over the separating gel. Distilled water was layered over spacer gel solution to prevent a meniscus. The spacer gel was prepared by mixing the following solutions, 1 part solution II, 4 parts solution IV, 1 part solution V and 2 parts 8 M urea. The polymerization of the spacer gel required direct fluorescent light. For this, a fluorescent light was placed 30 cm above the gel until the gel turned opaque, usually 30 minutes. The light was then lowered to a position directly over the gels and further polymerization allowed for 15 minutes. The water was then decanted and sample gel, prepared as the spacer gel, except containing the protein solution, was layered over the spacer gel. This did not have to be polymerized at this point but could be layered

with buffer and electrophoresed. However, since manipulations were easier if the sample gel was polymerized, this was done by placing the lamp directly above the gels for 15-30 minutes. The sample gel, 0.3 ml, contained 0.020-0.20 ml of protein solution with 75-200 mg of protein.

Figure 2 shows the electrophoresis apparatus and a gel tube ready for electrophoresis. The electrophoresis buffer was a 1:5 dilution of the beta-alanine-acetic acid buffer of Reisfeld (78). This was prepared by mixing 31.2 gm beta-alanine and 8 ml glacial acetic acid per liter giving a pH of 4.5. The electrophoresis was run in the cold with precooled buffer at 2.5 ma/tube using a constant current power supply. The lower reservoir was filled with buffer to the top and the small reservoirs above the tubes were filled with buffer. Platinum electrodes were placed in the reservoir above the gels and an electrode was in the lower reservoir. The anode was at the top and cathode at the bottom. The electrophoresis was carried out in the cold till the buffer front, which could be seen in the gel, reached the bottom of the tube. This usually took 4-4.5 hours.

After the gels were removed from the tubes by means of a No. 26 Yale needle with water passing through it, they were stained for 1 hour in 1% Buffalo Black NBR dissolved in 7% acetic acid. The gels were then transferred to 60 ml 7% acetic acid and allowed to elute stain overnight. The

Figure 2. Polyacrylamide gel electrophoresis apparatus.

Figure 2

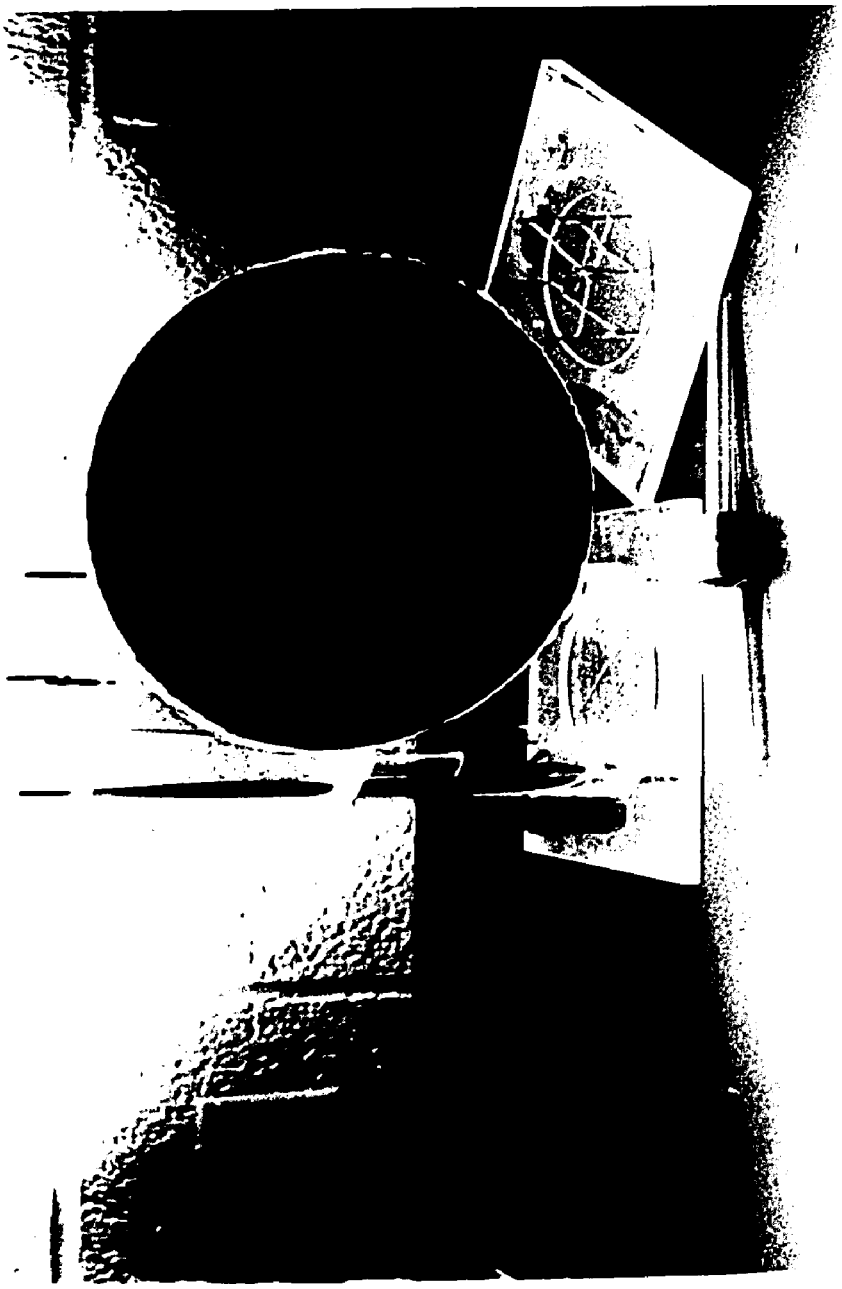
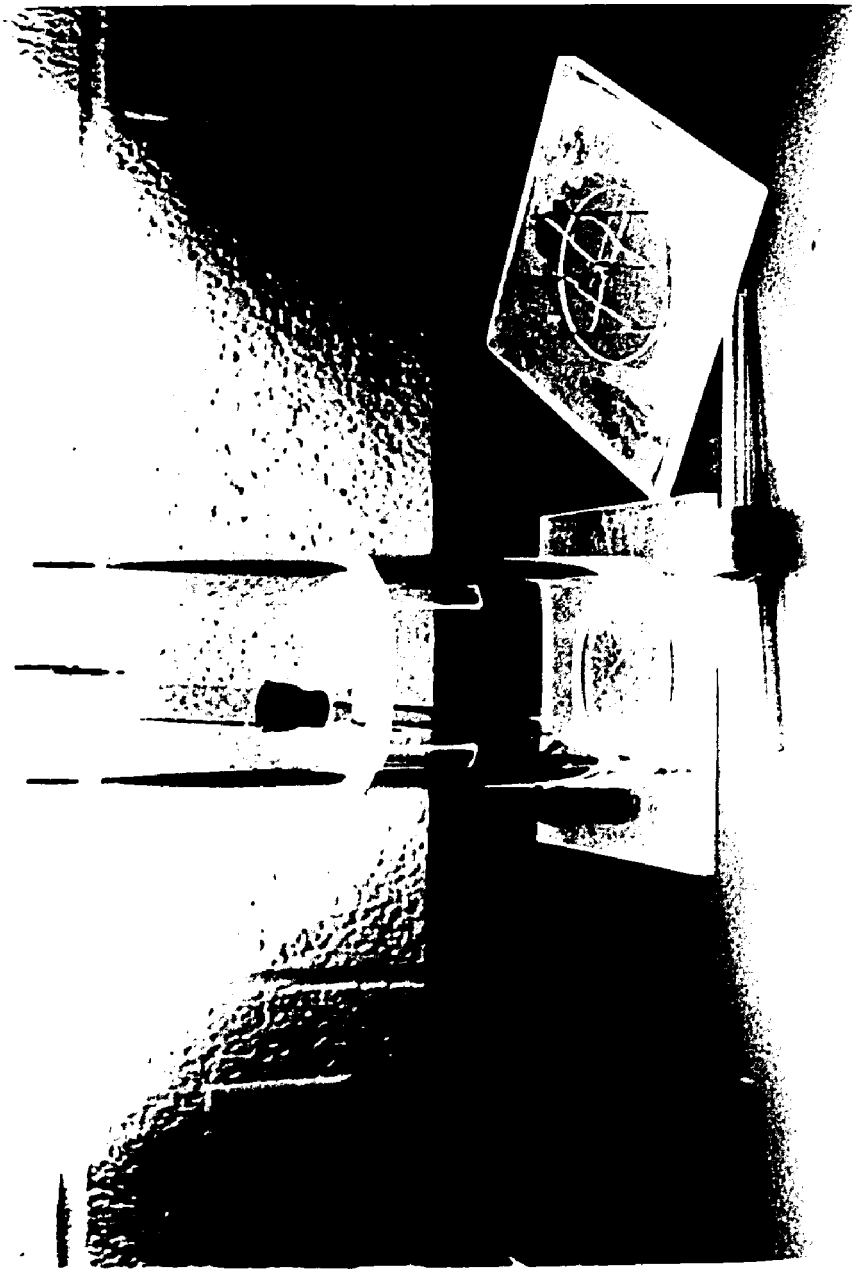


Figure 2



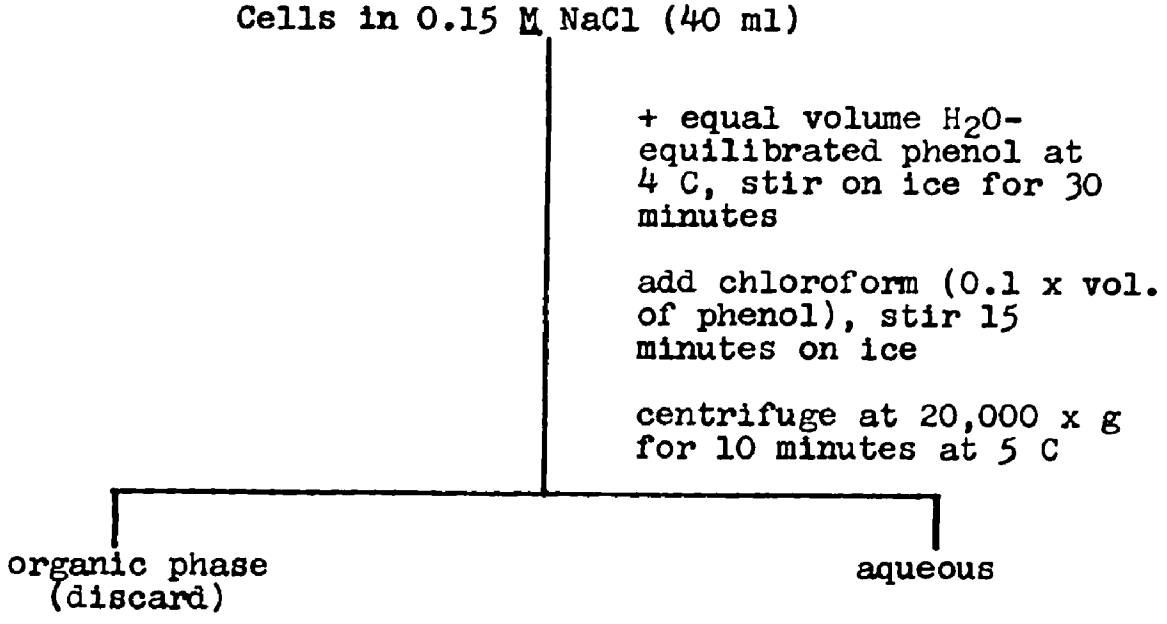
remaining stain was then removed electrophoretically by destaining at 8 ma/tube for 3-4 hours or until all residual stain was removed. The electrophoretic destaining was accomplished by placing a gel plug, usually the same as the separating gel, in a glass tube 1 x 14 cm. The stained gel was then placed in the tube. The destaining was done in 7% acetic acid with the anode at the top, since the stain was negatively charged it will migrate to the top and could be decanted. The gels with their stained protein bands were stored in 7% acetic acid and appeared to be stable indefinitely.

