A Study of Ribosome Stability as a Function of Ionic Conditions

Thomas G. Wood
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A STUDY OF RIBOSOME STABILITY AS A FUNCTION OF IONIC CONDITIONS

by

Thomas G. Wood

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Doctor of Philosophy

Western Michigan University
Kalamazoo, Michigan
August 1976

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Thomas G. Wood
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INTRODUCTION

Bacteria can be divided roughly into three groups on the basis of the temperature ranges in which they grow: psychrophilic bacteria grow in the range of -5° to 30°C, with an optimum at 10° to 20°C; mesophilic bacteria grow in the range of 10° to 45°C, with an optimum at 20° to 40°C; and thermophilic bacteria grow in the range of 25° to 80°C, with an optimum at 50° to 60°C. (1).

Thermophilic bacteria have been isolated from hot springs and numerous other sources (2). Several main theories have been advanced to explain the phenomenon of thermophily (3). The theory that has received most support so far attributes thermophily to physical and chemical differences of macromolecules from thermophiles as compared to similar macromolecules from mesophiles (4).

Early studies compared similar components from thermophilic strains of Bacillus to those of Escherichia coli. Since such comparisons do not exclude the possibility of intergeneric differences, a systematic study of mesophilic and thermophilic strains of one genus, namely Bacillus, has been conducted in our laboratory (5, 6, 7, 8).

Ribosomes are characterized by their sedimentation coefficients; in the case of prokaryotes, the 70S monomer consists of a small 30S subunit and a large 50S subunit. The dimer of the
70S ribosome is a 100S ribosome. The ribosomes and their subunits are in an association-dissociation equilibrium, and their relative amounts are a function of the magnesium ion concentration. The polyamine, spermidine, functions in a similar manner to magnesium ions in these systems.

The effect of ionic conditions on ribosome conformation has been studied, for *E. coli* ribosomes, by means of analytical ultracentrifugation and thermal denaturation profiles (9). Ribosomal conformation was evaluated by the sedimentation coefficient and the melting out temperature (Tm). The Tm is that temperature at which one half of the maximum change in the absorbance is obtained when the absorbance is measured as a function of increasing temperature. It was found that ribosomal stability and conformation depended only on a quantity denoted R. The R value is defined as the ratio of monovalent to divalent cation concentrations in the solution. The conformation of *E. coli* ribosomes was characterized by three phases as a function of increasing R value. At low R value, there was no conformational change in the 70S ribosome; at higher R values, there was a progressive unfolding of the ribosome. The R values in the first phase corresponded to the ionic conditions required for the correct functioning of the ribosomes in a cell free amino acid-incorporating system.
The present work was undertaken with the aim of carrying out a similar study for both thermophilic and mesophilic 70S ribosomes of the genus *Bacillus*. Specifically, the work involved the isolation of 70S monomers from *B. licheniformis* and *B. stearothermophilus* and measurement of their conformational changes as a function of the ionic composition of the medium.
MATERIALS AND METHODS

Organisms and Growth Conditions

A mesophile, *B. licheniformis* (NRS 243), and a thermophile, *B. stearothermophilus* 10, were used for this study. The mesophile was grown at 37°C and the thermophile was grown at 55°C.

Stock cultures were grown in a medium containing 1% tryptcase (BBL), 0.2% yeast extract (Difco), 10 ppm (w/w) manganese, and 2% Bactoagar (Difco). From these stock cultures, transfers were made to slants containing the above medium, but no manganese. Several transfers were made to assure that the organisms were growing well. Finally, three slants were incubated for 12 hours, in the case of the mesophile, and for 10 hours, in the case of the thermophile. Five ml sterile water were added to each slant and the cell suspension was used as an inoculum for a seed flask containing 2 liters of sterile medium (1% trypticase and 0.2% yeast extract). The seed flask was aerated and incubated in a water bath. When the absorbance (540 nm) had increased to 0.3 - 0.4 (approximately 3-6 hours), the liquid culture was used to inoculate 23 liters of sterile medium (1% trypticase and 0.2% yeast extract) in a 25 liter fermentor (New Brunswick Scientific, Model MF-128S). To prevent foaming, 1 ml of silicone antifoam (Union Carbide Corporation, SAG-471) was added. When an
absorbance of 1.0 at 540 nm was attained, the culture was chilled and the cells were collected by means of a continuous-flow system (Sorvall RC-2 with KSB continuous-flow attachment) at 30,000 x g, at 4°C, and at a flow rate of 250 ml/min. The cells were suspended in about 400 ml of buffer I (0.01 M Tris-HCl, pH 7.4, 0.01 M magnesium acetate, and 0.06 M NH₄Cl) and centrifuged at 11,700 x g for 20 min. The cells were then frozen in liquid nitrogen and stored at -20°C. The yield of cells (wet weight) was about 40 g for _B. stearothermophilus_ and 30 g for _B. licheniformis_.

**Isolation of Ribosomes**

The cells were thawed in a water bath and washed by centrifuging them in buffer I; they were then suspended in 2 volumes (w/v) of buffer II (buffer I plus 0.006 M 2-mercaptoethanol and 0.006 M spermidine), pH 7.4. These cells were disrupted in a French press (Aminco, Model 5-596) at 18,000 lb/in². Deoxyribonuclease (200 mg/25 g of cells) was added to hydrolyze the DNA, and the cell debris was removed by two 30 min centrifugations at 30,000 x g (Sorvall, Model RC-2). The first supernatant fluid was collected in its entirety, and the second supernatant fluid was collected to within 1 cm above the pellet.

