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Studies on the Ribosomal Ribonucleic Acids from Mesophilic and Thermophilic Bacteria

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STUDIES ON THE RIBOSOMAL RIBONUCLEIC ACIDS FROM MESOPHILIC AND THERMOPHILIC BACTERIA

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INTRODUCTION

Certain organisms have been known to live at temperatures at which cellular constituents ordinarily sensitive to heat are destroyed. These organisms, called thermophiles (heat-loving), normally live at higher temperatures than the mesophiles which live in the temperature range of the biokinetic zone (10^{o}- 45^{o}C).

Several types of explanations have been given to account for the ability of thermophiles to withstand high temperatures. In his studies on flagella of thermophilic and mesophilic bacteria, Koffler (10) showed that the cytoplasmic proteins of thermophiles are markedly more stable to heat than those of mesophiles. Allen (2) believes that the reason lies in the rapid resynthesis of damaged cell constituents. Some thermophilic bacteria withstand high temperatures because they appear to synthesize enzymes more rapidly than the rate at which the high temperature destroys them (1). Militzer and Tuttle (18) showed that some possess enzymes which continue to function normally at high temperatures. Gaughran (8) found that lipids of thermophiles remain constant in quantity and degree of saturation, whereas the lipid content of mesophiles decreases as the temperature is increased, and the lipid becomes more saturated.

Previous studies on thermophiles and mesophiles have been done mostly on their protein content (5, 10, 11, 12) and some on the deoxyribonucleic acid (DNA) from a thermophile (17). This present work attempts to find out any differences between these two types of organisms by studying their ribosomal ribonucleic acids.

The bulk of the ribonucleic acid (RNA) of cells is found in the cytoplasm. About 85% of this cytoplasmic RNA is incorporated into discrete particles called ribosomes, and the rest is distributed throughout the cytoplasm without being localized in any preformed body.

Ribosomes are organelles occurring in the cytoplasm of cells of various animals, plants and microorganisms. They are electron-dense particles of macromolecular dimensions (diameter, 100-150 Å). While they are usually attached to the outer surface of the endoplasmic reticulum and to the vesicles in the cytoplasm of animal cells, they occur free in bacterial extracts. They are roughly spherical in shape and contain a high proportion of RNA accounting for about half their mass. It is believed that the RNA is attached to protein forming ribonucleoprotein.

Isolation of ribosomes is readily accomplished by fractional centrifugation of tissue homogenates or of bacterial extracts. Bacteria in the exponential phase of growth are one of the richest sources of ribosomes.

In order to compare these two types of organisms, this study was done on the ribosomal RNA of 3 species of mesophiles and 3 strains of thermophiles, all belonging to the genus Bacillus. Three major experiments were carried out on the different ribosomal preparations: (1) isolation of ribosomal RNA, (2) determination of the base composition of the various RNA's, and (3) fingerprinting (or mapping) of the nucleotides in the RNA's.

MATERIALS AND METHODS

Organisms. Three species of mesophiles (B. pumilus NRS 236, B. licheniformis NRS 243 and B. sp. X-1) and three strains of thermophiles (B. stearothermophilus 2184, B. stearothermophilus 10 and B. stearothermophilus FJW) were used for this study.

Media. The bacterial cultures were grown in slants and petri dishes on a medium consisting of 2% Bacto agar (Difco), 1% Trypticase (BBL) and 0.2% Yeast extract (Difco). Slants for growing the stock cultures contained in addition to the above 10 p.p.m. of manganese.

Buffers. Tris buffer was made up of 0.01 M Tris(hydroxymethyl)aminomethane-0.01 M magnesium acetate, adjusted to pH 7.4 with 1 N hydrochloric acid.

Acetate buffer was made up of 0.01 M sodium acetate-0.1 M sodium chloride, adjusted to pH 4.6 with 1 N hydrochloric acid.

Phosphate buffer was prepared by mixing 33.0 ml. of 0.2 M monobasic sodium phosphate solution and 67.0 ml. of 0.2 M dibasic sodium phosphate solution, and diluting to a total of 200 ml. giving a solution of pH 7.1.

Chemicals. Aluminum oxide, chromatographic grade (Merck).

Sodium dodecyl sulfate was washed in cold ethanol and ether to remove ultraviolet absorbing material. A 10% (w/v) aqueous solution was prepared as stock solution.

Phenol was redistilled immediately before use, and a 90% (v/v) aqueous solution was used.

Perchloric acid, 70-72%, analytical grade (Mallinckrodt Chemical Works).

Formic acid, 97-100% (Matheson Coleman & Bell).

Toluene, A.C.S., analyzed reagent (Matheson Coleman & Bell).

Guanine, cytosine, adenine, uracil and thymine were obtained from Nutritional Biochemicals Corporation.

Guanylic acid, cytidylic acid, adenylic acid and uridylic acid were obtained from California Foundation for Biochemical Research.

Yeast RNA was obtained from Worthington Biochemical Corporation.

Crystalline pancreatic ribonuclease "A" Type XI-A and deoxyribonuclease were obtained from Sigma Chemical Company.

Reagents.

(a) Protein determination

The reagents used were those given for the estimation of protein with the Folin Ciocalteu reagent (14).

(b) Base Composition

The solvent system used for paper chromatography of the bases was 2 N hydrochloric acid in 65 volumes of isopropanol and 35 volumes of water (4).

(c) Fingerprinting

The electrophoresis buffer and chromatography solvent system used were those given by Rushizky and Knight (21).

Apparatus. All ultraviolet spectrophotometric measurements were made in silica cells with a 1-cm. light path in a Zeiss model PMQ II spectrophotometer and are expressed as optical density (OD).

A Sorvall model RC-2 refrigerated centrifuge was used for low speed centrifugations (10,000 r.p.m. and below), and a Spinco model L preparative ultracentrifuge was used for high speed centrifugations (above 10,000 r.p.m.).

Lyophilization was carried out in a Virtis apparatus.

A Savant model FP-22 Flat Plate instrument was used for the electrophoretic determinations.

Isolation of Ribosomes. Starting from a manganese slant, cultures were transferred twice on slants. The second transfer was grown to the logarithmic phase (about 6½ hours for the mesophiles and 6 hours for the thermophiles).

The growth of each slant was suspended in 6-8 ml. of sterile distilled water and about 1.2-2.0 ml. was used as an inoculum for one large petri dish (diameter, 6 in.). Cells were harvested from the petri dishes at the logarithmic stage of growth. Both slants and petri dish cultures of the mesophiles were incubated at 37°C and those of the thermophiles at 55°C. Ribosomes were prepared according to Tissieres et al (23) with some modifications.