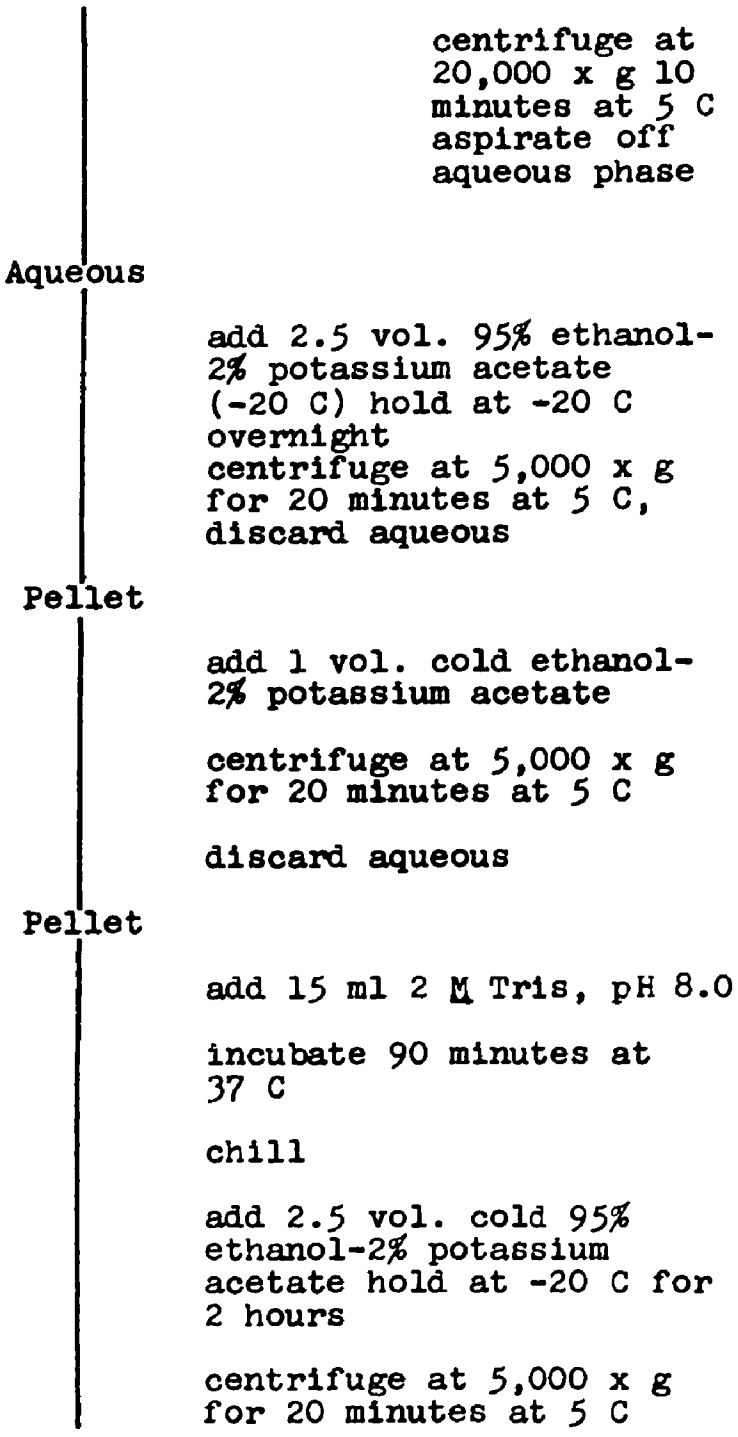
If split gels were desired the gel solutions were prepared as above. For split gels the glass tubes must not be fire-polished on the end into which the separator would be placed. To make the separator, white cardboard, a 5 x 7 file card, was cut into strips 7-9 mm x 20 mm. They were then coated with paraffin and allowed to dry. The separating gel was added to the gel tubes and allowed to polymerize. The spacer was added and the separator cardboard now inserted in the top of the tube through the spacer gel until it touched the separating gel. Care was taken to make sure the separator touched both sides of the tube so that there would be no leaks, which would allow the two protein solutions to mix. Distilled water was then layered over the spacer gel and polymerization accomplished as before. The sample gel, 0.15 ml, with one protein sample in it was placed on one side and the other sample gel with the second protein sample for

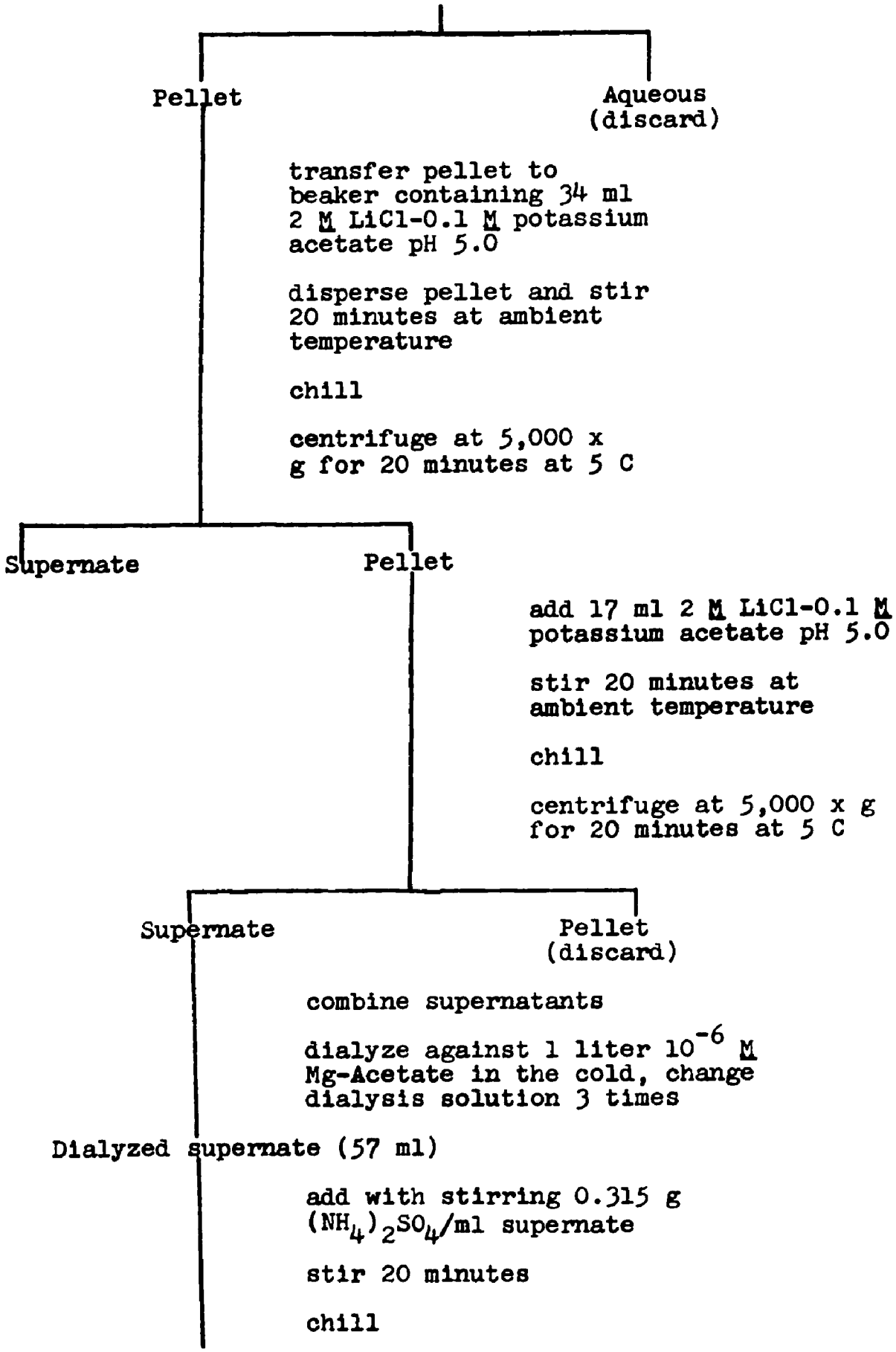
comparison was placed on the other side. One-half the amount of protein was used as that that would be used on a single gel. The sample gel was polymerized and the electrophoresis and staining were performed as described above. This method allowed the direct comparison of ribosomal proteins. This was not possible by the comparison of two separate gels since they did not always give identical protein patterns.

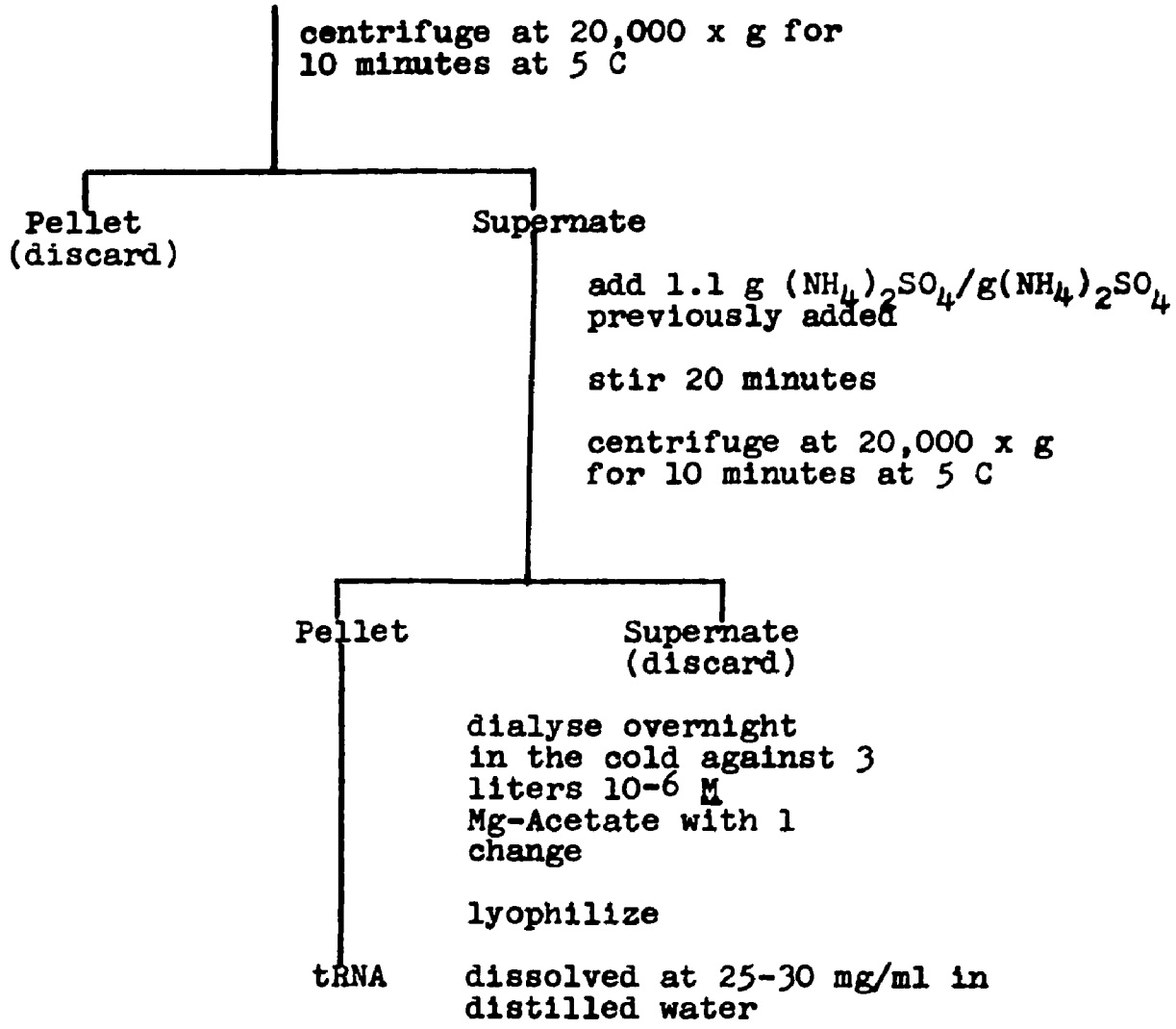
Preparation of Transfer RNA

The transfer RNA was prepared from E. coli D_{10} (RNase I^-) strain. The cells were grown in 12 liter batches in a fermenter to O. D. of 1.0-2.0. The cells were centrifuged at room temperature and washed once in 0.15 M NaCl and suspended at O. D. 200 in 0.15 M NaCl. The procedure for extracting the tRNA was that of Avital and Elson (3). The following flow sheet describes in detail the extraction and purification procedure used.




centrifuge at 20,000 x g 10 minutes at 5 C aspirate off aqueous phase
Aqueous
add 2.5 vol. 95% ethanol-2% potassium acetate (-20 C) hold at -20 C overnight
centrifuge at 5,000 x g for 20 minutes at 5 C, discard aqueous
Pellet
add 1 vol. cold ethanol-2% potassium acetate
centrifuge at 5,000 x g for 20 minutes at 5 C
discard aqueous
Pellet
add 15 ml 2 M Tris, pH 8.0
incubate 90 minutes at 37 C
chill
add 2.5 vol. cold 95% ethanol-2% potassium acetate hold at -20 C for 2 hours
centrifuge at 5,000 x g for 20 minutes at 5 C


Pellet
Aqueous (discard)
transfer pellet to beaker containing 34 ml 2 M LiCl-0.1 M potassium acetate pH 5.0
disperse pellet and stir 20 minutes at ambient temperature
chill
centrifuge at 5,000 x g for 20 minutes at 5 C
Supernate
Pellet
add 17 ml 2 M LiCl-0.1 M potassium acetate pH 5.0
stir 20 minutes at ambient temperature
chill
centrifuge at 5,000 x g for 20 minutes at 5 C
Supernate
Pellet (discard)
combine supernatants
dialyze against 1 liter 10^{-6} M Mg-Acetate in the cold, change dialysis solution 3 times
Dialyzed supernate (57 ml)
add with stirring 0.315 g $(NH_4)_2SO_4$/ml supernate
stir 20 minutes
chill



This extraction yielded approximately 15 mg tRNA per 40 ml cells of O.D. 200. The tRNA recovered by this procedure was uncharged by the incubation in 2 M Tris-HCl at 37 C. The LiCl step was to precipitate any contaminating ribosomal RNA and the first ammonium sulfate precipitation was to remove any molecules larger than tRNA. The second was to precipitate the tRNA itself. The tRNA solution was frozen in small aliqouts and stored at -40 C.

The tRNA may be thawed and refrozen without any effect on its ability to participate in *in vitro* protein synthesis.

In Vitro ^{14}C-phenylalanine Incorporating System

The polyuridylic acid (poly U)-directed incorporation of L-^{14}C-phenylalanine required ATP, GTP, $MgCl_2$, NH_4Cl, tRNA and a means for generating additional ATP. Creatine phosphate and creatine kinase were used as the ATP generating system. The creatine kinase was a valuable enzyme since the reaction catalysed by it, ADP→ATP, was irreversible in the presence of excess creatine phosphate.

The following stock solutions were prepared for the *in vitro* system:

1 M Tris-HCl pH 7.8

0.013 M GTP in 0.01 M Tris pH 7.8

0.051 M ATP in 0.01 M Tris pH 7.8

0.66 M creatine phosphate in 0.01 M Tris pH 7.8

2.5 mg/ml creatine kinase in 0.01 M Tris pH 7.8-0.1% bovine serum albumin

0.05 M L phenylalanine in distilled water

2 M NH_4Cl

0.2 M $MgCl_2$

1.43 M 2-mercaptoethanol

2 mg/ml potassium salt of poly U in distilled water

The ATP and creatine phosphate solutions were made fresh. The other solutions were stable over prolonged periods when stored at 4 C. All components were added individually to the reaction mixture.