This second supernatant was then centrifuged for two hours at 105,000 x g (Spinco, Model L) to sediment the ribosomes. The ribosomes were suspended in 25% of the original volume of buffer II.
in a Potter-Elvehjem homogenizer. This crude ribosome preparation was then purified by alternate cycles of low and high speed centrifugations (5 min at 10,000 x g followed by two hours at 105,000 x g) and resuspension of the pellet. After two such cycles, the ribosomes were suspended in 10% of the original volume of buffer II. The ribosome preparation (in 4 ml plastic tubes) was frozen in the vapor of liquid nitrogen and stored in liquid nitrogen.

Density Gradient Centrifugation

A linear 15-30% sucrose gradient (w/v in buffer II) was prepared using a Buchler linear gradient maker, a Buchler poly-staltic pump, and a Buchler stirrer. A volume of 16 ml of 15% sucrose was delivered from a burette into the left chamber of the gradient maker and 16 ml of 30% sucrose was delivered into the other mixing chamber. The mixed sucrose solution was pumped into a 32 ml Beckman polyallomer centrifuge tube. The resulting linearity of the gradient was checked by means of a refractometer.

The ribosome suspension (estimated absorbance of about 300-600 at 260 nm) was diluted 1:5 with buffer II, and 0.15 ml was then layered with an Eppendorf micropipette at 4°C onto each of three gradient tubes. The tubes were then centrifuged in a Spinco SW 21.1 swinging bucket rotor at 21,000 rpm for 15 hours (10). The gradients were then fractionated and the material absorbing at 254 nm was determined with the aid of a continuous fractionating
and recording system (ISCO density gradient fractionator, Model D, and ISCO ultraviolet analyzer, Model UA-2). A colored chase solution (2 drops of food coloring per 100 ml of a 40%, w/v, sucrose solution) was used with the fractionater to calibrate the volume of the delivery tube and the gradient was fractionated at the rate of 0.5 ml/min. The 70S peak was collected on the basis of this calibration and by following the tracing of the recorder.

The disposable monoject 20 gauge one inch length hypodermic needle would often become clogged because of a core. This core could be removed by forcing water through the needle or inserting a fine wire. The Beckman polyallomer centrifuge tubes were resealed by heating a glass rod in a flame and then applying it at the point of puncture, first to the outside, and then to the inside of the tube. In this fashion, a tube could be reused many times.

Thermal Denaturation Profiles

The 70S fraction, collected from the sucrose density gradient, was diluted with buffer I and centrifuged at 105,000 x g for 2 hours. The supernatant was carefully removed with a pipette, and the sedimented, washed 70S ribosomes were suspended in a buffer having the desired R value. Several of these solutions were checked by means of refractive index measurements for their sucrose content and were found to contain 0.2% or less of sucrose.
this sucrose solution had no absorbance at 260 nm.

The ribosome suspension (3 ml) was placed in a cuvette (1 cm light path, Teflon stopper) and the absorbance at 260 nm was measured against a buffer blank using a Zeiss (Model PMQ-II) spectrophotometer connected to a circulating water bath (Haake, Model F). A calibration curve was made to relate the temperature in the circulating bath to the temperature in the cuvette.

Absorbance readings were taken at approximately 5 degree increments from about 25° to 98°C. The absorbances were corrected for the thermal expansion of water and then divided by the initial absorbance at 25°C. These absorbance ratios were then plotted as a function of temperature, using a Digital Equipment Corporation Computer (Model PDP-10), a Time Sharing Peripherals plotter (Model 212), and the SPLINE subroutine program (11). This served to remove random errors from the drawing of the curves and improve the precision of the calculated Tm values. The Tm was taken to be the temperature at which one half of the absorbance change between 25° and 90°C was observed.

Viscosity Measurements

Viscosities of ribosome preparations (absorbance of 0.2-9.0 at 260 nm) were measured in an Ostwald viscometer having a 5 ml bulb. The viscometer was immersed in a water bath and the temperature was varied from 25° to 85°C. Viscosities were measured on 5 ml aliquots and the outflow time at each temperature
was measured three times. Measurements were made at 25°C, 35°C, at 5°C intervals between 35° and 65°C, and at 85°C. The viscometer was cleaned between runs by rinsing it with dichromate cleaning solution, distilled water, and distilled acetone.

Circular Dichroism Spectra

Circular dichroism spectra were determined for unfractionated ribosomes, suspended in buffer I, over the range 230-400 nm. A 5 ml, jacketed, teflon stoppered cell was connected to a circulating water bath (Haake, Model F). The temperature of the cell was calibrated against the temperature of the bath. Spectra were measured with a spectropolarimeter (Cary 60, Model 6003) interfaced to an IBM 1800 computer and the data were plotted by means of an IBM 1627 II plotter as molar rotation versus wavelength. The molar rotation was measured over a temperature range of 25°-65°C, at approximately 5°C intervals. The molar rotation of the buffer was subtracted from that of the ribosomes. The rotations were not corrected for changes in the concentration of the solution due to the thermal expansion of the solvent.