The cells from about 120 petri dishes were harvested in about 500-1000 ml. of cold Tris buffer and centrifuged for 20 minutes at 12,000 x g with either the GSA or the SS-34 Sorvall rotor to bring down the cells. The well-packed cells were broken by grinding in a cold mortar for 3-5 minutes with 3 parts by weight of aluminum oxide and extracted with 10 volumes of cold Tris buffer. Deoxyribonuclease (1 ug./ml.) was added to the mixture of aluminum oxide, broken cells and buffer and the mixture incubated for 30 minutes at 22°C in a water bath to depolymerize the viscous bacterial DNA.

The mixture was centrifuged for 15 minutes at 12,000 x g as above to bring down cell debris and aluminum oxide. The supernatant was decanted from the sediment and centrifuged for 65 minutes at 105,000 x g or for 95 minutes at 78,500 x g. The resulting pellet was gently suspended in cold Tris buffer with a policeman and kept in chipped

ice in the refrigerator overnight to allow it to go into suspension. The suspension was centrifuged once more at 12,000 x g for 20 minutes to bring down any cell debris and aluminum oxide still present, and the supernatant centrifuged for 65 minutes at 105,000 x g. The pellet was again allowed to go into suspension in the buffer overnight while being kept in chipped ice in the refrigerator. The low and high speed centrifugation was repeated once more, and the final pellet was suspended in about 25 ml. of cold Tris buffer to provide the final ribosomal preparation.

EXPERIMENTAL

Isolation of Ribonucleic Acid by Phenol Treatment

The preparation of RNA from ribosomes was a modified procedure of the method used by Kurland (13).

The suspension of ribosomes in Tris buffer was made 0.2% in sodium dodecyl sulfate (SDS) and incubated at room temperature for 5 minutes. An equal volume of aqueous phenol solution was added to the SDS-treated ribosomal solution and shaken for 8 minutes at 30-40°C in a Burrell wrist-action shaker (setting, 5). The mixture was centrifuged for 10 minutes in a clinical centrifuge and the upper aqueous phase was pipetted from the lower phenol phase with a medicine dropper equipped with a capillary tip. The operation was repeated two more times with smaller volumes of phenol (approximately 2/3 and 1/3, respectively, of the original volume used).

The RNA was precipitated from the final aqueous phase by adding two volumes of absolute ethanol. The flocculent precipitate was brought down by centrifuging in a clinical centrifuge for 10 minutes. The supernatant containing the alcohol-soluble phenol was discarded, and the precipitate suspended in cold acetate buffer. The precipitation with absolute ethanol, centrifugation and suspen-

sion in buffer was repeated two more times. The precipitate in the last centrifugation was resuspended in cold acetate buffer and dialyzed against distilled water overnight in the refrigerator. The dialyzed RNA suspension was then lyophilized and stored at -10°C.

Determination of the Ultraviolet-Absorption Spectrum of a Ribonucleic Acid Preparation. The absorption spectrum of the ribosomal RNA from strain B. stearothermophilus 2184 was determined by measuring its optical density against distilled water. Readings were recorded at 10-mu intervals from 220 mu to 300 mu. Optical densities were plotted against the corresponding wavelengths, and an absorption spectrum characteristic of RNA was obtained as shown in Fig. 1. An absorption maximum was observed at 260 mu. This is the wavelength at which nucleic acids strongly absorb ultraviolet light.

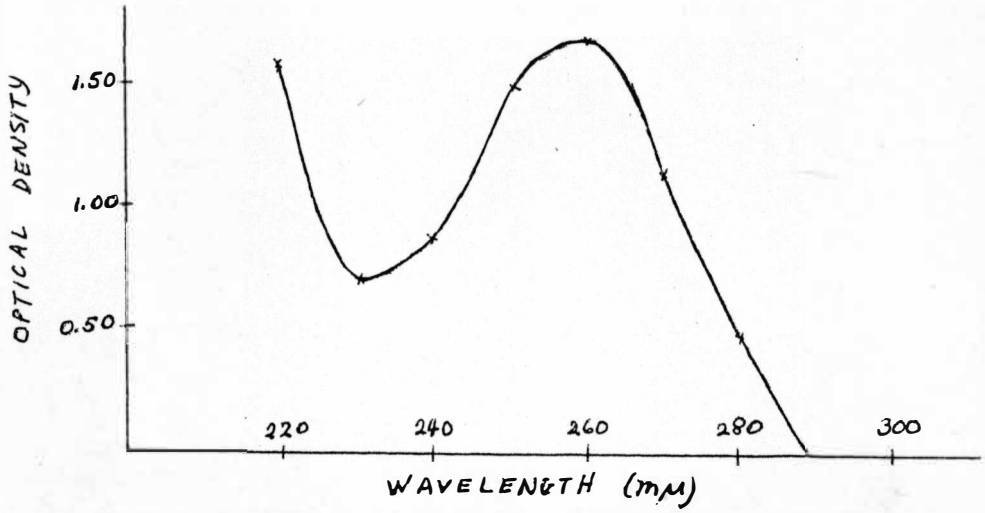

Fig. 1. Ultraviolet-absorption spectrum of RNA from B. stearothermophilus 2184

Determination of the Presence of Deoxyribonucleic Acid in a Preparation of RNA. The presence of DNA in the RNA of B. stearothermophilus 2184 was determined by treating an aliquot of RNA with deoxyribonuclease (DNAase), 10 ug. per ml., and incubating for 30 minutes at 37°C in a water bath. Another aliquot served as a control under the same conditions. Both suspensions were then dialyzed against distilled water overnight in the refrigerator. The absorption spectra of the two suspensions were determined according to the method used in the preceding experiment. There was no difference in the absorption spectrum between the DNAase-treated and the control (Fig. 2) showing that no DNA was present in the RNA preparation.