The *in vitro* incorporation mixture had the following components in μmoles/ml; volume of stock suspension added for 1 ml of reaction mixture is given in parenthesis.

50 μmoles Tris HCl pH 7.8	(0.05 ml)
0.26 μmoles GTP	(0.02 ml)
1.5 μmoles ATP	(0.03 ml)
13.2 μmoles creatine phosphate	(0.02 ml)
50 μg creatine kinase	(0.02 ml)
0.2 μmoles L-^{12}C-phenylalanine	(0.02 ml)
80 μmoles NH_4Cl	(0.07 ml)
14 μmoles $MgCl_2$	(0.03-0.04 ml)
14.3 μmoles 2-mercaptoethanol	(0.01 ml)
1.0 mg tRNA	(0.04 ml)

L-^{14}C-phenylalanine, a 1:5 dilution of stock label, was added to give the desired specific activity. S-100 was added at 2.4 mg/ml reaction. The ribosomes were added at varying concentrations. Poly U was added at 200-250 μg/mg ribosomes.

If the incorporation was to take place at 25 C, the $MgCl_2$ concentration was maintained at 14 μmoles/ml; however for those experiments run at 3.5 C, the $MgCl_2$ concentration was lowered to 7 μmoles/ml. The length of the incorporation time was variable and will be described in the results. The individual reaction volumes were either 0.25 ml or 0.125 ml depending on whether the duplicate sample size was 0.1 ml or 0.05 ml.

Assay of L-^{14}C-phenylalanine Incorporation

The assay of amino acid incorporation was done on filter papers, similar to the assay of Mans and Novelli (56). The samples, either 0.1 ml or 0.05 ml, were placed on 2.5 cm squares of Whatman 3MM filter paper supported on pins. The filter papers (FP) were then placed face down in cold 10% trichloroacetic acid (TCA) (10ml/FP), immediately after the sample had been absorbed into the filter paper. The filter papers were handled with tweezers. The filter papers were held 10 minutes in the TCA and then were transferred to boiling 5% TCA (5 ml/FP) for 10 minutes. Care was taken to make certain that the filter papers remained submerged during the boiling. After boiling, the filter papers were transferred to 1:1 ether-ethanol (5ml/FP), draining on the side of the TCA container before transfer. They were held in the ether-ethanol for 10 minutes at room temperature and placed face up on paper towels at 80 C for 1/2 hour. The filter papers were transferred to glass scintillation vials containing 15 ml toluene-liquifluor counting solution. Counting was done in a Packard Tri-carb Liquid Scintillation Spectrometer. The liquifluor was prepared by mixing 100 g PPO and 1.25 g POPOP in 1 liter toluene. The cocktails contained 42 ml liquifluor per liter toluene. The counting efficiency of ^{14}C-phenylalanine on the filter papers was 67.5%.

EXPERIMENTAL RESULTS

Comparison of Ribosomes by Urea Denaturation, Thermal Denaturation and Ribosomal RNA Composition

We employed these parameters to detect gross differences in ribosomal conformation or ribosomal RNA (rRNA) base composition between mesophilic and psychrophilic ribosomes. Precedents in the literature are as follows. Pace and Campbell (73) used thermal denaturation (Tm) and rRNA base composition of a number of different psychrophilic, mesophilic and thermophilic ribosomes to correlate maximum growth temperature and ribosome heat stability. Stenesh and Holazo (93) found a similar correlation between heat stability and maximum growth temperature using mesophilic and thermophilic Bacillus species rRNA. They also employed the effect of urea on rRNA as an additional parameter.

The ribosomes used in this study were prepared by the PM buffer method described in the Materials and Methods, and were predominantly 70S ribosomes. This was due to the effect of quick cooling of cells on ice and rapid sonication. Analysis in 5-20% sucrose density gradients in SW40 Ti rotor showed a single peak in the 70S region for these ribosome preparations.

A. Thermal Denaturation of 70S Ribosomes

A comparison of thermal denaturation (Tm) temperatures of 70S ribosomes from different species has been shown to reflect the degree of interaction between rRNA and protein in the ribosome (73). Table 2 shows that Tm's of wild type mesophilic ribosomes were consistently higher than those with wild type psychrophilic ribosomes. The Tm of 70S ribosomes was taken as the mid-point of the increase in absorbancy with temperature at 260 nm. Tm's were reproducible with an experimental error of less than ± 1.0 C. The Tm's were run in 10^{-2} M Mg^{+2}. The values reported were the average of at least 3 separate determinations. The wild type psychrophiles as a group had an average Tm of 66.2 C. Wild type mesophiles had an average Tm of 68.1 C. Thus, our studies support the correlation between growth temperature range and heat stability of wild type mesophilic and psychrophilic ribosomes as observed by others (73, 94). However, the Tm's of psychrophilic mutant and transductant ribosomes were not similar to the wild type psychrophilic ribosomes as expected but resembled those of their mesophilic progenitors. The psychrophilic mutants and transductants had an average Tm of 67.9 C which is typical of mesophiles and not wild type psychrophiles.

B. Hyperchromicity Changes in 8 M Urea

Exposure to 8 M urea induces an increase in absorbancy of ribosomes that may reflect the relative degree of

TABLE 2

THERMAL DENATURATION OF 70S RIBOSOMES

Organism	Growth Temperature Range	Tm (C)
P. aeruginosa (PAT38)	mesophilic	67.8
P. aeruginosa (PTO629)	mesophilic	68.9
M. freudenrechii	mesophilic	66.4
M. sodonensis	mesophilic	68.5
M. lysodeikticus	mesophilic	69.0
P. putida (A.3.12)	psychrophilic	66.8
P. fluorescens (PFO12)	psychrophilic	66.4
P. fluorescens (PFO14)	psychrophilic	66.7
P. geniculata (PGO50)	psychrophilic	65.8
M. cryophilus	psychrophilic	65.3
PTOPM22	psychrophilic-mutant	68.0
PTOPM23	psychrophilic-mutant	68.0
PFT293	psychrophilic-transductant	67.7
PFT300	psychrophilic-transductant	68.0

hydrogen bonding within the ribosome. Thus, ribosomes used in this study were compared in this regard. They were exposed to 8 M urea for 20 hours to allow dissociation to go to completion. Shaking was necessary to prevent aggregation of the ribosomes. The per cent hyperchromicity change observed upon exposure to 8 M urea for 20 hours is shown in Table 3. These values with their standard deviations, represent the average of 9-15 determinations for each organism. The per cent hyperchromicity was determined using the untreated ribosomes as controls. Table 3 shows that the psychrophilic mutant and transductant ribosomes resembled those of their wild type mesophilic progenitors and not ribosomes from wild type psychrophiles. Therefore,

the data show a greater degree of hydrogen bonding in ribosomes of wild type mesophiles than wild type psychrophiles coincident with the higher growth temperature range of the organisms. However, this does not appear to be true for psychrophiles produced by transduction or mutation.

TABLE 3

PER CENT UREA HYPERCHROMICITY

Organisms	Growth Temperature Range	% Hyperchromicity
P. aeruginosa (PAT38)	mesophilic	13.4 ± 3.0
P. aeruginosa (PT0629)	mesophilic	11.4 ± 1.4
M. freudenrechii	mesophilic	13.9 ± 1.9
M. sodonensis	mesophilic	12.3 ± 2.1
M. lysodeikticus	mesophilic	12.0 ± 2.0
P. putida (A.3.12)	psychrophilic	19.5 ± 2.8
P. fluorescens (PF012)	psychrophilic	16.7 ± 1.6
P. fluorescens (PF014)	psychrophilic	20.7 ± 2.6
P. geniculata (PG0050)	psychrophilic	18.1 ± 2.9
M. cryophilus	psychrophilic	19.8 ± 3.1
PTOPM22	psychrophilic-mutant	14.4 ± 1.8
PTOPM23	psychrophilic-mutant	11.4 ± 2.2
PFT293	psychrophilic-transductant	13.3 ± 2.8
PFT300	psychrophilic-transductant	11.1 ± 2.6

C. Analysis of Ribosomal RNA Base Composition

A correlation between increasing guanine-cytosine (G-C) content of rRNA and increasing maximum growth temperature has been shown by Pace and Campbell for a number of psychrophiles, mesophiles and thermophiles (73). Table 4 shows that the wild type organisms analyzed here also

TABLE 4

BASE COMPOSITION OF RIBOSOMAL RNA

Organism	Mole percent				percent G + C
	Guanine	Adenine	Cytosine	Uracil	
P. aeruginosa (PAT38)	32.9	26.3	23.8	16.7	56.7
P. aeruginosa (PT0629)	29.2	26.5	25.5	18.6	54.7
M. freudenreichii	32.3	25.2	24.5	17.8	56.8
M. sodonensis	35.8	22.9	24.0	17.3	59.8
M. lysodeikticus	34.2	22.7	26.9	16.1	61.1
P. putida (A.3.12)	30.7	26.0	24.3	19.0	55.0
P. fluorescens (PF012)	31.5	26.9	23.4	17.7	54.9
P. fluorescens (PF014)	31.4	27.9	23.2	17.5	54.6
P. geniculata (PG050)	30.9	27.3	23.8	18.0	54.7
M. cryophilus	30.2	26.9	23.0	20.6	53.2
PTOPM22	32.5	25.7	23.8	18.0	56.3
PTOPM23	33.4	25.4	24.9	16.3	58.3
PFT293	32.9	26.5	22.6	18.2	55.5
PFT300	32.3	26.0	23.7	18.2	56.0

showed a similar correlation; that is, that the wild type mesophiles had a higher G-C content than wild type psychrophiles. However, the ribosomal RNA G-C composition of psychrophilic mutant and transductant ribosomes did not resemble that of wild type psychrophiles, but was similar to wild type mesophiles and their mesophilic progenitors. These results combined with those from the above analyses indicate that differences observed by other workers using similar parameters with wild type mesophiles, psychrophiles and thermophiles may be trivial to the determination of the maximum or minimum growth temperatures of an organism. Thus these differences may be the result of growth within a specific temperature range rather than the cause of growth in a specific temperature range. Possibly some other facet of the ribosome, such as protein composition, may be important to the functioning of the ribosome at various temperatures and ribosome involvement as one of the factors in the determination of the growth temperature range of an organism.

Polyacrylamide Gel Analysis of 70S Ribosomal Protein

Analysis of 70S ribosomal proteins in polyacrylamide gels at pH 4.5 has been shown to resolve 20-25 protein bands (26). Therefore using this technique, a comparison of 70S ribosomal proteins from a mutant and transductant with their wild types should point out any

gross differences in protein composition of the ribosomes. At pH 4.5, the fastest moving bands will be the most basic proteins. PTOPM22 and PFT300 were the mutant and transductant selected for further analysis. It must be pointed out that in the analysis of 70S proteins in polyacrylamide gels, there may be many over-lapping bands and the absence or presence of some proteins may be obscured. However, the analysis could point up some differences which would indicate a need for further analysis of ribosomal subunits.

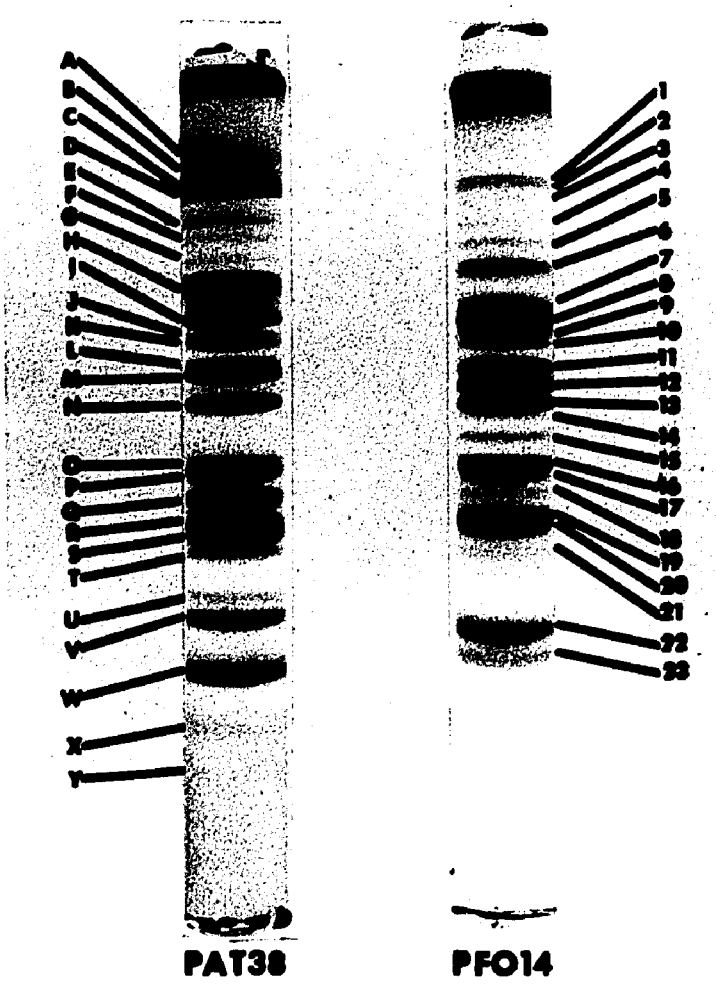
The 70S ribosomes used for protein analysis were prepared by the PM buffer method. A number of methods for the preparation of protein were tried. The acetic acid method of Waller and Harris (103) gave poor preparations of wild type, transductant and mutant ribosomal proteins that had inconsistent protein banding on polyacrylamide gels. The 4 M LiCl-8 M urea method of Leboy (50) gave good preparations of wild type 70S proteins but not transductant and mutant 70S proteins. Finally, the 2-chloroethanol procedure of Fogel and Sypherd (17) was determined optimal for the preparation of all 70S ribosomal protein.