Analytical Ultracentrifugation

Ultracentrifugation analyses were conducted in a Spinco (Model E) analytical ultracentrifuge equipped with rotor temperature indicator control and Schlieren optics. Sedimentations were performed at 4° to 6°C. Patterns were enlarged with a micro-
comparator (Nikon, Model 6C). The peaks were traced, cut out, and weighed in order to compare the areas under the peaks. Sedimentation coefficients were corrected to 20°C for the viscosity of water.

When necessary, the concentrations of magnesium ion and spermidine were changed by dialysis at 4°C for 24 hours.

Titration of Spermidine

Spermidine trihydrochloride was titrated with standard NaOH (carbonate free) using a Corning expanded scale research pH meter (Model 12). The NaOH was made carbonate free by preparing a saturated solution of NaOH in hot distilled water, and letting the carbonate precipitate out. The NaOH solution was stored in an air tight container and standardized by titration to a phenolphthalein end point, using dried potassium acid phthalate as the primary standard. For the titration, 25 to 30 mg of spermidine trihydrochloride were dissolved in 50 ml of distilled water. Nitrogen gas was continually passed over the solution and the NaOH was delivered from a 10 ml, class A burette.
Chemicals

Ammonium chloride - Baker Chemical
Bactoagar - Difco Laboratory
Deoxycholic acid - Sigma Chemical
Deoxyribonuclease - Worthington Biochemicals
Dodecyl sodium sulfate (SDS) - Sigma Chemical
Magnesium acetate - Baker Chemical
Mercaptoethanol - Eastman Organic Chemicals
Phenolphthalein - Baker Chemical
Potassium Acid Phthalate - Mallinckrodt
Silicone antifoam - Union Carbide SAG-471
Spermidine Trihydrochloride - Nutritional Biochemicals
Sucrose (Ribonuclease Free) - Mann Research Laboratories
Tris (hydroxymethyl) aminomethane (Tris) - Sigma Chemical
Trypticase - Baltimore Biological Laboratory
Urea - Matheson Coleman and Bell
Yeast extract - Difco Laboratory
RESULTS AND DISCUSSIONS

Density Gradient Centrifugation

The linearity of the density gradient was checked by measuring the sucrose concentration (refractometry) in 2 ml fractions recovered from the gradient. The results are shown in Fig. 1. As can be seen, the gradient was linear except for a deviation at the top and bottom of the gradient tube which can be ascribed to the cone-shaped design of the gradient maker. As a result, a relatively large amount of the concentrated solution must be withdrawn before the pressure drops sufficiently for the dilute solution to flow in. A similar effect occurs at the other end of the gradient. The gradient was stable for at least 24 hours.

Density gradient centrifugation of the ribosomes resulted in a profile consisting of four peaks which were numbered consecutively in the order of their recovery from the tube (i.e. from the top to the bottom of the tube). By using a colored, 40% (w/v) sucrose chase solution, the end of the gradient was indicated by a sharp rise in the absorbance. This rise in absorbance (peak 5) could, therefore, be used as a marker.

Typical density gradient profiles of the ribosome preparations are shown in Fig. 2. It can be seen that the 70S peak (peak no. 3) was larger than the 30S, 50S, and 100S peaks (peaks no. 1, 2, and 4).
Figure 1. Linearity of the Density Gradient

Ordinate: Fraction Number

Abscissa: Sucrose Concentration (% w/w)
This was achieved by washing the ribosome pellet, in the process of isolating the ribosomes, with buffer I which removed some of the adherent 30S and 50S subunits.

Additionally, the 70S peak was prevented from dissociating into 30S and 50S subunits during the density gradient centrifugation by including spermidine in the buffer used to make the gradient. The omission of spermidine led to patterns in which the 70S peak was greatly decreased (Fig. 3).

Tracings of the 70S and 100S peaks from the density gradient profiles were cut out and weighed. The ratio of weights, i.e. the relative abundance, of the 70S:100S ribosomes was 1.4 for *B. licheniformis* and 2.2 for *B. stearothermophilus*. The relative amount of 70S ribosomes was somewhat greater and less well separated from the 100S ribosomes in *B. stearothermophilus* as compared to *B. licheniformis*.

Peaks 1-4 were shown to represent 30S, 50S, 70S, and 100S ribosomes, respectively, and were identified in the following three ways: (a) Comparison of the patterns for unfractionated ribosomes obtained by density gradient centrifugation and by analytical ultracentrifugation (Fig. 4). Since the patterns contained an identical number of components, the sedimentation coefficients calculated from analytical ultracentrifugation were used to define the peaks obtained by density gradient centrifugation. (b) Collection of peaks 1-4 from the density gradient, followed by analytical ultracentrifugation and determination
Figure 2. Density Gradient Profile of Ribosomes in the Presence of Spermidine

Ordinate: Absorbance (254 nm) from 0 to 2.5

Abscissa: Effluent Volume

(a) *B. licheniformis*

(b) *B. stearothermophilus*
of the sedimentation coefficients of the four components. Sedimentation coefficients of 30S, 50S, 70S, and 100S were obtained.

(c) Collection of the third peak (70S) from the density gradient, followed by analytical ultracentrifugation; a single component, having a sedimentation coefficient of 70S was obtained. For this experiment, the 70S component was isolated from three gradients to give a final volume of about 3 ml. By measuring the refractive index of this solution, it was found that the sucrose concentration was 21.2% (w/w); the observed sedimentation coefficient was then corrected to the sedimentation coefficient in water ($S_{20,w}$) in the usual manner.