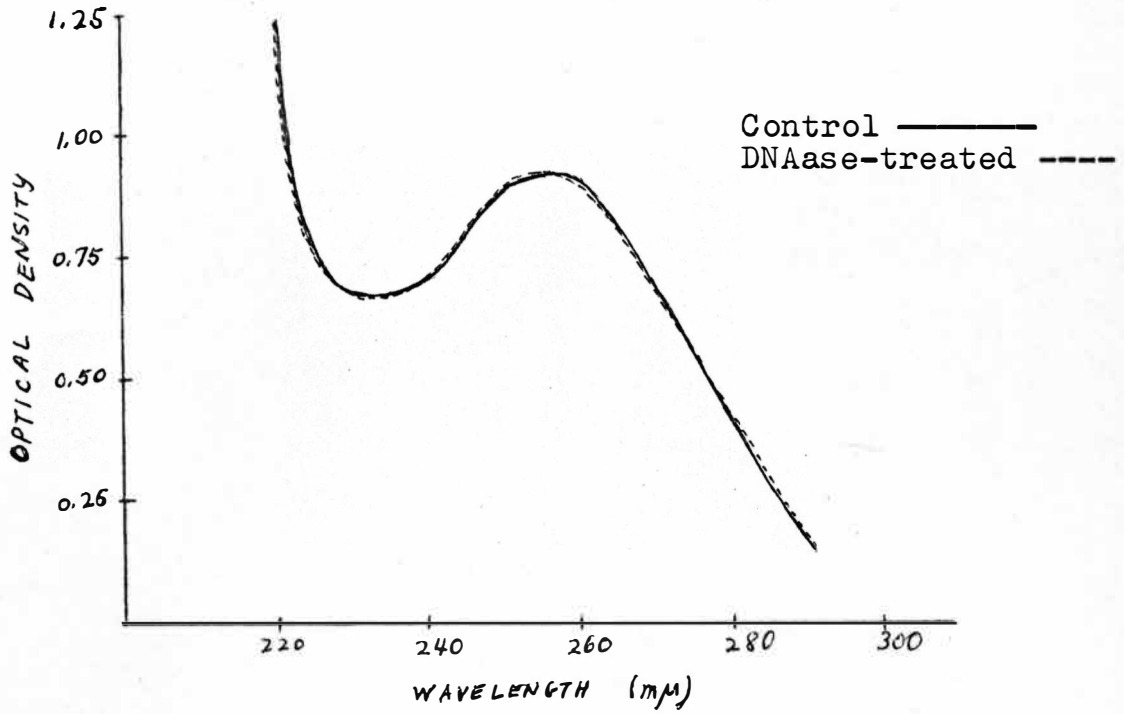

Fig. 2. Ultraviolet-absorption spectra of DNAase-treated and untreated RNA preparation of B. stearothermophilus 2184.

Estimation of the Protein Content of the RNA Preparations. The protein content of the RNA preparations was estimated by the Folin-Ciocalteu reagent (14). The readings at OD_{750} were almost identical to those for the reagent blank indicating that the amount of protein present in the RNA was negligible (less than 0.5%).

Determination of the Percentage Recovery of RNA from Ribosomes. The amount of RNA was calculated from the optical density at 260 mu of the original ribosome suspension and the RNA suspension (prior to lyophilization) from the equation (9):

$$\text{mg. RNA/ml.} = 0.0396 \times OD_{260}$$

and the measured volumes of the suspensions.

The amount of RNA recovered was also estimated from the dry weight of the lyophilized RNA and the initial amount of RNA in the ribosomal preparation calculated from the optical density as described above. A summary of the data on the isolation of RNA from the ribosomes (Table I) showed that the recovery varied between 62% and 85% based on OD_{260}, and 79% and 93% based on the dry weight.

Table I. Summary of Data on the Isolation of RNA from Ribosomes of Mesophiles and Thermophiles

	Mesophiles		
	B. Pum.	B. Lich.	B. X-1
Wt. of cells (g)	26	31	50
Wt. of RNA in ribosomes from OD_{260} (mg.)	18.8	25.3	13.0
Wt. of recovered RNA (mg.) Based on OD_{260}	-	17.2	9.5
Based on dry weight	17.5	23.4	11.1
Percentage recovery of RNA Based on OD_{260}	-	68.0	72.8
Based on dry weight	93.0	92.7	85.2
	Thermophiles		
	B. 10	B. FJW	B. 2184
Wt. of cells (g)	22	25	19
Wt. of RNA in ribosomes from OD_{260} (mg.)	33	57	27.8
Wt. of recovered RNA (mg.) Based on OD_{260}	28.3	35.5	19.0
Based on dry weight	30.5	44.9	18.1
Percentage recovery of RNA Based on OD_{260}	85.6	62.3	68.5
Based on dry weight	92.4	78.7*	65.2*

* Low recovery of RNA from B. FJW and B. 2184 was due to loss of some dry RNA during the transfer from the lyophilizing flask to the vial.

Abbreviations: B. Pum., B. Pumilus NRS 236; B. Lich., B. Licheniformis NRS 243; B. X-1, B. sp. X-1; B. 10, B. stearothermophilus 10; B. FJW, B. stearothermophilus FJW; B. 2184, B. stearothermophilus 2184.

Characterization of Ribonucleic Acid by Base Composition

The method of Bendich (4) was followed for determining the base composition of the RNA preparations. Preliminary experiments were first done on known bases and on yeast RNA before attempting to work on the RNA samples.

Preliminary Experiments on Known Bases.

1. Determination of R_f values and absorption spectrum of individual bases. Stock solutions of the five bases (adenine, cytosine, guanine, thymine and uracil) were made at concentrations of approximately 20 mg. per 10 ml. of 2 N perchloric acid. Melting point capillaries, open at both ends, were used to apply each of the base solutions on strips of Whatman No. 1 filter paper. A blank strip containing only 2 N perchloric acid was run simultaneously with the samples. Development of the chromatograms was carried out for 19 hours after which the papers were dried and the bases located on the paper under a short wave ultraviolet lamp (Mineralight model R-51). R_f values were calculated for each of the bases and compared with reported values (4) (Table II).

The outlined spots were cut out into small pieces and eluted with 5 ml. of 0.1 N hydrochloric acid for 2 hours at room temperature with constant shaking in a

Burrel wrist-action shaker (setting, 8). The eluates were decanted, centrifuged for 10 minutes in a clinical centrifuge to remove the shreds and the optical density read against the corresponding eluates of the blank. Readings were recorded at 10-mu intervals from 220 mu to 300 mu (Table III), and the wavelength of maximum absorption (λ_{max}) was determined. The observed maximum absorption of each base corresponded to the reported λ_{max} (3) for these bases (Table IV).

Table II. R_f Values of Standard Bases

Base	Calculated R_f	Reported R_f* (4)
Adenine	0.40	0.36
Cytosine	0.50	0.47
Guanine	0.31	0.25
Thymine	0.76	0.77
Uracil	0.68	0.68

* Solvent system: 2 N hydrochloric acid in 65 volumes isopropanol and 35 volumes of water.