A. Comparison of Ribosomal Protein from Transductant and Wild Type Ribosomes

In Figure 3, the genetic donor (PF014) and genetic recipient (PAT38) have their 70S ribosomal proteins labeled. The resolution of protein bands was greatest when 100-150 µg of protein in 10-40 µl was layered on the gels. For split gels one-half this amount was used. The anode was at the top

Figure 3. Polyacrylamide gels of genetic donor, PF014, and genetic recipient, PAT38, 70S ribosomal proteins.

Figure 3



and cathode at the bottom of the gel. The sample was applied at the anode end. If one were to compare only the wild types, they would have approximately 5 proteins in common.

Figure 4 details split gels of the genetic donor (PF014) and genetic recipient (PAT38) with the transductant (PFT300) ribosomal protein. The numbered or lettered bands on the outside of the gels indicate wild type proteins. Those labeled on the inside of the gels indicate the transductant proteins which were homologous with those from the wild type. From this kind of analysis it should be possible to predict the source of 70S proteins of the transductant. Figure 5 shows a gel pattern of transductant ribosomal proteins. The bands labeled on the left indicate proteins which may have originated with the genetic recipient, those on the right indicate proteins from the genetic donor. Proteins labeled T and T' could not be assigned to either wild type genetic donor or recipient. A number of polyacrylamide gels were used for assigning nomenclature to the proteins bands shown in Figure 5. There were 5 proteins in common between the genetic donor and genetic recipient. These were I-8, J-9, Q-18, R-19 and S-20. If one assumed that these proteins were originally present in the genetic recipient then 6 protein bands in the transductant were derived from the genetic donor and 12-13 from the genetic recipient. Analysis of this type consistently resolved 18-19 protein bands.

Figure 4. Split gels of genetic donor, PF014, and transductant, and genetic recipient, PAT38, and transductant.

Figure 4

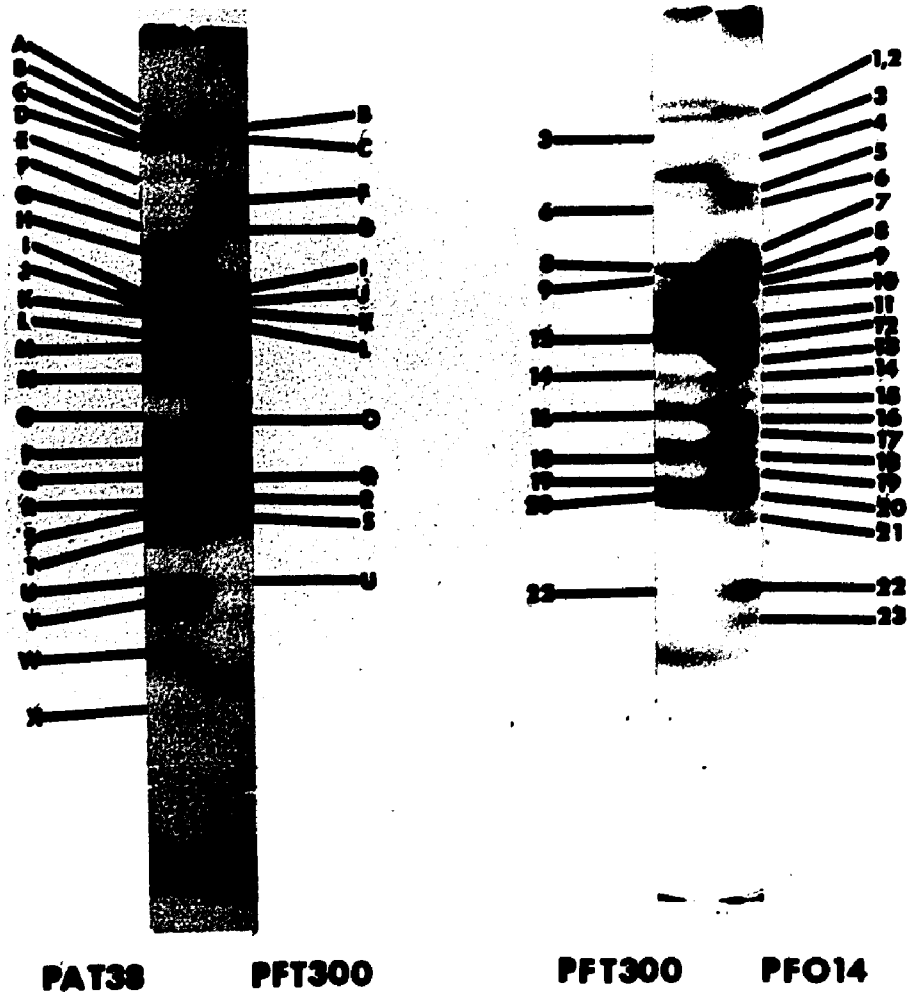
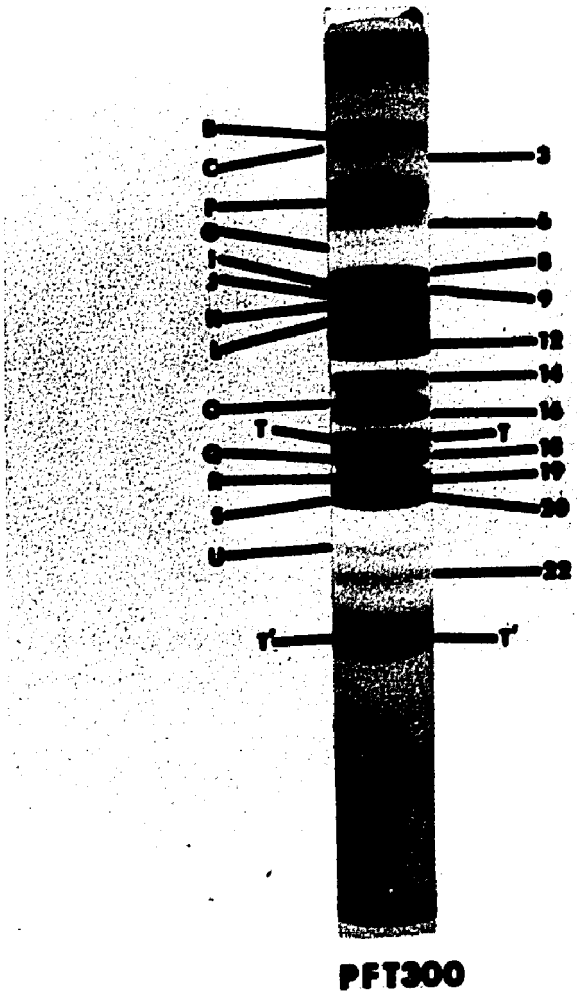


Figure 5. Polyacrylamide gel of transductant 70S ribosomal protein.

Figure 5



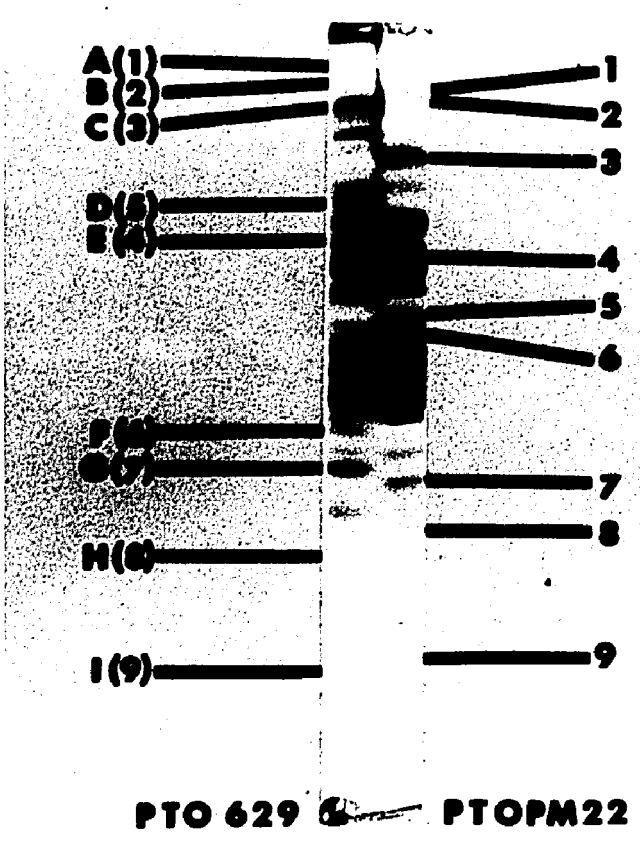
B. Comparison of Ribosomal Proteins from Mutant and Wild Type Ribosomes

We considered that a comparison of the mutant 70S ribosomal proteins with wild type 70S proteins might show less changes than observed with the transductant 70S proteins. This was expected on the basis of a more limited genetic change occurring in psychrophiles produced by mutation. This contention was further strengthened by isolation of revertants of the psychrophilic mutants. No bacteria capable of growing at higher temperatures were isolated from psychrophilic transductants, an expected finding, since they probably had a greater genetic change than the mutants. The isolation of mesophilic revertants of psychrophiles produced by mutation indicated that the genetic change in the production of the mutants was probably a point mutation. However, as Figure 6 shows, there were many altered proteins in the mutant ribosomes.

PTOPM22 was selected for ribosomal protein analysis, since it was the easiest to grow in large batches. The numerals shown in Figure 6 identify protein bands from mutant cells that were altered in mobility when compared with wild type. Letters identify proteins of the wild type that have no parallel protein in the mutant. The numerals in parenthesis refer to the altered proteins of the mutant that may correspond to the wild type proteins. There were a total of 9 altered proteins in the mutant. It was possible to identify these with 9 proteins in the wild type; however the identification of proteins 5 and 6 of the mutant

Figure 6. Split gel of mutant, PTOPM22, and wild type, PT0629, 70S ribosomal proteins.

Figure 6



was done solely on the basis of band morphology. This was necessary, since there were no closely aligned or nearby bands of the wild type in the gel to which bands 5 and 6 could be assigned. These interpretations were made after analyzing many single and split gels with different sample quantities. In this regard, protein concentration was increased to identify faint bands and decreased to clarify large bands. The gel presented here represented all changes as clearly as possible. Analysis of the wild type and mutant consistently resolved 23-25 proteins.

Comparison of Mutant and Wild Type Ribosomal Subunit Proteins by Polyacrylamide Gels

We considered that analysis of ribosomal subunits would delineate most of the proteins involved in the formation of 70S ribosomes. Therefore, a comparison of the ribosomal proteins from mutant and wild type ribosomal subunits would allow further identification of the altered proteins already observed in the comparison of 70S ribosomal proteins. Hopefully, the comparison of ribosomal subunit proteins coupled with *in vitro* protein synthesis data at 25 C and 3.5 C would provide a correlation between structural alteration and a change in functional capabilities of mutant ribosomes with regard to temperature.

A. Gradient Analysis of Subunit Preparations

PTOPM22, a psychrophilic mutant, was selected for further analysis and comparison with its progenitor *P. aeruginosa* (PTO629). Ribosomal subunits prepared here were

used for protein comparisons on polyacrylamide gels and *in vitro* protein synthesis. Analysis in sucrose density gradients was done to measure the purity of these subunits. For this, 0.400-0.600 A_{260} units of ribosomes were layered on 5-20% gradient as described in the Materials and Methods Section. Figure 7 shows representative sedimentation profiles of 50S and 30S subunit preparations. There was little contamination of the 50S preparation with 30S, likewise there was no apparent contamination of 30S subunit preparations with 50S ribosomes. These profiles were representative of the purity of all of the subunit preparations. Analysis by sucrose gradients only gives an indication of the degree of purity of the subunit preparation, for a more quantitative analysis it would be necessary to employ an analytical centrifuge. Although this was not done, the gradients shown here indicate no gross contamination of of either subunit with the other.

B. Comparison of Mutant and Wild Type 50S Ribosomal Proteins

Proteins of the 50S subunit were prepared by the 2-chloroethanol method of Fogel and Sypherd (17). Gels were run as for the 70S proteins with anode at the top and cathode at the bottom. Approximately 40-60 µg of wild type and mutant protein were used for split gels. Figure 8 shows a split gel of mutant and wild type proteins. The mutant is on the right, the wild type on the left. The mutant protein bands which had an altered electrophoretic mobility

Figure 7. Sucrose density gradient analysis of purified ribosomal subunits.