In the course of these studies, the possibility was investigated that the ribosome preparation may be altered sufficiently by changes in the ionic composition of the medium to allow preparation of 70S particles other than by density gradient centrifugation.

For this purpose the unfractionated ribosome preparation was dialyzed against and/or diluted with appropriate buffers in order to lower the concentration of magnesium and/or spermidine. The resulting ribosome suspensions were then analyzed by analytical ultracentrifugation for changes in the relative amounts of the various ribosomal particles.

As can be seen from Table I, none of these treatments gave 70S preparations of acceptable purity. A few test runs with $B. licheniformis$ ribosomes gave similar results. Because of
Figure 3. Density Gradient Profile of Ribosomes in the Absence of Spermidine

Ordinate: Absorbance (254 nm) from 0 to 2.5
Abscissa: Effluent Volume

(a) *B. licheniformis*

(b) *B. stearothermophilus*
Table I

Effect of Magnesium and Spermidine Concentration on the Distribution of 70S and 100S Ribosomes

<table>
<thead>
<tr>
<th>Method</th>
<th>Magnesium (M)</th>
<th>Spermidine (M)</th>
<th>Mercaptoethanol (M)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.01</td>
<td>-----</td>
<td>-----</td>
<td>Components as in Figure 4 (b)</td>
</tr>
<tr>
<td>heat^X</td>
<td>0.01</td>
<td>-----</td>
<td>-----</td>
<td>Components as in Figure 4 (b)</td>
</tr>
<tr>
<td>dialysis</td>
<td>0.001</td>
<td>-----</td>
<td>-----</td>
<td>Precipitate formed §</td>
</tr>
<tr>
<td>dialysis</td>
<td>0.005</td>
<td>-----</td>
<td>-----</td>
<td>Precipitate formed §§</td>
</tr>
<tr>
<td>dialution</td>
<td>0.007</td>
<td>-----</td>
<td>-----</td>
<td>Large amount of dissociation of 70S peak</td>
</tr>
<tr>
<td>dialution</td>
<td>0.0015</td>
<td>0.003</td>
<td>0.006</td>
<td>Large amount of dissociation of 70S peak</td>
</tr>
<tr>
<td>dialysis</td>
<td>0.005</td>
<td>0.006</td>
<td>0.006</td>
<td>100S peak was decreased by about 50%</td>
</tr>
<tr>
<td>dialution</td>
<td>0.005</td>
<td>0.003</td>
<td>0.006</td>
<td>Precipitate formed §§§</td>
</tr>
<tr>
<td>dialution</td>
<td>0.0054</td>
<td>0.0035</td>
<td>0.006</td>
<td>100S peak was decreased by about 50%</td>
</tr>
<tr>
<td>dialution</td>
<td>0.005</td>
<td>0.0035</td>
<td>0.006</td>
<td>Precipitate formed §§§</td>
</tr>
<tr>
<td>dialution</td>
<td>0.005</td>
<td>0.0041</td>
<td>0.006</td>
<td>100S peak was decreased by more than 70%</td>
</tr>
<tr>
<td>dilution</td>
<td>0.005</td>
<td>0.0045</td>
<td>0.006</td>
<td>About the same as above</td>
</tr>
</tbody>
</table>
Table I
(Continued)

A The precipitate would not go back into solution when dialyzed against buffer I or II.

AA The precipitate would go into solution when dialyzed against either buffer I or II; there was little or no change in the relative amounts of the various ribosomes when the redissolved material was examined by analytical ultracentrifugation.

AAA The precipitate would not go into solution when dialyzed against buffer I or II.

≠ B. stearothermophilus

X Ribosome solution kept at 25°C for 4 hours, then cooled to 4°C.
Figure 4. Ultracentrifuge Patterns of Ribosomes
(Sedimentation is from left to right)

(a) *B. licheniformis*

(b) *B. stearothermophilus*
these findings, the 70S ribosomes were subsequently isolated exclusively by density gradient centrifugation.

**Thermal Denaturation Profiles**

Thermal denaturation profiles were obtained as a function of the R value which is defined as the ratio of the concentration of monovalent ions (Tris and ammonium) to the concentration of divalent ions (magnesium).

Representative thermal denaturation profiles are shown in Fig. 5. After obtaining a particular profile, the ribosome suspension was kept in the spectrophotometer and allowed to cool down overnight from 90-100° to 25°C. The absorbance of the renatured solution was about 20% higher than that of the original solution at 25°C.

R values were chosen first such that the magnesium concentration was kept constant and ammonium and Tris⁺ were varied but the concentration ratio of ammonium to Tris⁺ was also kept constant. These values were based on buffer I which has an R value of 7 and contains 0.01 M Mg²⁺, 0.06 M NH₄⁺, and 0.01 M Tris⁺. R values were next obtained by lowering the magnesium concentration and keeping the ammonium to Tris concentration ratio constant. Finally, R values were obtained by changing the concentration ratio of ammonium to Tris. As shown from the data in Table II, the melting temperature is determined by the absolute R value and does not depend on
how this value was obtained, disregarding the presence of SDS and urea. The standard incubation mixture used in amino acid incorporation experiments for both of these species of Bacillus had an R value of 15.5 (12). This is located on the first line in Fig. 6 (a and b).