Table III. Optical Densities of Standard Bases

Wavelength (mu)	Guanine	Adenine	Cytosine	Uracil	Thymine
220	0.108	0.155	0.187	0.130	0.181
230	0.113	0.088	0.068	0.064	0.067
240	0.195	0.159	0.039	0.143	0.073
250	0.239	0.311	0.080	0.262	0.147
260	0.174	0.412	0.163	0.316	0.219
270	0.156	0.358	0.244	0.216	0.214
280	0.142	0.152	0.240	0.056	0.119
290	0.085	0.013	0.124	0.003	0.024
300	0.019	-0.002	0.014	0.001	0.002

Table IV. Optical Densities of Standard Bases at λ_{max}

max (mu)	Guanine	Adenine	Cytosine	Uracil	Thymine
248.5	0.240				
262.5		0.418			
276			0.259		
260				0.316	
264.5					0.230

2. Determination of the resolving power of the solvent system for different bases in a mixture. A mixture of the five bases was chromatographed to determine whether the bases would be completely resolved by the solvent systen being used. The spots obtained were distinctly separate from each other and could easily be identified from their relative positions on the paper. Optical densities of the eluates were taken over the range of the ultraviolet spectrum and the ratios of the optical density at one wavelength to that at 260 mu were calculated. The spots were numbered from the starting line down to the tip of the paper as spot #1, spot #2, spot #3, spot #4 and spot #5. The OD ratios of these eluates and of the eluates from the individual bases obtained in the preceding experiment were compared to literature values of OD ratios (3)(Tables V and VI).

Table V. OD Ratios of Eluates from Mixture

Spot #	250/260	280/260
1	1.26	0.91
2	0.77	0.40
3	0.49	1.51
4	0.75	0.09
5	0.70	0.61

Table VI. OD Ratios of Standard Bases and Reported Values

Base	250/260		280/260	
	Observed	Reported	Observed	Reported
Guanine	1.38	1.37	0.82	0.84
Adenine	0.76	0.76	0.37	0.375
Cytosine	0.49	0.48	1.47	1.53
Uracil	0.83	0.84	0.177	0.175
Thymine	0.67	0.67	0.54	0.53

There were some discrepancies between the OD ratios of the mixture eluates and the literature values, but identification of the bases was still possible as the relative magnitudes of these OD ratios remained unchanged. Thus, spot #1 is guanine; spot #2, adenine; spot #3, cytosine; spot #4, uracil and spot #5, thymine.

3. Determination of the recovery of the bases. To check for the recovery of the bases applied, 0.10 ml. of each base were mixed together and 20 ul. of the mixture was chromatographed. The optical densities at 260 mu of the eluates and of known dilutions of the unchromatographed bases were measured and the percentage recovery calculated from the OD_{260} (Table VII).

Table VII. Percentage Recovery of Bases from OD_{260}

Base	"Units" Applied	"Units" Eluted	Percentage Recovery
Guanine	0.509	0.420	82
Adenine	0.799	0.830	103
Cytosine	0.449	0.440	98
Uracil	0.559	0.600	107
Thymine	0.517	0.485	94

Preliminary Experiments on Yeast Ribonucleic Acid.

A weighed sample of yeast RNA was hydrolyzed in a glass stoppered centrifuge tube according to the procedure of Bendich (4) and 10 ul. of the hydrolysate was chromatographed. A control volume containing the five known bases was run simultaneously with the hydrolysate and the blank. Four spots, distinctly separated from each other, were obtained in the sample lane and corresponded to the first four spots on the control lane. The R_f values calculated for the spots agreed very closely with the R_f values of the bases from the control lane (Table VIII).

The optical densities of the eluates of the four spots from the hydrolysates were measured over the en-

tire range of the ultraviolet spectrum and the OD ratios compared with the OD ratios of the individual bases given in Table VI. Both the R_f measurements and the OD ratios indicate that spot #1 is guanine; spot #2, adenine; spot #3, cytosine; and spot #4, uracil (Table IX).

Table VIII. R_f Values of Bases from Yeast RNA Hydrolysate

Spot #	Sample Lane	Control Lane
1	0.34	0.34
2	0.45	0.46
3	0.58	0.58
4	0.73	0.73

Table IX. OD Ratios of Bases from Yeast RNA Hydrolysate

Spot #	250/260	280/260	Base	250/260	280/260
1	1.35	0.83	Guanine	1.38	0.82
2	0.75	0.40	Adenine	0.76	0.37
3	0.41	1.55	Cytosine	0.49	1.47
4	0.80	0.196	Uracil	0.83	0.177

Determination of the Base Composition of the RNA Samples.

About 2.5 mg. of each of the RNA preparations were hydrolyzed with concentrated perchloric acid according to the procedure used for the yeast RNA. The hydrolysate was made up to a total volume of 0.25 ml. and 20 ul. were used for each chromatographic run.

The developed papers all showed four distinct spots which were identified from their R_f values (Tables X and XI), the absorption maxima and the OD ratios (Tables XII and XIII) of their eluates. The spots were numbered from the starting line downward, and were identified as guanine, adenine, cytosine and uracil, respectively. The optical density of the eluates was measured over the entire range of the ultraviolet spectrum, and the λ_{max} of each spot agreed with the theoretical λ_{max} (3) of the corresponding base.

Table X. R_f Values of RNA Preparations from Mesophiles

Spot #	B. Pum.	B. Lich.	B. X-1	Known Bases	
1	0.36	0.39	0.33	Guanine	0.31
2	0.49	0.52	0.43	Adenine	0.40
3	0.58	0.61	0.54	Cytosine	0.50
4	0.74	0.76	0.72	Uracil	0.68

Table XI. R_f Values of RNA Preparations from Thermophiles

Spot #	B. FJW	B. 2184	B. 10	Known Bases	
1	0.34	0.31	0.32	Guanine	0.31
2	0.46	0.42	0.44	Adenine	0.40
3	0.55	0.52	0.54	Cytosine	0.50
4	0.74	0.71	0.72	Uracil	0.68

Table XII. OD 250/260 of RNA Preparations from Mesophiles

Spot #	B. Pum.	B. Lich.	B. X-1	Known Bases	
1	1.39	1.40	1.36	Guanine	1.38
2	0.77	0.77	0.77	Adenine	0.76
3	0.47	0.50	0.44	Cytosine	0.49
4	0.88	0.85	0.78	Uracil	0.83

Table XIII. OD 250/260 of RNA Preparations from Thermophiles

Spot #	B. FJW	B. 2184	B. 10	Known Bases	
1	1.41	1.40	1.35	Guanine	1.38
2	0.78	0.75	0.74	Adenine	0.76
3	0.48	0.50	0.44	Cytosine	0.49
4	0.78	0.85	0.77	Uracil	0.83

The concentrations of the bases in the RNA hydrolysates were calculated by two methods (4):

(1) Absorption maxima technique

micromoles base = OD units x micromoles/OD unit

An OD unit is defined as the optical density at the λ_{max} of the eluate multiplied by the total volume in milliliters. The micromoles/OD unit for each base is a constant and has the following values (4): adenine, 0.0794; guanine, 0.0901; uracil, 0.123; and cytosine, 0.100.