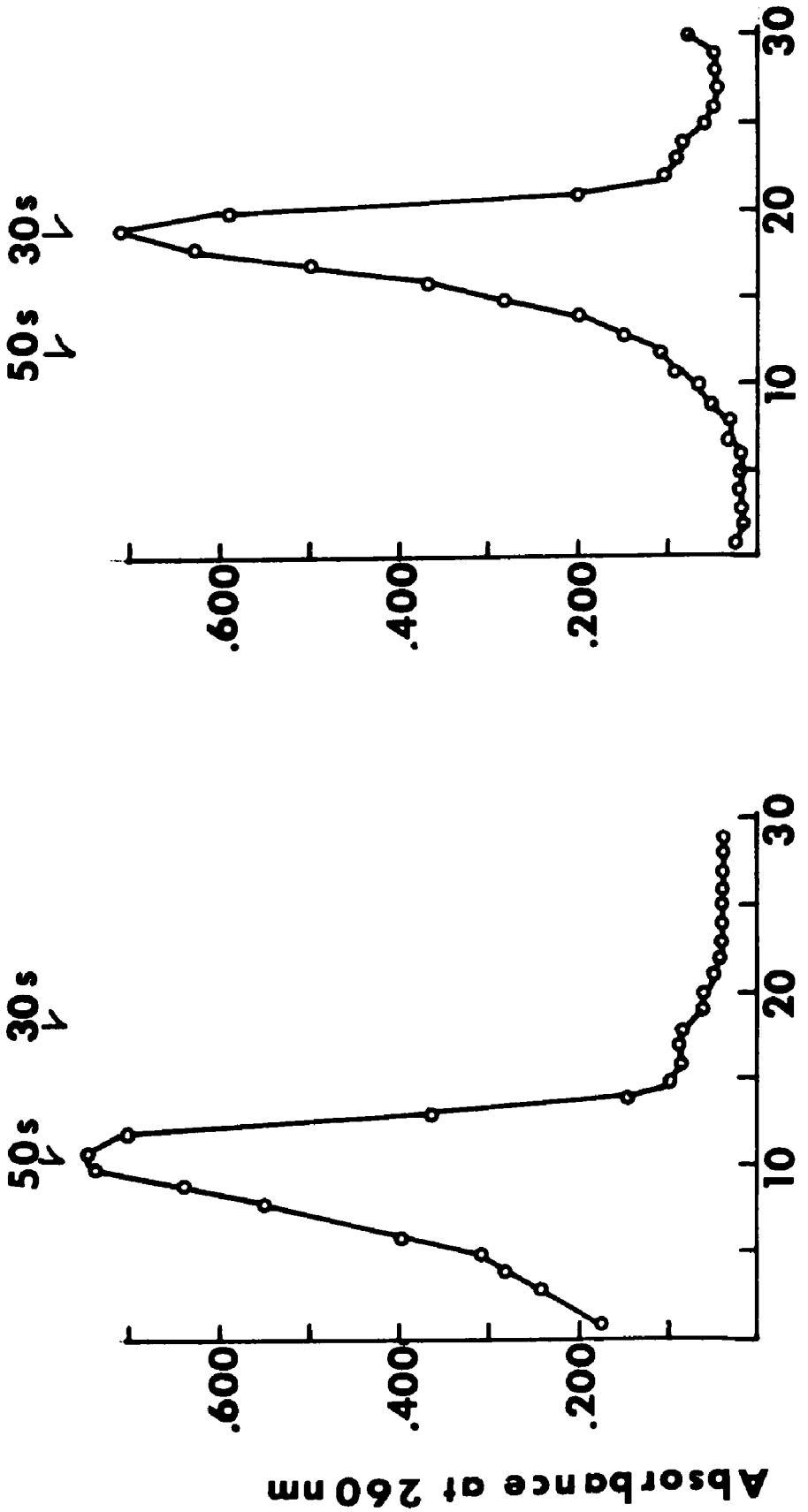
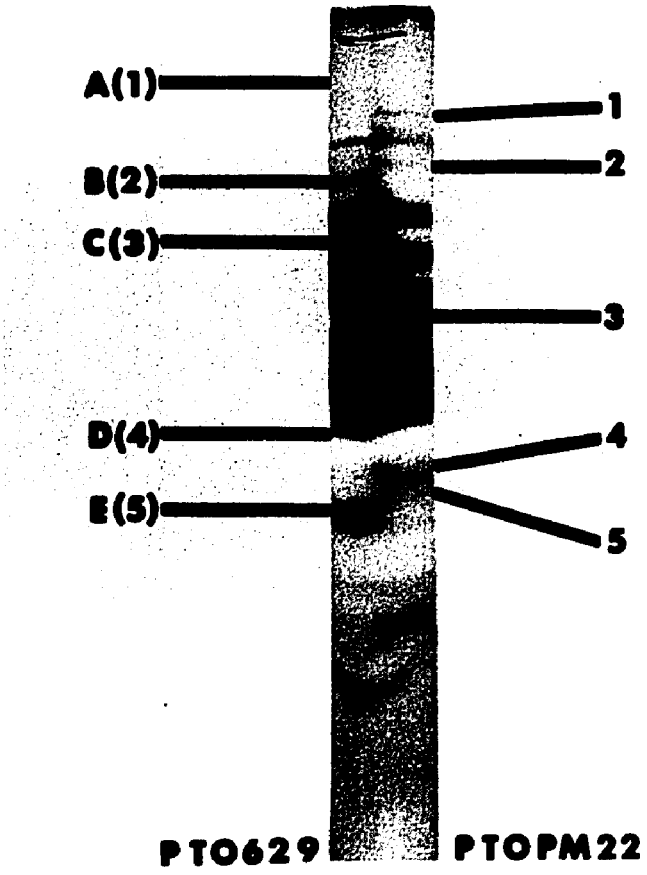


Figure 7

Figure 8. Split gel of mutant, PTOPM22, and wild type, PT0629, 50S ribosomal proteins.

Figure 8



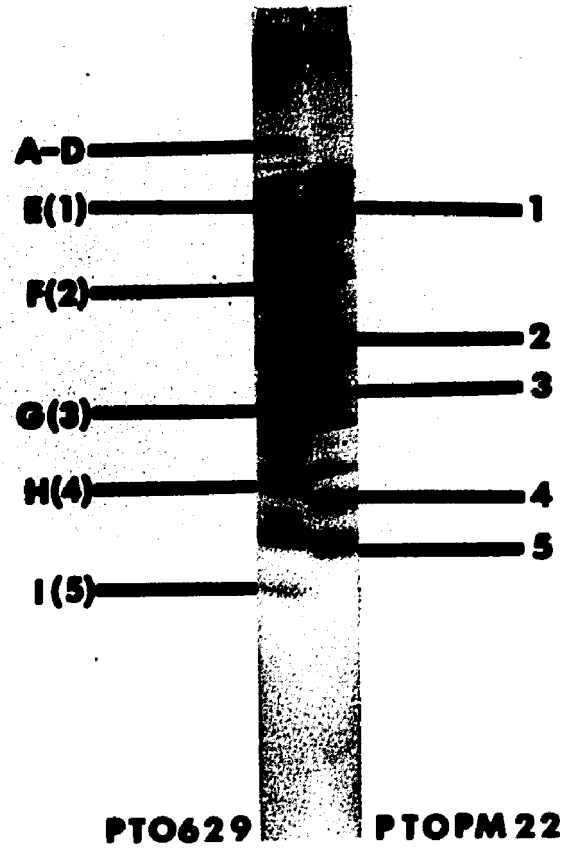
are numbered on the left. Letters identify the wild type protein bands that have no parallel band in the mutant half of the gel. Numbers in parentheses identify the altered mutant proteins that possibly correspond to the wild type proteins. It is significant to note that the changes in electrophoretic mobility of the mutant proteins were both to less basic and more basic electrophoretic properties than the corresponding wild type proteins. A total of 5 altered proteins were resolved. The protein preparations used here was from subunits isolated from a 70S preparation that was not washed with a 1 M sucrose -0.5 M NH_4Cl solution. However, no significant differences were noted when sucrose-NH_4Cl washed subunits were used. As before, the analysis presented here represented a study of many gels of which this one was representative.

C. Comparison of Mutant and Wild Type 30S Ribosomal Proteins

It was necessary to prepare the 30S ribosomal protein by the method of Hardy et al (35). Their method of preparation and gel analysis resolved 19-20 30S proteins of E. coli. As Figure 9 shows, we were able to resolve 19-20 wild type (left side) and mutant proteins (right side). Different gel concentrations and protein concentrations were repeatedly tried but the gel shown in Figure 9 was the best resolution attainable. There were a total of 5 altered mutant proteins, enumerated on the right. Five wild type proteins, E-I are labeled on the left. The altered mutant

Figure 9. Split gel of mutant, PTOPM22, and wild type, PT0629, 30S ribosomal proteins.

Figure 9



bands appeared to correspond to these wild type proteins. However, a large number of small bands, A-D, on the top of the wild type half of the gel had no corresponding bands in the mutant. Perhaps these are factors involved in protein synthesis which have a greater binding affinity for the wild type 30S than for the mutant 30S subunit. If so, possibly they were lost in the process of isolating the mutant 30S subunits. The absence of these may have affected the protein synthesizing capabilities of the mutant 30S subunit, as will be shown in the next section. The altered mutant 30S proteins were changed to both less and more basicity, as were the 50S alterations.

Analysis of the 50S and 30S ribosomal proteins has revealed a total of 40-45 wild type and 35-40 mutant proteins. Nine proteins were shown to be altered in the comparison between wild type and mutant 70S proteins. However, subunit analysis pointed out 10 major changes in mutant 50S and 30S ribosomal proteins and the possible absence of 4 other proteins in the mutant 30S subunit.

Studies of Polyuridylic Acid Directed Incorporation of ^{14}C-phenylalanine by Wild Type and Mutant Ribosomes

Krajewska and Szer (47) and Das and Goldstein (13) have shown *in vitro* that the defect in protein synthesis by *E. coli* at low temperatures appears to involve ribosomal functions. This could be a factor in determining the minimum temperature of growth. Studies of this kind have not been

conducted with Pseudomonas. Therefore, analysis of in vitro protein synthesis by mutant and wild type ribosomal subunits at 3.5 C should indicate if wild type Pseudomonas ribosomes were limiting protein synthesis at low temperature. This would give additional data on functional alterations in the mutant ribosomes with respect to temperature when compared with the wild type. These studies would not tell the nature of the alteration that had taken place on the mutant ribosomes, but should indicate which subunit(s) was involved. These results could then be compared with the changes in ribosomal protein already described.

Since an in vitro incorporating system for Pseudomonas has only been described once (47), we felt it was necessary to determine some characteristics of the system employed here. Certain characteristics such as Mg^{+2} concentration requirements of the poly U directed system were not determined since these were well detailed previously (47).

A. Basic Components of Polyuridylic Acid Directed ^{14}C-phenylalanine Incorporating System

It might be appropriate at this time to describe the ribosomes used in the in vitro incorporating system. The sedimentation analysis of the subunits has already been described. The 70S ribosomes used, were suspended in TNB and sedimentation analysis of the suspension showed that the 70S ribosomes had dissociated into 50S and 30S subunits in

this buffer. This suspension was called the 70S suspension and was used for preliminary *in vitro* studies and the preparation of the purified subunits. Table 5 describes some of the properties of the ribosomes used. In most cases, a ratio of 60-65% RNA to 35-40% protein was maintained. However, PTOPM22 70S ribosomes appeared to be contaminated with some non-specific protein and there was a persistent yellow color to all preparations of PTOPM22 70S ribosomes. This could not be completely removed, even in the purified subunits and may account for the higher protein concentration of the PTOPM22 70S ribosomes. In general, it appeared that 100 μg of ribosomes were present per A_{260} unit. However, since this value varied considerably with PTOPM22 ribosomes, all data was expressed in terms of mg of ribosomal protein as determined by the Lowry method, though this also could have been affected by the contaminating substance. The degree of contamination by the non-specific substance in PTOPM22 ribosomes may also have affected determination of absorbancy at 260 nm. Therefore, wild type PT0629 70S were used for most of the preliminary studies.

For the calculation of the amount of phenylalanine polymerized, it was necessary to assume certain molecular weights for the ribosomes. A molecular weight of 3×10^6 was assumed for 70S ribosomes and 1.8×10^6 for 50S ribosomes (89). Total weight of the ribosome was estimated from the data in Table 5.

TABLE 5

CHARACTERISTICS OF RIBOSOMES USED FOR *IN VITRO* PROTEIN SYNTHESIS

Organism	Ribosomes	% RNA	% Protein	260/280	µg Ribosomes/ A_{260} unit
PT0629	70S	60	40	1.85	105
PT0629	50S	67	33	2.00	95
PT0629	30S	65	35	2.00	115
PTOPM22	70S	55	45	1.75	125
PTOPM22	50S	65	35	1.96	105
PTOPM22	30S	60	40	1.94	105

Nirenberg (66) showed that ATP, GTP and an ATP generating system were needed for optimum *in vitro* incorporation. This was also true for the system used in this investigation. Deletion of any reaction mixture components resulted in decreased or negligible incorporation.

It was necessary to determine the effect of poly U and tRNA on the *in vitro* system. For this, an experiment was conducted in which the effect of the addition of these components on total incorporation could be determined. In this experiment, 0.1 ml duplicate samples were taken at 45 minutes using 0.2 ml TD pipettes. The specific activity of ^{14}C-phenylalanine was 1.5 mC/mM. For all reactions at 25 C, a 10 minute preincubation of all components was done before the addition of poly U. All components were present

except for poly U and tRNA which were added as described in Table 6. The poly U was added at 180 µg/mg ribosomal protein and the tRNA at 1 mg/ml reaction. Table 6 shows the data obtained from this kind of experiment. This showed the requirement not only for poly U as exogenous mRNA, but also for exogenous tRNA. The complete system incorporated on the average 10-15 phenylalanine residues/ribosome. This was slightly lower than the value obtained by Gilbert (27) with an E. coli in vitro protein synthesizing system. However, if only 20-30% of our ribosomes were active as Gilbert showed for his system, then incorporation per active ribosome could have been as high as 60-80 phenylalanine residues. The incorporation by PTOPM22 was about one-third that by PTO629 perhaps, by analogy, indicating a much lower number of active ribosomes in those preparations.

In the course of developing the in vitro system, it was necessary to change from the millipore filter assay of Nirenberg (65) to the filter assay of Mans and Novelli (56). Millipore filters gave poor reproducibility between duplicate samples. Duplicate samples varied by as much as 20-30%. However, the filter paper method gave a variation of around 5% for most duplicate samples. Filter paper assay results also demonstrated lower backgrounds and greater reproducibility between experiments than did the millipore assay.