Generally speaking, thermal denaturation profiles at low and medium R values (e.g. a, b, and f in Fig. 5) were characterized by a steeper transition and a higher final absorbance than those at high R values (e.g. c and h in Fig. 5). Thermal denaturation profiles for samples containing urea or SDS (e.g. d, e, i and j in Fig. 5) were also generally characterized by a gradual transition much as the curves for high R values. In the presence of SDS or urea, the absorbance did not level off at the high temperatures as well as it did in the absence of these compounds.

It can be seen from Table II that, in the presence of urea, the melting temperatures were lower for both organisms. This is expected in view of the known hydrogen bond breaking properties of urea (13). The decrease in Tm amounted to about 5°C for the 2M urea, and to about 14°C for the 6M urea solutions. A graphical presentation of the data for the solutions containing urea would yield a plot as in Fig. 6 with the two lines being parallel to, lower than, and intersecting at the same R value as for the solutions without urea.

In the presence of 0.45% SDS, the Tm was increased over that of the control without SDS. In the presence of 1% SDS, the Tm was essentially equal to that of the control and in the presence
Figure 5. Thermal Denaturation Profiles of 70S Ribosomes

Ordinate: Absorbance Ratio at 260 NM
(Absorbance at given temperature)
(Absorbance at 25°C)

Abscissa: Temperature (°C)

(a) B. licheniformis, R = 1.925
(b) B. licheniformis, R = 9.6
(c) B. licheniformis, R = 7000
(d) B. licheniformis, R = 100, 2M urea
(e) B. licheniformis, R = 1, 1.0% SDS
(f) B. stearothermophilus, R = 1.925
(g) B. stearothermophilus, R = 7
(h) B. stearothermophilus, R = 630
(i) B. stearothermophilus, R = 1, 2M urea
(j) B. stearothermophilus, R = 1, 2.0% SDS

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of 2% SDS, the Tm was slightly lower than that of the control. These results were obtained for solutions having an R value of 1 as well as for those having an R value of 100.

Since SDS is known to bind strongly to proteins (14), the increase in Tm at low SDS concentrations may be due to a stabilization of the ribosomes as a result of such binding. At higher SDS concentrations, the destabilizing detergent effect of SDS probably outweighs the stabilizing binding effect so that the Tm is decreased. Both of these effects are apparently less pronounced in solutions of high ionic strength (R = 100).

It can be seen from Fig. 5 that at R values above 300 (c, h) the transition was less steep and started at a lower temperature than was the case for those at lower R values (a, b, f, and g). This effect could be produced if the 70S ribosomes were already partially denatured at the high R values even before they were heated. In order to check for this possibility, a sample of ribosomes was isolated from each organism, divided into two portions, having an R value of 7 and 354, respectively, and the absorbance measured as a function of time. It can be seen from Table III that prolonged exposure of the ribosomes to the buffer of high R value did not lead to significant changes in the absorbance. This suggests that the high ionic strengths of these buffers were insufficient by themselves to produce an unfolding of the ribosomes.
<table>
<thead>
<tr>
<th>R</th>
<th>Γ/2</th>
<th>Tm B. stearo.</th>
<th>Tm B. lich.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.070</td>
<td>0.0306</td>
<td>73.16</td>
<td>66.50</td>
</tr>
<tr>
<td>0.105</td>
<td>0.0309</td>
<td>78.06</td>
<td>68.23</td>
</tr>
<tr>
<td>0.210</td>
<td>0.0318</td>
<td>76.93</td>
<td>70.32</td>
</tr>
<tr>
<td>0.350</td>
<td>0.0331</td>
<td>71.29</td>
<td>70.13</td>
</tr>
<tr>
<td>0.525</td>
<td>0.0346</td>
<td>72.90</td>
<td>68.87</td>
</tr>
<tr>
<td>0.700</td>
<td>0.0361</td>
<td>73.58</td>
<td>68.97</td>
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<td>0.963A</td>
<td>0.0288</td>
<td>73.06</td>
<td>69.35</td>
</tr>
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<td>1.925</td>
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<td>72.00</td>
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<td>90.50AA</td>
<td>0.6014</td>
<td>70.94</td>
<td>65.54</td>
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<td>110.0</td>
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<td>67.90</td>
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<td>0.7796</td>
<td>64.03</td>
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<td>3.3944</td>
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<td>0.0387</td>
<td>57.19</td>
<td>58.39</td>
</tr>
<tr>
<td>20.0 (6M urea)</td>
<td>0.2048</td>
<td>59.19</td>
<td>51.29</td>
</tr>
<tr>
<td>100.0 (6M urea)</td>
<td>0.9039</td>
<td>58.65</td>
<td>55.65</td>
</tr>
<tr>
<td>1.0  (2M urea)</td>
<td>0.0387</td>
<td>70.00</td>
<td>65.10</td>
</tr>
<tr>
<td>100.0 (2M urea)</td>
<td>0.9039</td>
<td>60.65</td>
<td>59.68</td>
</tr>
<tr>
<td>20.0 (0.45% SDS)</td>
<td>0.2050</td>
<td>------</td>
<td>68.83</td>
</tr>
<tr>
<td>1.0  (0.45% SDS)</td>
<td>0.0389</td>
<td>83.50</td>
<td>74.50</td>
</tr>
<tr>
<td>100.0 (0.45% SDS)</td>
<td>0.9041</td>
<td>71.34</td>
<td>70.48</td>
</tr>
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</table>

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Table II  
(continued)