(2) Differential extinction technique

The concentration is calculated from the difference (Δ) in the optical density values read at the absorption maximum and at another wavelength. For standard solutions containing 10 ug. of base per ml. of 0.1 N hydrochloric acid solution, the following Δ values have been determined:

Adenine:	$E_{262.5} = 0.930$	$E_{290} = 0.030$	$\Delta = 0.900$
Guanine:	$E_{249} = 0.737$	$E_{290} = 0.262$	$\Delta = 0.475$
Uracil:	$E_{260} = 0.738$	$E_{280} = 0.148$	$\Delta = 0.590$
Cytosine:	$E_{276} = 0.910$	$E_{300} = 0.047$	$\Delta = 0.863$

Hence: $$x = \frac{10 \Delta_x}{\Delta}$$

where: x = ug. of base in 1 ml. of eluate

Δ_x = observed OD difference of eluate

The results of the base analysis of ribosomal RNA from the six strains studied are given in Tables XIV, XV, XVI and XVII.

Table XIV. Base Composition of RNA Preparations from Mesophiles

	Micromoles per 100 micromoles base (calculated by absorption maxima technique)		
Base	B. Pum.	B. Lich.	B. X-1
Guanine	32.7 ± 0.40	31.9 ± 0.30	31.7 ± 1.25
Adenine	29.0 ± 0.35	28.5 ± 0.30	30.8 ± 1.00
Cytosine	21.1 ± 1.10	22.2 ± 0.60	24.0 ± 0.55
Uracil	17.2 ± 0.40	17.4 ± 0.60	13.6 ± 1.10

	Micromoles per 100 micromoles base (calculated by differential extinction technique)		
Base	B. Pum.	B. Lich.	B. X-1
Guanine	32.7 ± 0.45	32.9 ± 0.30	32.7 ± 0.60
Adenine	28.3 ± 0.25	27.7 ± 0.00	30.4 ± 0.70
Cytosine	21.1 ± 1.00	22.1 ± 0.20	23.8 ± 0.80
Uracil	18.0 ± 0.30	17.3 ± 0.10	13.1 ± 1.00

Table XV. Base Composition of RNA Preparations from Thermophiles

	Micromoles per 100 micromoles base (calculated by absorption maxima technique)		
Base	B. FJW	B. 2184	B. 10
Guanine	34.4 ± 0.25	33.2 ± 0.60	33.9 ± 1.75
Adenine	25.4 ± 0.35	25.1 ± 0.40	25.8 ± 0.15
Cytosine	24.7 ± 0.50	25.8 ± 0.50	24.0 ± 0.65
Uracil	15.8 ± 0.30	15.9 ± 0.50	16.5 ± 1.25

	Micromoles per 100 micromoles base (calculated by differential extinction technique)		
Base	B. FJW	B. 2184	B. 10
Guanine	34.9 ± 0.30	34.9 ± 0.80	34.1 ± 1.85
Adenine	26.5 ± 0.30	25.1 ± 0.45	26.7 ± 1.50
Cytosine	25.4 ± 0.60	25.2 ± 0.60	25.0 ± 1.90
Uracil	13.3 ± 0.65	14.9 ± 0.65	14.3 ± 1.55

Table XVI. Summary of Data on the Base Composition of RNA Preparations from Mesophiles and Thermophiles

	Micromoles per 100 micromoles base*					
	Mesophiles			Thermophiles		
Base**	B. Pum.	B. Lich.	B. X-1	B. FJW	B. 2184	B. 10
G	32.7	32.4	32.2	34.2	34.1	34.0
A	28.7	28.1	30.6	26.0	25.1	26.3
C	21.1	22.2	23.9	25.1	25.5	24.5
U	17.2	17.4	13.4	14.6	15.4	15.4
A + G	61.4	60.5	62.8	60.2	59.2	60.3
C + U	38.3	39.6	37.3	39.7	40.9	39.9
G + C	53.8	54.6	56.1	59.3	59.6	58.5
A + U	45.9	45.5	44.0	40.6	40.5	41.7

* Values are averages of 4 calculations representing 2 runs per strain except for B. X-1 where 4 runs were made and calculated by the two methods.

**G, A, C and U, stand for guanine, adenine, cytosine and uracil, respectively.

Table XVII. Base Ratios of RNA Preparations

	Mesophiles			Thermophiles		
Base	B. Pum.	B. Lich.	B. X-1	B. FJW	B. 2184	B. 10
$\frac{G + C}{A + U}$	1.17	1.20	1.28	1.46	1.47	1.40
$\frac{A + U}{G + C}$	0.85	0.83	0.78	0.68	0.68	0.71

Characterization of Ribonucleic Acid by Fingerprinting

The mapping procedure for the nucleotides in the RNA preparations was based on the method described by Rushizky and Knight (21) except for the technique involved in running the electrophoresis because the instrument used in this experiment was of a different type. Some preliminary experiments were first done on yeast RNA to determine the efficiency of the procedure.

Fingerprinting of yeast RNA. Digestion of yeast RNA by ribonuclease (RNAase) Type XI-A was carried out under 3 different conditions: A - no indicator, no toluene; B - no indicator, with toluene; C - with indicator and toluene. Indicator was added in order to follow the change in pH during the digestion period, and toluene served to retard microbial growth. These 3 treatments were carried out to determine the effect of the indicator and/or toluene on the digestion of yeast RNA.

Bromthymol blue, 0.01 ml., was added to a small centrifuge tube containing 3 mg. of RNA and 0.15 mg. of RNAase dissolved in 0.75 ml. of 0.04 M phosphate buffer, pH 7.1. One hour after digestion was started, 2 drops of toluene was added and the digestion was carried out at 37°C for a total of 24 hours with shaking in a recip-

rocal water bath shaker. The toluene was extracted with a small volume of peroxide-free ether after the digestion and the residual ether evaporated by a gentle air current passed through a cotton wad.