TABLE 6

EFFECT OF THE ADDITION OF POLY U AND tRNA ON INCORPORATION OF ^{14}C-PHENYLALANINE BY PT0629 70S RIBOSOMES

Reaction Constituents	cpm/mg Ribosomal Protein	nmoles Phenylalanine/mg Ribosomal Protein
no poly U no tRNA	185	0.08
plus poly U no tRNA	4,640	2.1
no poly U plus tRNA	380	0.18
plus poly U plus tRNA	17,650	8.0

B. Polyuridylic Acid Dependence of ^{14}C-phenylalanine Incorporation

Krajewska and Szer (47) showed with a psychrophilic Pseudomonas that poly U did not saturate the system except at very high levels, (150-250 µg/mg of ribosomes). This phenomenon was not temperature dependent, but was intrinsic to the system. This level of poly U was 2-3 times that optimal in their E. coli system. Since they used a psychrophilic Pseudomonas, it was necessary to determine if the same result would be observed by us with a mesophilic Pseudomonas.

PT0629 ribosomes were used at 1 mg protein/ml reaction mixture. Poly U was added at varying concentrations to the preincubated reaction mixture and duplicate 0.10 ml samples

were taken after 45 minutes incubation at 25 C. The specific activity of ^{14}C-phenylalanine in the reaction mixture was 1.5 mC/m M. Figure 10 illustrates the saturation curve with poly U for the PT0629 system at 25 C. The specific activity was 5 mC/mM. 0.05 ml duplicate samples were taken at 60 minutes. There does not appear to be a finite optimum concentration. Since it appears from Figure 10 that a plateau was reached in poly U saturation around 250 µg/mg ribosomal protein, the incorporation for three concentrations in this range was determined. Table 7 illustrates that incorporation was constant over the range 200-350 µg/mg ribosomal protein. Therefore, 200-250 µg of poly U per mg ribosomal protein was routinely used in the reaction mixtures. Since Krajewska and Szer (47) found no effect of temperature on the amount of poly U necessary for maximum incorporation, no other temperature was studied. Analysis of the poly U saturation of the system containing PTOPM22 ribosomes was not done. However, varying the concentration from 200-300 µg did not show inhibition or gross stimulation of the reaction over that at 250 µg poly U/mg ribosomal protein.

C. Rate of Polyuridylic Acid Directed ^{14}C-phenylalanine Incorporation at 25 C and 3.5 C

It should be stated at this point that S-100 from PT0629 was used in all experiments with either PT0629 or PTOPM22 ribosomes, except where noted otherwise. This was necessary because the S-100 of PTOPM22 lost activity rapidly,

Figure 10. Poly U dependence of ^{14}C-phenylalanine incorporation at 25 C.

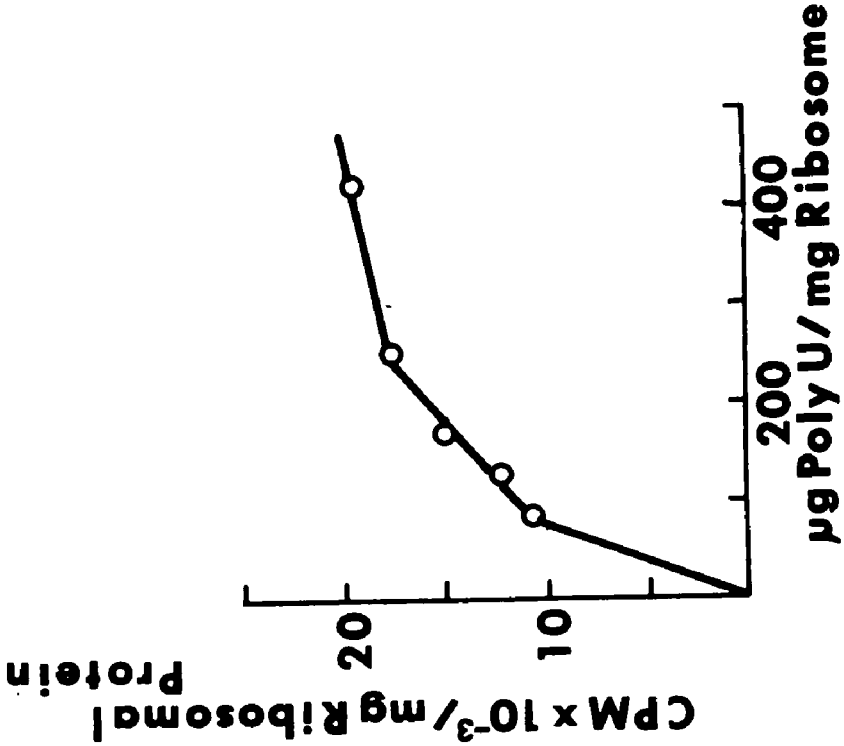

CPM x 10⁻³/mg Ribosomal Protein
20
10
200
400
µg Poly U/mg Ribosome

Figure 10

whether stored at 4 C or frozen at -40 C. The significance of this will be discussed later.

TABLE 7

POLYURIDYLIC ACID DEPENDENT INCORPORATION OF ^{14}C-PHENYLALANINE

Poly U µg/mg Ribosomal Protein	cpm/mg Ribosomal Protein
210	20,100
250	20,800
330	19,600

The rate at 25 C was determined using 260 µg of poly U/mg ribosomal protein. The specific activity at 25 C was 5 mC/mM. Ribosomes were added at 0.9 mg protein/ml reaction. 0.05 ml samples were taken at the times indicated in Figure 11a. Figure 11a illustrates the rate of incorporation of ^{14}C-phenylalanine at 25 C. The PT0629 reaction reached completion at 30 minutes, whereas the PTOPM22 reaction was complete in 15 minutes. However, the incorporation per mg of ribosomal protein with PTOPM22 ribosomes was only 40% of that observed with PT0629 ribosomes. This difference may reflect inaccuracy in the determination of the amount of ribosomes present in PTOPM22 70S preparations, again due to contaminating proteins. If there were fewer ribosomes present then the incorporation values would be lower per mg of protein. As

will be shown later, using purified subunits, a difference of this magnitude was not observed.

The rate of phenylalanine incorporation at 3.5 C was determined using the same concentration of poly U and ribosomes. However, the specific activity was raised to 10 mC/mM. Samples, 0.05 ml, were taken at the times indicated in Figure 11b. Completion of the reaction was not reached even at 180 minutes with either PT0629 or PTOPM22 ribosomes. However, at about 90 minutes the reaction began to slow down. Though there was still a difference between incorporation by PT0629 and PTOPM22 ribosomes it was not as great as at 25 C. In this regard, Krajewska and Szer (47) stated that for use of poly U below 6 C the ribosomes and poly U must be preincubated at 20-25 C and then chilled to the desired low temperature followed by addition of other prechilled components. This was necessary to prevent a conformational alteration of the poly U at low temperature that was inhibitory to the reaction. Table 8 shows that preincubation of the ribosomes and poly U at 25 C before shifting to 3.5 C did increase the incorporation. The specific activity was 1.5 mC/mM, and samples were taken in duplicate at 180 minutes. The non-preincubated reaction had all components prechilled at 3.5 C and then mixed at 3.5 C. Although preincubation was to stabilize poly U conformation, the effect of preincubation actually could have been an activation of the mesophilic ribosomes, similar to that observed by Das and Goldstein (13). However,

Figure 11a. Rate of ^{14}C-phenylalanine incorporation at 25 C by PT0629 and PTOPM22 70S ribosomes.

Figure 11b. Rate of ^{14}C-phenylalanine incorporation at 3.5 C by PT0629 and PTOPM22 70S ribosomes.

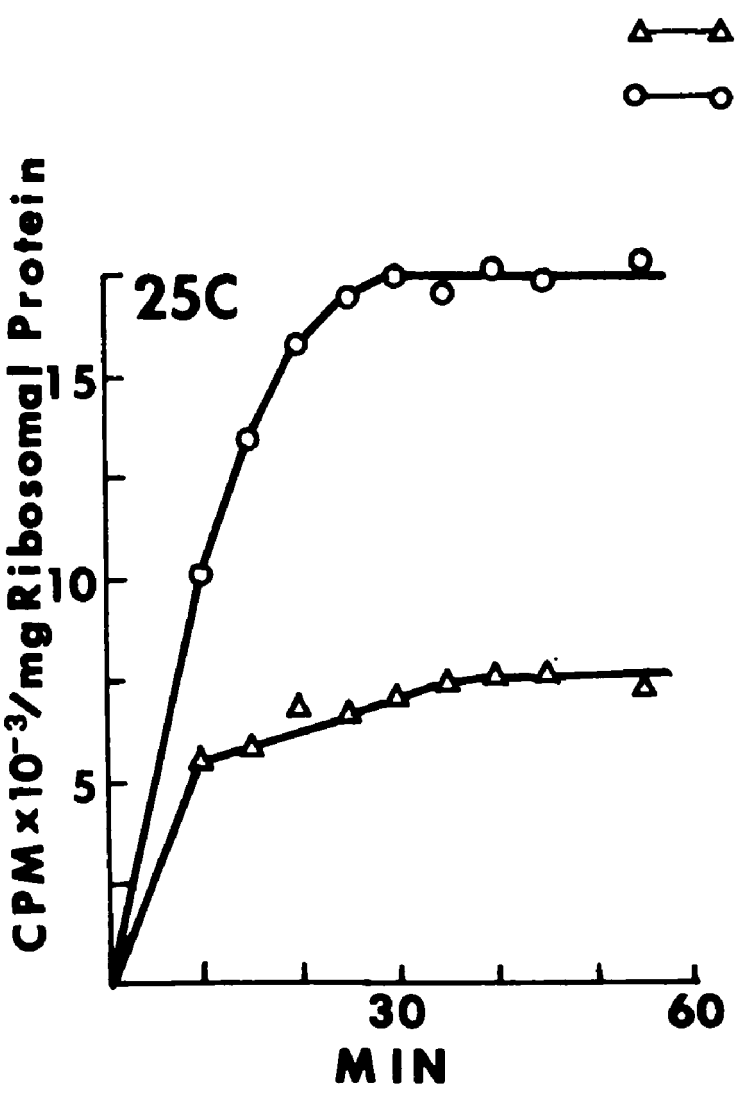


Figure 11a

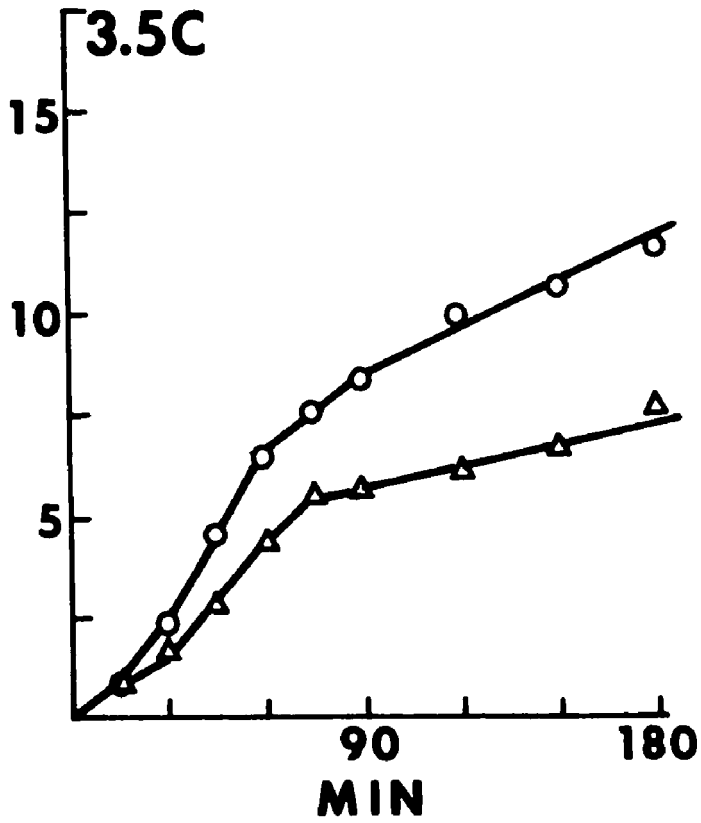


Figure 11b

Krajewska and Szer (47) observed no activation of mesophilic ribosomes following preincubation at 20 C.

TABLE 8

NECESSITY OF PREINCUBATION AT 25 C FOR POLY U DIRECTED INCORPORATION OF ^{14}C-PHENYLALANINE AT 3.5 C

Reaction Conditions	cpm/mg Ribosomal Protein
preincubated at 25 C for 10 minutes	6,560
not preincubated at 25 C for 10 minutes	3,500

Therefore, from studies of the rates at 25 C and 3.5 C, and analysis of preincubation requirements for poly U directed incorporation of phenylalanine at 3.5 C, end points of 45-60 minutes at 25 C and 180 minutes at 3.5 C were used. Also, for any experiments at 3.5 C, the ribosomes of both PT0629 and PTOPM22 were mixed with poly U and preincubated at 25 C for 10 minutes.

D. Heterologous Combinations of S-100 and Ribosomes from Mutant and Wild Type

Studies by Das and Goldstein (13) and Krajewska and Szer (47) showed that the ribosomes were limiting protein synthesis at low temperature in E. coli. However, we considered it necessary to determine if the wild type Pseudomonas S-100 would limit protein synthesis at 3.5 C, since this had not been determined previously for mesophilic Pseudomonas. The specific activity was 1.5 mC/mM, with

1.4 mg/ml PT0629 ribosomal protein and approximately 0.63 mg/ml PTOPM22 ribosomal protein. Incubation was for 60 minutes and samples were taken in duplicate. The efficiency of the heterologous combination was about 68% for mesophilic ribosomes and psychrophilic S-100. This was similar to the findings of Krajewska and Szer (47). However, they found 100% efficiency with psychrophilic ribosomes and mesophilic S-100 when compared with homologous psychrophile system. However, in this system, the psychrophilic mutant ribosomes in combination with mesophilic S-100 were only 65% as efficient as the homologous psychrophilic mutant combination. The mutant S-100 used here was from a large batch of cells that were lysed and the S-100 prepared rapidly. This S-100 was used as soon as the ribosomes were pelleted. However, this S-100 also was not stable upon storage and showed little activity 1 day later.