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>T/2</th>
<th>1.0 (1.0% SDS)</th>
<th>0.0390</th>
<th>73.20</th>
<th>70.60</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0 (1.0% SDS)</td>
<td>R</td>
<td>T/2</td>
<td>0.9042</td>
<td>71.00</td>
<td>69.03</td>
<td></td>
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<tr>
<td>1.0 (2.0% SDS)</td>
<td>R</td>
<td>T/2</td>
<td>0.0394</td>
<td>70.32</td>
<td>68.45</td>
<td></td>
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<tr>
<td>100.0 (2.0% SDS)</td>
<td>R</td>
<td>T/2</td>
<td>0.9046</td>
<td>69.68</td>
<td>70.32</td>
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<tr>
<td>No Mg$^{2+}$</td>
<td>R</td>
<td>T/2</td>
<td>0.0612</td>
<td>57.50</td>
<td>57.20</td>
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</tr>
<tr>
<td>.01 M Tris$^+$</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>.06 M NH$_4^+$</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Magnesium concentration lowered

** Ratio of NH$_4^+$ concentration to Tris$^+$ concentration changed

SDS is included in the R values; $\Gamma/2$ is the ionic strength.
A plot of Tm versus R value (computer drawn, least squares) is shown in Fig. 6. Though there is a considerable amount of scattering in the data, there is a definite indication of at least two stages of ribosome conformation as a function of increasing R value. The initial stage, represented by an almost horizontal line may reflect the dissociation of 70S ribosomes into 30S and 50S subunits; the second stage may reflect the unfolding of the subunits. For E. coli, similar data were interpreted to be indicative of three stages of ribosome conformation and the first stage was represented by a horizontal line (9).

The lines of Fig. 6 (a) intersect at R = 23.3 (+26, -12) and those of (b) intersect at R = 17.9 (+37, -12). The large propagated errors in the intersection of these lines renders the difference between the points of intersection of (a) and (b) meaningless. The lines for the thermophiles are at higher temperatures than those for the mesophiles as expected for these organisms (7, 8).

The Tm values are summarized in Table II as a function of R values and ionic strength (T/2). Using the Henderson-Hasselbalch equation, it was calculated that at pH 7.4, 11.17% of the Tris was in the ionized form. The ionic strengths were then calculated by using this value and the molar concentrations of magnesium and ammonium. Due to the fact that only a comparison of the ionic strengths was needed, molar concentrations were not converted to molal concentrations. It can be seen
Table III

Absorbance at 260 nm of 70S Ribosomes at Low and High R Values

<table>
<thead>
<tr>
<th>R</th>
<th>B. stearothermophilus</th>
<th>B. licheniformis</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.138</td>
<td>0.158</td>
</tr>
<tr>
<td>354</td>
<td>0.141</td>
<td>0.160</td>
</tr>
<tr>
<td>7A</td>
<td>0.139</td>
<td>0.160</td>
</tr>
<tr>
<td>354A</td>
<td>0.143</td>
<td>0.161</td>
</tr>
<tr>
<td>7AA</td>
<td>0.340</td>
<td>0.332</td>
</tr>
<tr>
<td>543AA</td>
<td>0.349</td>
<td>0.337</td>
</tr>
</tbody>
</table>

* solution kept in the buffer for one hour at room temperature

**AA solution (different sample) dialyzed against buffer overnight at room temperature

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from Table II that the melting temperature depended almost entirely on the R value and not on the ionic strength.

Viscosity Measurements

The thermal denaturation profiles presented in Fig. 5 show that the absorbance for the *B. stearothermophilus* ribosomes at low R values (f, g) decreases significantly in the range of 25-55°C and only increases at temperatures above 55°C. This was a general phenomenon for the *B. stearothermophilus* preparations at low R values (R = 0.07 - R = 20.0) and was not observed for the corresponding preparations from *B. licheniformis* (Fig. 5, a, b). Conceivably this effect could be produced by a conformational change of the ribosomes. In an attempt to determine the applicability of this interpretation, the ribosomes were studied over this temperature range by means of viscosity, light scattering, and circular dichroism measurements.

Results of the viscosity measurements are summarized in Table IV. Ribosome preparations from the mesophile and the thermophile had essentially the same concentration and can, therefore, be compared directly regardless of concentration changes as a result of the thermal expansion of the sample.

It can be seen from Table IV that, for *B. stearothermophilus*, the relative viscosity decreased significantly as the temperature was raised from 25°C-35°C, and then remained essentially constant up to about 60°C. This was true for both the unfractionated and
Figure 6. Variation of Tm with R Value

Ordinate: R Value
Abscissa: Tm

(a) \textit{B. licheniformis}
(b) \textit{B. stearothermophilus}

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### Table IV

**Relative Viscosities\(^\Delta\) of Ribosomes**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th><em>B. licheniformis</em></th>
<th></th>
<th><em>B. stearothermophilus</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ribosomes(^\text{K})</td>
<td>70S(^\text{KK})</td>
<td>Ribosomes(^\text{#})</td>
<td>70S(^\text{##})</td>
</tr>
<tr>
<td>25</td>
<td>1.034</td>
<td>1.022</td>
<td>1.036</td>
<td>1.018</td>
</tr>
<tr>
<td>35</td>
<td>1.011</td>
<td>1.019</td>
<td>1.017</td>
<td>1.005</td>
</tr>
<tr>
<td>40</td>
<td>1.009</td>
<td>1.016</td>
<td>1.015</td>
<td>1.005</td>
</tr>
<tr>
<td>45</td>
<td>1.006</td>
<td>1.011</td>
<td>1.016</td>
<td>1.004</td>
</tr>
<tr>
<td>50</td>
<td>1.013</td>
<td>1.013</td>
<td>1.016</td>
<td>1.005</td>
</tr>
<tr>
<td>55</td>
<td>1.015</td>
<td>1.008</td>
<td>1.018</td>
<td>1.001</td>
</tr>
<tr>
<td>60</td>
<td>1.014</td>
<td>1.013</td>
<td>1.015</td>
<td>1.005</td>
</tr>
<tr>
<td>65</td>
<td>1.005</td>
<td>1.010</td>
<td>1.005</td>
<td>1.005</td>
</tr>
</tbody>
</table>