The digest was divided into two parts for electrophoresis, one subjected to high voltage (2000 volts, 40 volts per cm.) and the other to low voltage (350 volts, 7 volts per cm.). About 0.70 ml. of the digest was applied in a corner near the cathode edge of a sheet of Whatman 3MM paper by means of a lambda pipette while being dried with cold air from a hair dryer. The paper was placed on the electrophoresis plate which was lined with a plastic sheet and both edges of the paper were allowed to dip into the electrophoresis buffer contained in plastic trays on each side of the plate. The paper was wetted with the buffer using an atomizer, and a picric acid marker was placed on the same edge as the sample but near the opposite corner. Another plastic sheet was placed right on top of the paper and weighted down with a thick glass plate. The low voltage run was for 18 hours while the high voltage run took 3 hours to finish.

The papers were dried and placed in a chromatocab, saturated with the developing solvent made up of equal parts of the electrophoretic buffer adjusted to pH 3.8 and tert-butanol, and allowed to equilibrate for 1 hour before they were developed. After 36 hours the papers

were dried and examined under ultraviolet light.

To determine whether all the nucleotides would be retained during the chromatographic run, solutions of each of the four nucleotides (adenylic acid, cytidylic acid, guanylic acid and uridylic acid) in phosphate buffer, were chromatographed simultaneously with the yeast RNA digests.

The papers containing the yeast RNA digests all showed very intense streaking down from the origin and from some of the spots. No difference was observed among the different treatments, but the low voltage electrophoresis resulted in better fingerprint (more compact spots) than the high voltage run.

The control paper containing the nucleotides showed no streaking from any of the spots and all of the four nucleotides were retained during the 36-hour chromatographic run.

The following experiments were carried out to determine the probable cause of streaking from the origin.
(a) Diffusion of material from the origin before current is applied.

A sample of the yeast RNA digest was applied to the paper and the area around the origin was wetted for some time before applying the current. The electrophoresis was run at 2000 volts for 3 hours, and the paper viewed

under ultraviolet light after drying.

No streaking from the origin was observed indicating that the streak was not due to diffusion of material from the origin.

(b) Interference of indicator with movement of material during chromatography.

The paper was divided into 3 lanes - RNA digest with indicator was applied to the first lane, RNA digest without indicator to the second lane, and indicator alone to the third lane - and chromatographed for 12 hours.

Streaking was observed in both the first and second lanes, but no spots or streaks on the third lane. The indicator was observed to move with the solvent front. The results of this experiment showed that the streaking was not due to the presence of indicator in the material, and it did not interfere with the movement of the material.

(c) Ratio of RNA to RNAase in the digestion mixture.

Digestion was carried out using two types of RNAase at two different RNA/RNAase ratios: RNA/RNAase Type I-A, 20/1; RNA/RNAase Type I-A, 100/1; RNA/RNAase Type XI-A, 20/1; and RNA/RNAase Type XI-A, 100/1. Type I-A contains several fractions and Type XI-A is very homogeneous. Indicator and toluene were added to all and digestion was performed under the same conditions as before. The digests obtained were chromatographed directly without prior

electrophoresis.

All of the four treatments showed streaking down from the origin, and the intensity of the streaks was the same for all. It was previously thought that the streaking might be due to incomplete digestion or to high enzyme concentration, but the results showed that variations in the type of ribonuclease or its concentration relative to that of the RNA had no effect.

(d) Composition of the chromatographic solvent.

A sample of yeast RNA was digested according to the usual procedure, but was chromatographed using a solvent consisting of 87 parts of tert-butanol to 63 parts of electrophoresis buffer adjusted to pH 3.8. Rushizky (21) pointed out that this solvent gave equal or better resolution than the 50:50 solvent.

The results obtained with this experiment showed an improvement over the previous ones in that the origin showed a very narrow and short streak in contrast to the long and intense streaks formerly observed.

No further experiments were made after obtaining these results, and the succeeding fingerprinting of the RNA preparations were made using this solvent system.

Fingerprinting of RNA Preparations.

Attempts at quantitation of RNA fingerprints. To about 3 mg. of the RNA preparations in 0.30 ml. of 0.04 M phosphate buffer were added 0.16 ml. of RNAase Type XI-A solution (1 mg./ml., in 0.04 M phosphate buffer) and 0.01 ml. of bromthymol blue indicator. Two drops of toluene were added after 1 hour and the digestion carried out at 37°C for 24 hours with shaking.

A small amount of the digest was saved for optical density measurements, and the rest was applied to the paper and electrophoresis was run at 7 volts per cm. and a current of about 7.5 mA for 17-20 hours according to the procedure described under yeast RNA.

After the papers were dried they were chromatographed using the 87:63 tert-butanol:electrophoresis buffer solvent for 34 hours.

Examination under ultraviolet light showed very slight trailing from the origin and the spots were found to be nicely separated from each other. However, the material in the spots appeared to be diffused and not concentrated in a small area. The spots also varied in sizes.

The spots including the origin were cut out into small pieces and eluted with 10 ml. of 0.01 N hydrochloric acid in a large test tube and shaken in a wrist-action shaker for 6 hours (setting, 10). Blanks corresponding to the smallest and biggest spots were cut out from the

paper and eluted similarly. The eluates were decanted from the bulk of the paper and centrifuged for 10 minutes in a clinical centrifuge to remove any residual fibers.

Percentage recovery was calculated from the optical densities of the eluates (corrected for eluates of blank spots of comparable size) and the optical densities of the digest aliquots which had not been fingerprinted. Allowance was made for the amount of digest used in a given fingerprint experiment.

The percentage recovery obtained for duplicate runs of samples of B. Licheniformis and one run of B. Pumilus RNA was very low: 15% and 14% for B. Licheniformis and 23% for B. Pumilus. These low recoveries posed a problem for the quantitative interpretation of the results.

Several short experiments were run to determine the cause of these low recoveries:

(a) The pH of the eluates and the digest not fingerprinted were checked and found not to have changed drastically.

(b) A comparison of the OD_{260} eluted in 6 hours from different sizes of paper, but all of which had received the same amount of nucleotide, showed that the paper contributes appreciably to the optical density measured. The optical density was found to increase with increasing size of the paper being eluted and was higher than that of the unchromatographed material.

(c) In an attempt to cancel the reading due to the paper, two sizes of paper, one a blank and one containing nucleotide, were eluted and the optical density measured. After correcting for the blank the sample gave an optical density reading but this was less than the optical density applied to the paper.

(d) To determine the effect of the solvents used in electrophoresis and chromatography on the elution of the nucleotide from the paper, the same amount of nucleotide was applied to equal sizes of new filter paper and to one cut from paper previously used for fingerprinting. The papers were eluted for 4 hours and the optical density read against a blank. The results showed a higher recovery (82%) from the new paper and a low recovery (63%) from the used paper.