TABLE 9

EFFICIENCY OF HETEROLOGOUS COMBINATIONS OF S-100 AND RIBOSOMES AT 25 C

Ribosomes	S-100	cpm/mg Ribosomal Protein	% of Homologous
PT0629	PT0629	13,300	(100)
PT0629	PTOPM22	8,980	67.5
PTOPM22	PTOPM22	4,220	(100)
PTOPM22	PT0629	2,730	64.7

Table 10 shows the results of analysis of the S-100 and ribosome combinations at 3.5 C. S-100 from PT0629 was more efficient than that from PTOPM22 regardless of the source of the ribosomes. This may have something to do with the rapid loss of activity of PTOPM22 S-100 on storage. The low incorporation levels in the PTOPM22 system were unexplained. However, in later experiments, using purified subunits the activity of PTOPM22 ribosomes at 3.5 C was 70% that at 25 C. This was comparable to that found by Krajewska and Szer (47). Possibly gross contamination by the yellow substance in PTOPM22 70S preparations caused the loss of activity at 3.5 C.

TABLE 10

EFFICIENCY OF HETEROLOGOUS COMBINATIONS OF S-100 and RIBOSOMES AT 3.5 C

Ribosomes	S-100	cpm/mg Ribosomal Protein	% of Homologous
PT0629	PT0629	6,250	(100)
PT0629	PTOPM22	1,020	16.3
PTOPM22	PTOPM22	157	(100)
PTOPM22	PT0629	610	382

E. Determination of Endogenous Phenylalanine in PT0629 S-100

The specific activities that have been given so far did not include the endogenous pool of ^{12}C-phenylalanine, only the ^{12}C-phenylalanine added to the reaction.

Since the S-100 of PT0629 used in these experiments was not dialyzed, there could be a significant endogenous pool of ^{12}C-phenylalanine. It was necessary to determine this pool to obtain the true amount of phenylalanine polymerized in the system.

The basic reaction mixture contained 1.0 µc of ^{14}C-phenylalanine/ml and 0.08 ml S-100 (2.80 mg)/ml reaction. Table 11 shows the effect on ^{14}C-phenylalanine incorporation of adding ^{12}C-phenylalanine to the reaction. Using the data from this table it was possible to calculate the endogenous pool of ^{12}C-phenylalanine in the S-100. This was done by setting up the following ratio:

$$\frac{\text{µmoles endogenous phenylalanine}}{\text{µmoles added + µmoles endogenous}} = \frac{\text{cpm/ml reaction with added phenylalanine}}{\text{cpm/ml reaction without added phenylalanine}}$$

The contribution of phenylalanine by the ^{14}C-phenylalanine suspension was negligible. Employing this equation an average value of 0.043 µmoles phenylalanine/0.08 ml S-100 was obtained. This was equivalent to 15.0 nmoles phenylalanine/mg total S-100 protein. Therefore, taking this value into account, the maximum incorporation by PT0629 at 25 C was approximately 12-18 phenylalanine residues/ribosome. One-third this value was obtained when 70S PT0PM22 were used.

TABLE 11

DILUTION OF ISOTOPE FOR DETERMINATION OF ENDOGENOUS PHENYLALANINE IN PT0629 S-100

Amount ^{12}C-phenylalanine added/ml reaction	cpm/ml Reaction
none	69,300
0.1 µmole	21,700
0.2 µmole	11,600

F. Efficiency of in vitro Protein Synthesis by Heterologous Ribosomal Subunit Combinations at 25 C

Using heterologous combinations of mesophilic and psychrophilic ribosomal subunits, Greenwald and Szer (29) were able to show that the E. coli 50S was defective in in vitro protein synthesis from 2-9 C. Since it has been shown here that the S-100 of the mesophilic wild type, PT0629, did not appear to be limiting protein synthesis at low temperature, analysis of the functional capabilities of the ribosomal subunits would indicate if a wild type subunit was limiting protein synthesis at low temperature. This would also indicate if the protein alterations already observed in mutant subunits were associated with a functional change.

The subunits used in these experiments have already been described. The specific activity in these experiments was 1.5 mC/mM not including the endogenous ^{12}C-phenylalanine pool. When the endogenous pool was included in the calculation

of specific activity it was 1.23 mC/mM. This value was used to calculate the amount of phenylalanine polymerized. In these reactions, the 50S subunits were limiting. Therefore, the data was expressed as cpm/mg 50S subunit protein. The protein amounts of subunits were as follows, PT0629 50S 0.670 mg/ml, PT0629 30S 0.600 mg/ml, PTOPM22 50S 0.580 mg/ml and PTOPM22 30S 0.560 mg/ml. Samples (0.10 ml) were taken at 60 minutes at 25 C and 180 minutes at 3.5 C in duplicate. The usual preincubations were done at 25 C for the reaction at 3.5 C.

Table 12 shows the results for incorporation of phenylalanine by heterologous and homologous subunits at 25 C and 3.5 C. The data shown are the average of 2 experiments. At 25 C all of the combinations were functional, however, it did appear that the 30S of PTOPM22 may be limiting to some extent at 25 C. This may be due to the absence of certain proteins already mentioned. When the same combinations of subunits were analyzed at 3.5 C a quite different result was observed. Combinations of subunits containing wild type mesophilic (PT0629) 50S had a much lower level of incorporation at 3.5 C than observed at 25 C. Incorporation at 3.5 C was about 25% that observed at 25 C. However, combinations of mutant 30S and 50S, and wild type 30S and mutant 50S incorporated at 3.5 C approximately 70% of that observed at 25 C. This ratio was similar to that observed by Krajewska and Szer (47) for wild type psychrophile ribosomes at 20 C and 3 C. Therefore,

this indicated that in this system the wild type mesophile 50S ribosomal subunit was in some way limiting protein synthesis at 3.5 C. Though a limitation was observed by the 50S mesophile subunit, the incorporation by the homologous mesophile (PT0629) subunit combination was considerably higher than that observed by Krajewska and Szer for E. coli ribosomes at 3 C. They observed 0.05 nmoles phenylalanine incorporated/mg 70S ribosome with a ratio of 0.016 for 3 C/20 C. Therefore, preincubation at 25 C may be activating some of the wild type ribosomes used in this study.

TABLE 12

HETEROLOGOUS SUBUNIT INCORPORATION OF ^{14}C-PHENYLALANINE AT 25 C AND 3.5 C

Subunits		nmoles phenylalanine incorporated/mg 50S ribosomal protein		Ratio of 3.5/25 C Activity
50S	30S	25 C	3.5 C	3.5/25 C
PT0629	PT0629	3.7	1.0	.27
PT0629	PTOPM22	2.9	0.6	.21
PTOPM22	PT0629	3.8	2.4	.64
PTOPM22	PTOPM22	2.9	2.1	.72

G. Incorporation by PTOPM22 Ribosomes at 37 C

The mutant 50S ribosomal subunit was shown to have altered function(s) at low temperature when compared with

the wild type 50S subunit. This functional change in the mutant 50S may offer an explanation for the lowering of the minimum growth temperature of the wild type by mutation. However, the concomitant lowering of the maximum growth temperature with the minimum growth temperature is still unexplained. In this regard, the observation that there were protein changes in both the subunits, not just the 50S, may be important to the lowering of the maximum growth temperature. There could be a certain interaction of ribosomal subunits at elevated temperatures that is necessary for their activity, and possibly this interaction was impaired. Therefore, to answer this question, the *in vitro* activity of the mutant subunits was determined at 37 C. Table 13 shows that the mutant subunits were only 50% as efficient at 37 C as they were at 25 C. Wild type subunits were equally efficient at 25 C and 37 C. The incubation time was 30 minutes at 37 C, otherwise the reaction mixture was run as at 25 C. These results indicate that the mutant ribosomes may not be active at elevated temperatures *in vivo* and hence may explain the lowering of the maximum growth temperature concomitant with the lowering of the minimum, which was shown to be 50S dependent. This limitation of protein synthesis at higher temperatures for psychrophilic mutant ribosomes was contrary to the observation with wild type psychrophiles by Krajewska and Szer (47). They showed that wild type psychrophile ribosomes functioned equally well *in vitro* at both 20 C and 37 C. On the other hand,

this limitation in protein synthesis at 37 C by mutant subunits may only involve the protein alterations shown for the 30S subunit. In support of this concept, a mutation in the 30S subunit of E. coli was shown to cause a defect in protein synthesis at elevated temperatures (44). Therefore, the protein alterations already shown for the psychrophilic mutant 30S subunit, which may be trivial to the activity of ribosomes at low temperatures, may be reflected in a limitation of protein synthesis at high temperature which is non-permissive for the growth of the psychrophilic mutant.

TABLE 13

INCORPORATION AT 37 C BY PTOPM22 SUBUNITS

Temperature	cpm/mg 50S Ribosomal Protein
25 C	5150
37 C	2610

In summary, this study has shown, by comparing psychrophilic mutant and transductant ribosomes with their mesophilic wild type ribosomes, that certain physical differences observed previously by others between wild type mesophilic and psychrophilic ribosomes did not obtain here. Thus, these differences previously observed by others may be trivial to establishing the role of the ribosomes in the

determination of the growth temperature range of bacteria. On the other hand, it was found in this investigation that the protein composition of the ribosome may determine its functional capabilities in response to temperature fluctuations. In this regard, the protein changes observed for the 50S subunit correlated with the gain in activity by the mutant 50S subunit, at low temperature. Finally, limitation of protein synthesis at elevated temperatures normally permissive to the wild type may reflect the alteration of both the 50S and 30S subunits.

DISCUSSION

The physiological bases for psychrophily and mesophily have not been resolved. However, a central theme emerges from previous studies of the factors involved in the determination of bacterial growth temperature range. Namely, regulation of enzyme synthesis and function with temperature is a major factor in the determination of growth temperature range. The studies from which this theory resulted involved primarily the analysis and comparison of various enzyme systems from mesophiles and psychrophiles. These studies for the most part dealt with protein molecules, whose activity is defined by their structure and configuration. Protein structure is determined by its amino acid composition, which is prescribed by the genetic message. However, the configuration which a polypeptide assumes, and other macromolecules as well, is determined by many factors, such as ionic strength, pH and temperature (102). Of these factors, only temperature is relevant to our discussion.

A protein molecule will assume active and inactive configurations depending on the temperature (102). Most protein enzymes are active over a wide range of temperature; however, they are rapidly inactivated if the temperature becomes too high or too low. A protein must be able to

assume an active form at the temperature at which it is synthesized. Thus, an overriding consideration for bacterial growth is that enzymes be synthesized in a form which can assume a functional conformation at the incubation temperature. Therefore, within the growth temperature range of a cell, the site of protein synthesis, the ribosome, must function correctly. In this regard, certain ribosomal properties have been found to correlate with bacterial growth range. These properties can be divided into two groups, the structural and the functional aspects of the ribosome. The study of these properties by others indicates that the ribosome may be a significant factor in the control of protein synthesis with temperature and hence may influence the growth temperature range of a bacterium.

Pace and Campbell (73) showed that the degree of rRNA-protein interaction in the ribosomes, as measured by Tm, correlated with maximum growth temperature. Stenesh and Holazo (93) reported similar findings utilizing the effect of urea on isolated rRNA. However, results presented in this investigation show that these physical characteristics of the ribosome were trivial to growth temperature range. Therefore, some aspect other than the properties assessed by the above, may also be involved in the ribosome-dependent determination of growth temperature range.

With regard to the activity of ribosomes with temperature, Das and Goldstein (13), and Krajewska and Szer (47) showed that mesophilic ribosomes were limiting for protein synthesis at low temperature, whereas the psychrophilic ribosomes functioned at the same temperature. Greenwald and Szer (29) subsequently reported that the mesophilic 50S subunit limited protein synthesis at low temperature while the mesophilic 30S ribosomal subunit did not. Results presented in this investigation supported the involvement of the mesophilic 50S subunit in the limitation of protein synthesis at low temperature. In addition, we showed that the limitation of protein synthesis by the mesophilic 50S could be overcome by conversion to psychrophily.

Since protein composition determines the activity of the ribosomes, it may also specify their temperature response. Altered proteins may be demonstrated when comparing wild type ribosome with proteins from psychrophilic mutant ribosomes. This is particularly important, since the physical characterization of wild type and mutant ribosomes did not denote any significant differences between them.

No studies of ribosomal proteins from mesophiles and psychrophiles have been reported previously. However, many comparisons by polyacrylamide gel electrophoresis were reported for the identification of ribosomal protein mutations. Examples of these comparisons were: The work of

Leboy, Cox and Flaks (50) showing that E. coli K12 had a different ribosomal protein than other strains of E. coli; and Krembel and Apirion (48) who reported altered ribosomal proteins upon mutation in the lir locus, a locus which specifies sensitivity to lincomycin and erythromycin. Therefore, based on this precedent, polyacrylamide gel electrophoresis provided a valid means for comparing ribosomal proteins from a psychrophilic mutant or transductant with ribosomal proteins of the mesophilic progenitor. Comparisons of this type could denote alterations in the electrophoretic character of progenitor proteins upon conversion to psychrophily.

Our results with the psychrophilic transductant showed that at least six ribosomal proteins of the mesophilic genetic recipient were altered upon conversion to psychrophily. These six altered proteins were similar to six proteins in the psychrophilic genetic donor. The large number of altered proteins was not anticipated. However, in the comparison of the psychrophilic mutant and wild type proteins, we expected less genetic alteration. This seemed to be a logical assumption, since revertants of the mutant could be isolated, indicating the existence of a point mutation. However, contrary to expectations, our observations in this investigation indicated that there were at least 10 altered proteins in the mutant ribosomes.