\(\Delta\) Outflow time of ribosome solution divided by the outflow time for buffer I \((R = 7)\)

\(\text{K}\) Unfractionated ribosomes, absorbance of 9.0 at 260 nm

\(\text{KK}\) Purified 70S ribosomes, absorbance of 0.423 at 260 nm

\(#\) Unfractionated ribosomes, absorbance of 8.4 at 260 nm

\(\##\) Purified 70S ribosomes, absorbance of 0.417 at 260 nm
the purified 70S ribosomes.

For *B. licheniformis*, on the other hand, the relative viscosity decreased continuously as the temperature was raised from 25° to 45°C, then increased as the temperature was raised to 50°C, and thereafter remained approximately constant up to about 60°C.

There is a fair amount of scatter in the data because of the fact that even the more concentrated ribosome solution had only a low viscosity. The data do seem to indicate, however, a difference in the viscous properties of *B. licheniformis* and *B. stearothermophilus* ribosomes over the temperature range of 25°-65°C. That viscosity is a measure of ribosome conformation can be seen from the fact that the relative viscosity of *B. licheniformis* ribosomes at 65°C was 1.01 while at 85°C it was 1.30.

**Light Scattering**

A second method for evaluating possible conformational changes of the 70S ribosomes in the temperature range of 25°-65°C involved the use of light scattering. For this purpose, turbidimetric measurements of ribosome solutions were made at wavelengths at which ribosome solutions show little or no absorption (300-400 nm). Measurements were made at 20 nm intervals and at 5°C intervals in the temperature range of 25°-65°C. The 70S ribosomes were suspended in buffer I at essentially identical concentrations (apparent absorbance at 260 nm of 4.33 and 4.10
Figure 7. Light Scattering of 70S Ribosomes

Ordinate: Absorbance at a given temperature
Absorbance at 25°C

Abscissa: Temperature (°C)

(a) □ B. licheniformis at 320 nm
      △ B. stearothermophilus at 320 nm

(b) ○ B. licheniformis at 380 nm
      ♦ B. stearothermophilus at 380 nm

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for the ribosomes from *B. stearothermophilus* and *B. licheniformis*, respectively).

The relative absorbance changes were essentially the same for all wavelengths and hence only representative data are shown in Fig. 7. It can be seen that the nonspecific scattering of the *B. stearothermophilus* ribosomes increased sharply as the temperature was raised from 40° to 65°C while that of the *B. licheniformis* ribosomes increased only slightly over the same range of temperature. It is difficult to ascribe this increase in light scattering to hyperchromicity of the ribosomes as a result of denaturation since the Tm of the thermophilic ribosomes was consistently higher than that of the mesophilic ribosomes (Table II, Fig. 5). It is likely, therefore, that the changes in light scattering for *B. stearothermophilus* reflect some other type of conformational change in the ribosomes. That conformational changes of proteins and nucleic acids are reflected in changes in light scattering is well established (15).

**Circular Dichroism Spectra**

If 70S ribosomes from *B. stearothermophilus* undergo significantly different conformational changes over the temperature range of 25° to 65°C than those from *B. licheniformis*, then this should be reflected in differences of the circular dichroism spectra of the ribosomes. The results of such measurements are shown in Fig. 8. The data were calculated by using a molecular
weight of 1000 and a concentration of 1.0 mg/1 for the ribosome (16). Thus the curves indicate relative, rather than absolute, circular dichroism values. The circular dichroism spectra can be considered to be due solely to RNA since proteins do not contribute to the spectra at these wavelengths. For each organism the spectra in the range of 25°-65°C were very similar; comparing the spectra at 25° with those at 65°C indicates approximately the maximum change observed.

The circular dichroism spectra of the 70S ribosomes from the two organisms are quite different. One difference is that the B. licheniformis spectra are shifted about 30 nm toward longer wavelengths than those of B. stearothermophilus; thus B. licheniformis ribosomes show a strong positive peak at about 300 nm while B. stearothermophilus ribosomes have this peak at about 270 nm. This is puzzling since the ultraviolet absorption spectra of both types of ribosomes are almost identical and show very little absorption in the 300 nm area. A second difference in the spectra was the occurrence of a positive peak in the case of B. stearothermophilus, and two negative peaks, in the case of B. licheniformis, just toward the larger wavelength side of the 270-300 nm peak.