In view of the failure to get a good recovery of the material applied, attempts to do a quantitative analysis of the fingerprints were abandoned.

Qualitative Investigation of RNA Fingerprints. Duplicate samples of the RNA preparations were fingerprinted according to the procedure described in the preceding section. The number of spots and their relative positions on the paper were located under a strong ultraviolet lamp. The spots were numbered from top to bottom and left to right, and the origin and the distance reached by the

picric acid marker were taken as reference points.

The same number of discrete spots was obtained in the fingerprints of the different RNA preparations except for B. Pumilus which appeared to have one more weak spot. The respective spots were found in more or less the same position and the same spot number was given to the spot found in a particular area on the paper.

Fig. 3 shows the approximate position of the spots in the map. A line was drawn horizontally from the origin and was designated as the top margin, and another line drawn from the origin down to the serrated edge of the paper indicated the left margin. The distance travelled by the fastest spot was measured and used as the basis for calculating the value (R) which is the ratio of the distances moved during electrophoresis by the other spots and the fastest spot.

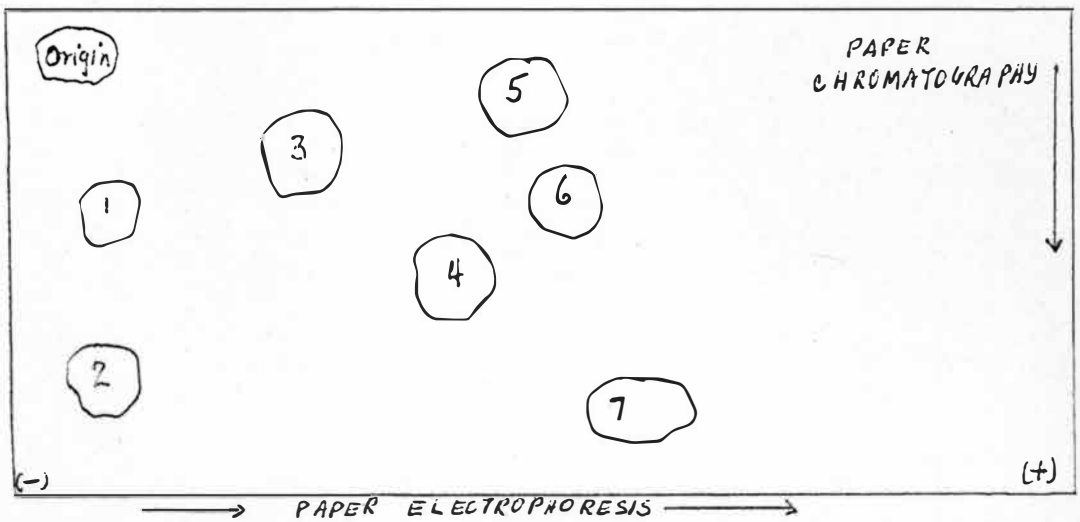

Fig. 3. Fractionation of ribonuclease digests of RNA from ribosomes of mesophiles and thermophiles

Results of the fingerprinting of the different RNA preparations are shown in Tables XVIII and XIX.

Table XVIII. Data on the Fingerprinting of RNA Preparations from Mesophiles

Spot No.	(R) during electrophoresis		
	B. Pum.	B. Lich.	B. X-1
1	0.02	0.02	0.03
2	0.00	0.00	0.01
3	0.44	0.36	0.43
4	0.73	0.67	0.74
5	0.84	0.76	0.91
6	0.93	0.86	0.99
7	1.00	1.00	1.00

Table XIX. Data on the Fingerprinting of RNA Preparations from Thermophiles

Spot No.	(R) during electrophoresis		
	B. FJW.	B. 2184	B. 10
1	0.04	0.04	0.04
2	0.03	0.00	0.03
3	0.48	0.42	0.47
4	0.77	0.75	0.77
5	0.89	0.84	0.88
6	0.95	0.91	0.93
7	1.00	1.00	1.00

DISCUSSION

Isolation of Ribosomal Ribonucleic Acid

The ultraviolet-absorption spectrum of ribosomal RNA from B. stearothermophilus 2184 shown on Fig. 1, is typical of the spectrum for nucleic acids where maximum absorption occurs at about 260 mu. This curve indicated the presence of RNA in the material isolated, but it did not exclude the presence of DNA in the preparation since DNA would also show the same maximum. Incubation of half the RNA suspension with deoxyribonuclease and subsequent dialysis against water produced optical density readings very close or equal to the readings of the untreated RNA. The spectra obtained for these two treatments (Fig. 2) show no change in the absorption spectrum and the absorption maximum indicating that the substance showing a strong absorption at this wavelength was not affected by the presence of the enzyme. A decrease in the peak would have been observed if DNA were present because of the loss of material as a result of enzymatic hydrolysis and subsequent dialysis. The absence of DNA has also been confirmed by Stenesh (22) by a chemical analysis.

Consideration was also given to the possible presence of protein in considerable amounts. Although protein has

a strong ultraviolet absorption band at 280 mu, the presence of small amounts cannot be detected from the absorption curve since nucleic acids also absorb at 280 mu. The amount of protein can be estimated by the Folin reagent which is very sensitive. These tests indicated that protein was absent or, at least, present in less than 0.5%.

In his work on E. coli ribosomes, Kurland (13) reported that sodium dodecyl sulfate in low concentrations (1% or less) splits the RNA from its protein component. The resulting RNA is stable and remains in the macromolecular state in this incubation mixture due to the simultaneous inhibition of latent ribosome ribonuclease (7) by 1% SDS. The removal of protein and the inhibition of the RNAase have been confirmed in this study.

Recovery of RNA isolated by the phenol treatment fell within the range reported by Kurland (13) for RNA from E. coli ribosomes. He reported a recovery varying between 70% and 90%. Except for B. stearothermophilus FJW and B. stearothermophilus 2184 which gave low recoveries due to mechanical loss, the percentage recovery (Table I) of the other four preparations based on the dry weight of RNA were in the upper limit of the reported values. However, the recoveries based on the OD_{260} were much lower than those based on dry weight. Using ribosomes from rabbit reticulocyte, Cox (6) was able to isolate RNA in better than 90% yield by precipitation as the

guanidinium salt. However, his yield of RNA using phenol was very low. Littauer and Eisenberg (15) were able to obtain yields of 81-93% using ethanol precipitation, and 69-88% using ammonium sulfate precipitation, in their work on the isolation of RNA from E. coli "protoplasts".