Observation of numerous altered proteins implied that the altered ribosomal activity and protein composition

might be the result of some generalized effect on translation of the genetic code. This would be consistent with the pleiotropic changes observed upon conversion to psychrophily. In this regard, Reid et al (76, 77, 23) reported the isolation of an ochre suppressor mutation in E. coli that resulted in multiple ribosomal protein changes. They characterized the mutation as a point mutation which altered tRNA. The suppressor mutation also resulted in an unusual physiology, high reversion rate and abnormal 30S ribosomal subunits. Apirion (2) isolated a strain of E. coli with a suppressor mutation that caused altered ribosomes, however he was not able to demonstrate altered ribosomal proteins. Thus, mutations can be isolated which act at the level of translation and cause altered ribosomal function and/or altered ribosomal proteins. However, no mutation has been reported which exhibited the magnitude of altered ribosomal proteins coincident with altered ribosomal activity that were observed in this investigation.

Though the genetic alteration responsible for the conversion of mesophiles to psychrophiles was unknown, it was possible to speculate on its nature. The first assumption was, as already stated, that the genetic event resulting in altered ribosomal proteins and hence altered ribosomal activity, must have affected the general translation of the genetic message. The second assumption was that the mutation to psychrophily was a point mutation. Therefore, it was possible to eliminate certain mutational

events as causes of our observations pertaining to altered ribosomal proteins. The isolation of psychrophilic mutant revertants eliminated a deletion as the genetic alteration.

E. coli ribosomal mutations have been most frequently studied although mapping has not revealed any concentration of loci large enough to be affected by a single mutation resulting in the number of alterations observed in this investigation (72). However, since we were dealing with Pseudomonas, comparisons with observations in E. coli may not be correct. In this regard, reports of the genetic map of P. aeruginosa showed that related genetic markers did not appear contiguously as in E. coli, but were scattered around the chromosome in clusters of 2 or 3 genes (38). This provided further circumstantial evidence that the mutation to psychrophily, resulting in many altered ribosomal proteins, must have affected some generalized cellular function, such as translation of the genetic message.

The most logical possibilities for the genetic alteration were factors which controlled fidelity of translation. These could be divided into two groups: (a) fidelity at the ribosome level; (b) fidelity at the level of tRNA. With regard to fidelity at the ribosome level, Rosset and Gorini (80) characterized a ribosomal ambiguity (ram) mutation of the 30S subunit of E. coli. This mutation caused the ribosomes to extensively misread the genetic

message *in vitro*. *In vivo* the mutation conferred on the cell the ability to suppress all nonsense codons non-specifically. This type of mutation could result in altered proteins in the cell and would be consistent with the pleiotropic nature of the mesophiles converted to psychrophily. Effects similar to those just described can be found upon mutation to streptomycin resistance, which affects the fidelity of codon recognition by the 30S ribosomal subunit (80).

The second possibility, involving the fidelity of translation at the level of tRNA, seemed to be even more plausible than the ambiguity mutation. This mutation would result in suppression of the translation of mRNA causing the wrong amino acid to be substituted at a certain codon. Suppressor mutations have been shown to cause pleiotropic effects that were not related to the suppressed phenotype (22). Similarly, suppressor mutations can cause temperature-sensitive bacterial growth (14). Apirion (2) reported a suppressor mutation which caused temperature-sensitive growth of *E. coli* and altered ribosomes. This indicated that the suppressor may have acted at the level of the ribosome. Reid *et al* (23, 76, 77) characterized an *ochre* suppressor mutation as a suppressor tRNA which caused a number of altered ribosomal proteins and a variety of pleiotropic effects. Therefore, a mutation resulting in a suppressor tRNA may account for the number of altered

ribosomal proteins and pleiotropic effects observed here for conversion to psychrophily. This mutation could be expressed by the alteration in the specificity of an amino-acylating enzyme. The latter seemed most likely since the electrophoretic changes in ribosomal proteins, observed here for the mutation to psychrophily, were both to more and less electrophoretic movement. If there had been a constant substitution of one specific amino acid for another, the electrophoretic change would have been undirectional. That is, if a neutral amino acid had substituted for a basic one, the protein would have been made less basic. However, as was the case here there was change to both more and less basicity. This would indicate a greater lack of specificity in the substitution of amino acids. This could occur by an alteration of a specific amino-acylating enzyme that resulted in multiple substitutions of amino acids by a specific tRNA. Or there could be a factor, analogous to the sigma factor of RNA polymerase, that aids in determining the specificity of amino-acylating enzymes and this factor may have been mutated so that there was a general loss of specificity for acylation of tRNA's. Another possibility is that there was more than one tRNA species which was mutated; however it seems unlikely that a point mutation caused a multitude of altered tRNA's.

A general loss of fidelity in translation, as described above, would be expected to affect the growth rate of the mutant cells. In this regard, the growth rate

of the mutant was observed to be considerably less than that of the wild type at temperatures permissible for both organisms.

Another possible explanation for the alterations in the electrophoretic mobilities of the ribosomal proteins of the mutant was the yellow substance contaminating most preparations of mutant ribosomes. This substance could have influenced the behavior of the ribosomal proteins of the mutant in polyacrylamide gels and might also have affected the activity of the mutant ribosomes with temperature. However, this seemed unlikely since there was no difference in polyacrylamide gel protein patterns between 50S mutant subunits which were washed with 1M sucrose-0.5 M NH_4Cl and those which were not. Visual observation of the sucrose washed ribosomes indicated little if any contaminating substance, though this did not exclude low levels of contamination. If the activity of the ribosomes was influenced by the contaminating substance then both subunits should have been affected since both preparations were contaminated. In this regard, the ratio of the mutant ribosome activity at 25 C and 3.5 C was comparable to that observed for wild type psychrophiles by Krajewska and Szer (47). This indicated that, though the ribosomes of the mutant were contaminated, their response to temperature was not affected by the contaminating yellow substance.

So far this discussion has involved the nature of the genetic alteration in the psychrophilic mutants;

however, the nature of the genetic change in the psychrophilic transductants must also be considered. For transduction to take place there had to be an insertion of donor genetic material into the recipient chromosome, which obviously resulted in the alteration of some function of the mesophilic recipient which allowed it to grow at low temperature. In this regard, a ram mutation or suppressor tRNA seemed possible explanations for the many altered proteins and the pleiotropic effects that occurred concomitant with transduction to psychrophily. A ram mutation could come about if a gene was transduced into the recipient which coded for a ribosomal protein controlling the fidelity of codon recognition on the 30S ribosome. If this was inserted with a concomitant deletion or inactivation of the normal gene for this function, there might be a high level of ambiguity induced. Another possibility was that a transduced ribosomal protein might not be entirely homologous with the recipients ribosomal components and thus result in a distortion of ribosome conformation and an ambiguity in translation.

A similar situation could exist for a suppressor tRNA. If a gene for an amino-acylating enzyme was transduced into the recipient that had a different specificity for tRNA than the normal enzyme, then the substitution of an amino acid could result. Another possibility was that a factor, previously mentioned as analogous to sigma factor, was transduced which affected the specificity of

amino-acylating enzymes. Then a whole spectrum of amino acid substitutions could result if this factor altered the specificity of the wild type enzymes. This factor might alter the specificity of tRNA recognition by the amino-acylating enzymes.

Therefore, we postulated that conversion to psychrophily by either mutation or transduction may have altered the fidelity of translation. This alteration would be the result of either a ribosomal change affecting codon recognition (a ram mutation) or a change involving tRNA resulting in the substitution of amino acids, i.e. a suppressor tRNA. Consequently, the lowering of the maximum and minimum growth temperature of mesophilic Pseudomonas may be an indirect result of the above postulated genetic changes.

An alteration in translation could explain the low level of activity and the instability observed for the mutant S-100 in this investigation. This instability could be explained by the synthesis of many altered or faulty proteins. If this were so, the lysis of the cells in the preparation of ribosomes and S-100 may destroy some necessary balance between active and inactive proteins. Possibly the continued synthesis of proteins is required because of the high turnover rate that might result from the instability of the proteins that are synthesized. Thus, when the S-100 is prepared, the altered proteins rapidly become unstable

and inactive without replacement proteins, accounting for the low activity of the S-100 on storage.

The results of this investigation, while showing an alteration of mesophile ribosome function with temperature upon conversion to psychrophily, have not elucidated the altered ribosomal function. However, it is possible to speculate as to the nature of the altered function. The best way to do this is to correlate *in vitro* results with *in vivo* results. In this regard, Friedman *et al* (21) studied the effect of low temperature on the polyribosome pattern of *E. coli*. Their results indicated that, if protein synthesis was initiated at temperatures permissive for cell growth, it would continue at temperatures below the minimum for growth. However, they showed that at low temperature the initiation of new protein synthesis was blocked. Comparing the above with the *in vitro* results, which showed that the mesophile 50S was limiting for protein synthesis at low temperature, one must conclude that the mesophile 50S was limiting initiation of protein synthesis *in vivo* at temperatures below the minimum for growth. This was interesting since Nomura, Lowry and Guthrie (67) showed that the initiation complex of formylmethionyl-tRNA-30S ribosomes-mRNA was an obligatory step for the initiation of protein synthesis. Their results indicated that the 50S subunit was not required until the initiation complex formed. Consequently, the limitation of protein synthesis in the case of mesophiles at low temperature might occur after the

formation of the initiation complex. The 50S could block the translocation of the formylmethionyl-tRNA to the peptidyl site from the aminoacyl site on the 30S subunit. This could occur if the peptidyl site of the mesophile 50S ribosome underwent a conformational change at low temperature that did not allow it to accept a tRNA molecule.

Another possible explanation for the limitation of protein synthesis at low temperature by the mesophile 50S is that the 50S subunit may affect the 30S subunit initiation complex. In this regard, Nomura et al (67) reported that the 50S subunit was a specific inhibitor for the formation of the initiation complex. The 50S subunit at low temperature could distort the initiation complex causing a release of the bound tRNA.

The enzymes of the 50S do not appear to be the limiting factor at low temperature, since Friedman et al (21) showed that if protein synthesis was initiated at permissive temperatures it continued at low temperature. Therefore, the 50S activity of peptide elongation and translocation of tRNA must have continued at low temperature. However, this did not rule out some hitherto unidentified enzymatic activity as the limiting aspect of the 50S subunit for protein synthesis at low temperature.

The fact that initiation was blocked in vivo and that the 50S appeared to be the limiting factor could account for the significant amount of protein synthesis observed for the wild type at 3.5 C in this investigation.

Polyuridylic acid which was used as messenger in our *in vitro* system required no initiation *per se*. Therefore, the preincubation at 25 C before shifting to 3.5 C may have resulted in the formation of mesophilic 50S and 30S couples on the poly U, which stabilized the conformation of the 50S. This would allow a limited number of ribosomes to synthesize protein at low temperature. This would be particularly true if, as postulated above, the conformation of the 50S was the limiting factor at low temperatures.

In summation, we conclude that the activity of ribosomes with temperature was contingent on their protein composition. Mesophile ribosomes possessed a protein composition that limited the activity of their 50S ribosomes at low temperature which, in turn, limited the growth of cells at low temperature. In this regard, psychrophilic growth may be contingent on a specific ribosomal protein composition that was selected for by exposure to low temperatures. Therefore, growth temperature range *per se* may be determined by the activity of ribosomes at various temperatures, which is in turn contingent on their protein composition.

SUMMARY

A mesophile, Pseudomonas aeruginosa, and several psychrophilic mutants and transductants were compared to detect any differences, compositional or functional, in their ribosomes.

Thermal denaturation, sensitivity to 8 M urea and rRNA base composition were used to compare a number of wild type psychrophiles, mesophiles and psychrophilic mutants or transductants with their mesophilic progenitors. We found that differences observed between wild type mesophiles and psychrophiles did not obtain when the psychrophilic mutants and transductants were compared with their mesophilic progenitors. Consequently, the differences observed for wild type mesophiles and psychrophiles may be trivial to the growth temperature of an organism.

Ribosomal proteins of a psychrophilic mutant and transductant were compared with those from their mesophilic progenitors by polyacrylamide gels electrophoresis. The psychrophilic transductant had at least 6 altered ribosomal proteins when compared with its mesophilic progenitor. In addition, the psychrophilic mutant showed at least 5 altered proteins in both the 50S and 30S subunit upon comparison with the mesophilic wild type. Hence, these protein changes indicate a possible involvement of ribosomal

protein composition in determination of the growth temperature range of a bacterium.

Ribosomal protein changes may not be significant unless a change in the functional capability of the ribosome can be shown. In this regard, we observed that the 50S ribosome of the mesophile was limiting for protein synthesis at low temperature, but the psychrophilic mutant 50S did not exhibit this limitation. Therefore, an alteration of 50S ribosome activity with temperature was shown coincident with ribosomal protein alterations upon conversion to psychrophily.

However, these results did not account for the 30S ribosomal protein alterations that were observed. We observed that the psychrophilic mutant ribosomes had a reduced activity at 37 C. This could be interpreted to mean that the 30S change may not affect ribosome activity at low temperature although it may be relevant to the lowering of the maximum temperature. Consequently, the ribosome may provide a link between maximum and minimum growth temperatures.

The observation that the wild type 50S subunit limited protein synthesis at low temperature, and that this limitation did not exist with the mutant 50S, was evidence for a relationship between the 50S protein alterations and altered ribosome activity at low temperature. Therefore, these results indicated that the protein composition of

the 50S ribosome may be a factor in determining the minimum temperature of growth for bacteria.

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