As the temperature was changed from 25° to 65°C, both types of spectra were shifted about 3-4 nm toward the larger wavelength side. This can be attributed to the dissociation of the 70S ribosomes into their subunits (17, 18). The B. licheniformis

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spectrum, over the range of 25° to 65°C, showed the following:
the large negative peak remained unchanged; the large positive
peak increased in height by about 5 units and the small negative
peak decreased in height by about 2-3 units. The B. stearothermo-
philus spectrum, over the same temperature range, showed the
following: a decrease in height by about 4-5 units for the
large positive peak; an increase in height by about 2-3 units
for the large negative peak; and a decrease in height by about
2 units for the small positive peak. Thus the large positive
peak increases in height for the 70S ribosomes from B. licheni-
formis but decreases in height for the 70S ribosomes from B. stearo-
thermophilus. This and the fact that the large negative peak
only changes for the 70S ribosomes from B. stearothermophilus
indicates that, over this range of temperatures, the thermophilic
70S ribosomes undergo a conformational change which differs
from that undergone by the mesophilic ribosomes. Thus all three
experimental approaches (viscosity, light scattering, and
circular dichroism) support the concept that, in the temperature
range of 25-65°C, the thermophilic ribosome undergoes a
different conformational change from that of the mesophilic
ribosome. The different conformational state is presumably
responsible for the decrease in absorbance observed in the thermal
denaturation profiles of the ribosomes from B. stearothermophilus
(Fig. 5).
Figure 8. Circular Dichroism Spectra of 70S Ribosomes

(a) \textit{B. licheniformis}, 25°C
(b) \textit{B. licheniformis}, 65°C
(c) \textit{B. stearothermophilus}, 25°C
(d) \textit{B. stearothermophilus}, 65°C
Titration of Spermidine

In order to determine the contribution of spermidine to the R value, it was necessary to know the charge on the molecule at the pH used. While spermidine is usually considered to be a divalent ion, often equivalent to Mg$^{++}$, a perusal of the literature revealed no data on the actual pK values of spermidine. It was, therefore, necessary to determine the pK's of spermidine. While this work was in progress a paper was published in which an evaluation of the pK's of spermidine was described (19).

For our experiments, spermidine was titrated with 0.03167 N NaOH (exps. 1 and 2) and with 0.06109 N NaOH (exps. 3 and 4). A typical titration curve is shown in Fig. 9.

The pK's were calculated from the following equation (20):

\[ g + (g-1)[H^+]K_1 + (g-2)[H^+]^2 K_2 + (g-3)[H^+]^3 K_3 = 0 \]

where \( g = m - a + (\frac{[OH^-] - [H^+]}{[T]}) \).

\[ a = \frac{(\text{ml base added}) (\text{N base})}{[T]}(\text{Total volume of solution}) \]

\[ [T] = \text{molar concentration of spermidine (all its forms) at a given point in the titration} \]

\[ m = \text{maximum number of possible charges on the molecule} \]

\[ [OH^-]; [H^+] \text{ calculated from the pH} \]

\[ K_1 = K_3, K_2 = K_3 K_2, K_3 = K_1 K_2 K_3 \]

\( K_1; K_2; K_3 \) are the three dissociation constants of spermidine.
Figure 9. Titration of Spermidine

Ordinate: pH

Abscissa: ml of 0.06109 N NaOH added
Equation 1 was used for each of three points of a particular titration. This set of three equations was then solved simultaneously for $K_1$, $K_2$, and $K_3$ by Cramer's Rule. The results are summarized in Table V. From these data, it can be calculated from the Henderson-Hasselbalch equation that, at pH 7.4, spermidine exists as a mixture of a trivalent cation (81.3%), a divalent cation (18.62%), a monovalent cation ($7.6 \times 10^{-2}\%$), and an uncharged molecule ($2 \times 10^{-5}\%$). The contribution of spermidine to the R values (all at pH 7.4) could then be calculated. It should be noted that the notion that spermidine exists predominantly or entirely as a divalent cation at physiological pH is incorrect.
Table V

pK Values of Spermidine

<table>
<thead>
<tr>
<th>Exp.</th>
<th>pK₁</th>
<th>pK₂</th>
<th>pK₃</th>
</tr>
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<tbody>
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<td>7.639</td>
<td>9.456</td>
<td>10.481</td>
</tr>
<tr>
<td>2</td>
<td>7.948</td>
<td>9.666</td>
<td>10.698</td>
</tr>
<tr>
<td>3</td>
<td>8.196</td>
<td>9.880</td>
<td>10.772</td>
</tr>
<tr>
<td>4</td>
<td>8.370</td>
<td>10.133</td>
<td>11.309</td>
</tr>
<tr>
<td>Mean ± s.d.</td>
<td>8.040 ± .27</td>
<td>9.79 ± .24</td>
<td>10.82 ± .30</td>
</tr>
<tr>
<td>Mean ± s.d.</td>
<td>8.34 ± .05</td>
<td>9.81 ± .02</td>
<td>10.89 ± .05</td>
</tr>
</tbody>
</table>

* s.d. = standard deviation; row 5 lists our experimental values; row 6 lists the literature values (19).
BIBLIOGRAPHY


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VITA

The author was born to Frances Beulah and Dale Arthur Wood on December 31, 1941 in Kalamazoo, Michigan. He received his elementary and secondary education in Michigan. He graduated from Ferris State College with a degree of Bachelor of Science in Pharmacy in June, 1965 and became licensed as a pharmacist in the state of Michigan in February, 1966. He was inducted into the United States Army in January, 1967 and was released in December, 1968. In February, 1968, he became licensed as a pharmacist in the state of California. He enrolled in the Chemistry Department of Western Michigan University in April, 1969 and in January of 1972 entered the Ph.D. program in biochemistry in that department.