Base Composition of Ribosomal Ribonucleic Acid

The term "base" refers to the purines and pyrimidines isolated from nucleic acids. In this study, hydrolysis of the ribonucleic acids by a strong acid resulted in the cleavage of the acid-labile glycosidic bonds liberating the purine and pyrimidine bases. The use of 70-72% perchloric acid at 100°C for 60 minutes insured the complete hydrolysis of RNA to the free bases, especially cytosine which is incompletely liberated at much lower temperatures (20).

Bendich (4) reported recovery within ± 2% or less with this method using artificial mixtures of purine and pyrimidine standards. The percentage recovery of the bases shown in Table VII gives values close to those of Bendich except for guanine. This low recovery of guanine may be attributed to incomplete elution of all the guanine in the spot. When viewed under ultraviolet light, guanine appears as a light blue fluorescent spot unlike the dark

spots of the other bases. Hence, some of the material might have been left on the paper when the spot was cut out due to the absence of a definite boundary.

The spots were identified by their R_f values and the OD ratios at 250/260 and 280/260 mu, and the λ_{max} of the eluates. In all cases only four discrete spots were found on the paper chromatograms, and these were identified as guanine, adenine, cytosine and uracil. No other bases were detected under the conditions of analysis.

Some differences have been observed between the base composition of the three species of mesophiles and three strains of thermophiles studied. As shown in Table XVI, the thermophiles have a consistently higher percentage of guanine and cytosine than the mesophiles, whereas the uracil (with the exception of B. sp. X-1) and adenine content of the mesophiles are higher than those of the thermophiles. The G+C/A+U ratio (Table XVII) shows a distinct difference between these two types of organisms. The thermophiles have a considerably higher G+C/A+U ratio than the mesophiles. In view of the importance of guanine and cytosine for the formation of hydrogen bonds in nucleic acids, it is tempting to speculate that a difference in hydrogen bonding may exist between the ribosomal ribonucleic acids of mesophiles and thermophiles.

Mangiantini *et al* (16) reported similar observations in their studies of one thermophilic organism, B. stearo-

thermophilus B, compared to the mesophilic E. coli. His values on the composition of B. stearothermophilus B and the results of this study are shown in Table XX.

Table XX. Base and Nucleotide Composition of Ribosomal RNA from Thermophiles

Base Composition (Average of 3 strains of B. stearothermophilus)		Nucleotide Composition B. stearothermophilus B (Mangiantini et al (16)	
Adenine	25.8 ± 0.47	Adenylic acid	25.9 ± 1.07
Guanine	34.1 ± 0.07	Guanylic acid	34.5 ± 0.72
Cytosine	25.0 ± 0.37	Cytidylic acid	22.0 ± 0.68
Uracil	15.1 ± 0.37	Uridylic acid*	17.5 ± 0.64
$\frac{A + U}{G + C}$	0.69		0.78

*plus pseudouridylic acid

The differences observed between the values reported by Mangiantini and those found in this study may be attributed to the differences in the methods of analysis used. Mangiantini reported nucleotide composition based on the direct alkaline hydrolysis of whole RNA in the intact ribosome, whereas this study reports base composition of the purified RNA isolated from the ribosomes. Moreover, their reported A+U/G+C ratio may be higher because it included a determination of pseudouridylic acid which accounted for about 3% of the total uridylic acid.

A comparison of the nucleotide composition of two mesophilic bacteria belonging to the genus Bacillus reported by Miura (19) with the base composition of the three mesophilic bacteria studied here is shown in Table XXI.

Table XXI. Base and Nucleotide Composition of Ribosomal RNA from Mesophiles

Base	B. Pum.	B. Lich.	B. X-1	Bacillus cereus (Miura (19)	Bacillus subtilis
G	1.16	1.19	1.03	1.26	1.31
A	1.00	1.00	1.00	1.00	1.00
C	0.75	0.80	0.78	0.87	0.93
U	0.64	0.63	0.44	0.84	0.83
G+C/A+U	1.16	1.22	1.25	1.16	1.22
G+U/A+C	1.03	1.01	0.83	1.12	1.11

The values are expressed as molar ratios, A = 1.00

The G+C/A+U ratios obtained for the three mesophiles studied here agree very closely to those reported by Miura. Based on his results, Miura concluded that the RNA's of the ribosomes of different bacterial species have the same nucleotide ratio. This study has shown that the nucleotide ratio may be similar only for organisms which have the same optimum temperature for growth, since a distinct difference was observed in the G+C/A+U ratio between the mesophiles and thermophiles.

Fingerprinting of Ribosomal Ribonucleic Acid

Although Rushizky and Knight (21) reported a recovery of 90% of the applied OD_{260} as characteristic fractions, the same fingerprint procedure gave a recovery of only about 20% in this study. Due to the failure to elute the bulk of the material applied, the study on the digestion of RNA by ribonuclease was done only qualitatively.

Maps of the RNA digests showed no apparent difference between the two types of organisms. The number and location of the spots on the paper appeared to be similar in both the mesophiles and the thermophiles. Tables XVIII and XIX show the R values of the spots calculated along the direction of electrophoresis. In all the runs, a very distinct and intense spot was always found in the area designated as spot #7.

Since the amount of nucleotides in the spots could not be measured, no conclusions can be drawn as to the relative proportions of these various nucleotides in the ribosomal RNA. The apparent absence of differences in the number and position of the spots indicates, but does not prove, that the corresponding spots are composed of identical nucleotides. This point can only be settled after the complete sequence of bases is determined for all these nucleotides.

SUMMARY

Ribosomes were isolated from three strains of mesophiles: B. pumilus NRS 236, B. licheniformis NRS 243 and B. sp. X-1, and from three strains of thermophiles: B. stearothermophilus 2184, B. stearothermophilus 10 and B. stearothermophilus FJW. Ribonucleic acid was isolated from the ribosomes in 70-90% yield by phenol treatment. The RNA preparations contained no detectable DNA and only traces of protein.

The base composition of the ribosomal RNA was determined by paper chromatography of the hydrolysate after acid hydrolysis. Distinct differences in base composition were observed between the mesophiles and the thermophiles. All the three thermophiles show a higher guanine and cytosine content but a lower percentage of uracil and adenine than the mesophiles. The average G+C/A+U ratio of the thermophiles was 1.44 and that of the mesophiles was 1.22.

Attempts to study quantitatively the fractionation of ribonucleic acid by enzymatic hydrolysis using fingerprinting techniques have failed. A qualitative determination showed seven discrete spots found in almost the same positions on the paper.

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VITA

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APPROVAL OF EXAMINING COMMITTEE

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(Chairman)

Date _____