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Immunochemical Studies of Ribosomes from Mesophilic and Thermophilic Bacteria

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IMMUNOCHEMICAL STUDIES OF RIBOSOMES FROM MESOPHILIC AND THERMOPHILIC BACTERIA

by

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INTRODUCTION

Many investigators have been intrigued by the phenomenon of thermophily. This phenomenon involves bacteria that survive and grow at temperatures that normally denature proteins and nucleic acids (about 55-80°), whereas mesophilic bacteria grow at lower temperatures (about 20-45°).

The first thermophilic bacterium was isolated by Miquerel in 1879 (1). Since Miquerel's initial discovery, thermophiles have been isolated from many sources in nature. Three major theories have been presented as partial explanations of the phenomenon of thermophily. The first theory, developed by Gaughran (1), suggests that thermophily may be explained by the presence of heat-stable lipids which protect cellular components. The second theory, proposed by Allen (2), attempts to explain thermophily on the basis of increased rates of synthesis and degradation. The third theory, which has received the most support so far, views thermophily as being due to physical-chemical differences of important macromolecules.

Most of the evidence for the latter theory comes from comparative studies of proteins and nucleic acids. These investigations have been summarized in two recent reviews (3,4). Comparisons of this type have usually involved a thermophilic strain of Bacillus and a mesophilic strain

of Escherichia coli. However, these findings can not rule out the possibility of intergeneric differences. In order to avoid this complication, comparative studies from this laboratory have involved both mesophilic and thermophilic strains of one genus, namely Bacillus. Previous papers from this laboratory have described differences between these mesophilic and thermophilic strains with respect to DNA (5), ribosomal RNA (6), ribosomes (7), and cell-free amino acid incorporating systems (8).

In view of these demonstrated differences between the two types of organisms, it was of interest to learn more about the structure of the ribosomes involved. The present research was undertaken with this in mind.

An immunochemical approach was selected as a means of assessing structural relationships between the various ribosomes. This was done by preparing rabbit antibodies to ribosomes and carrying out an antigen-antibody reaction, using the Ouchterlony immunodiffusion technique. These results were put on a quantitative basis using a spectrophotometric method and also correlated with results from amino acid incorporation experiments.

MATERIALS AND METHODS

Organisms and Growth Conditions

Three mesophilic species and three thermophilic strains of the genus Bacillus were used in this study. The organisms are listed below:

MESOPHILES	THERMOPHILES
B. licheniformis (NRS 243)	B. stereothermophilus (FJW)
B. pumilus (NRS 236)	B. stereothermophilus (10)
B. sp. (X-1)	B. stereothermophilus (2184)

The cells were grown in fermentors (New Brunswick Scientific Co., model MF-128S) with a medium consisting of 1% Trypticase (BBL) and 0.2% yeast extract (Difco); the medium also contained 1 ml of antifoam (Union Carbide Corp., SAG-471) per 25 liters. Mesophilic cells were grown at 37° and thermophilic cells were grown at 55°. The cells were harvested during the logarithmic growth phase (absorbance of 1.0 at 540 nm, approximately 2 g wet weight per liter). The culture was quickly chilled and the cells were collected by centrifugation at 30,000 X g using a continuous-flow system (Sorvall, model RC-2). The cells were washed with buffer I (0.01 M magnesium acetate, 0.06 M ammonium chloride, pH 7.4), frozen in liquid nitrogen, and stored at -20°.

When discussed later in this thesis, the organisms are abbreviated B. lich, B. pum., B. X-1, B. FJW, B. 10 and B. 2184.

Isolation of Subcellular Antigen Fractions

Ribosomes and supernatant fractions were isolated from frozen cells according to the procedure of Stenesh and Schechter (8). The cells were thawed, washed with buffer I and suspended in 1.5 volumes (W/V) of buffer I (also 0.006 M in spermidine and 0.006 M in 2-mercaptoethanol). The suspension was disrupted in a French press (Aminco, model 5-596) at 18,000 psi (9). Deoxyribonuclease was added (9) and cell debris was removed by two 30-minute centrifugations at 30,000 X g in the Sorvall. The first supernatant fluid was collected in its entirety and the second supernatant fluid was withdrawn to within about 1 cm above the pellet.

The ribosomes were collected by one 2-hour centrifugation at 105,000 X g (Spinco, model L). The supernatant from this centrifugation was designated as S-100 fraction. The ribosomal pellet was suspended in 25% of the original volume of buffer I (also 0.006 M in 2-mercaptoethanol and and 0.006 M in spermidine). These ribosomes were designated as crude ribosomes (CRs). A portion of the CR fraction was then purified by two cycles of low and high speed centrifugations. This involved 5 minutes at 10,000 X g followed by 2 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 I (also 0.006 M in 2-mercaptoethanol and 0.006 M in spermidine)

and centrifuged for 5 minutes at 20,000 X g to give the final preparation of washed ribosomes (WRs). The three antigen fractions (CR, WR and S-100) were frozen at -20° in small vials and diluted to a concentration of 2 mg protein per ml with buffer I (also 0.006 M in 2-mercaptoethanol and 0.006 M in spermidine) prior to use.

Protein in these fractions was determined by the method of Lowry (10) using bovine serum albumin as standard. Growth of the cells and isolation of the subcellular fractions was carried out by N. Schechter in this laboratory.

Preparation of Antigen Emulsions

Reagents and Equipment

Freund's adjuvant (complete and incomplete, Pentex)

16 gauge 1½ inch disposable aluminum hub Luer-Lok needles

20 gauge 1½ inch disposable aluminum hub Luer-Lok needles

Polyethylene tubing (Intramedic PE 320, ID 0.106 inch)

Glass Luer-Lok hypodermic syringes (10 ml, Becton, Dickinson)

Double-gang needle assembly

Procedure

Double-gang needle assembly

The double-gang needle assembly was prepared as follows. Two disposable 16 gauge needles were ground to remove the points and then deburred by holding the needle against the side of a grinding wheel at an angle of 45°. The two needles were connected by means of a short piece of polyethylene tubing, leaving 0.5 inch of the tubing between the needle tips. This assembly was then securely taped to a wood splint so that when syringes were attached to the assembly, one syringe could be held in the hand during the emulsification procedure without breaking the small polyethylene tubing section of the apparatus. A double-gang needle assembly was prepared for each of the different antigen samples to be emulsified.

Emulsification of antigen samples (11)

A 10 ml syringe with a 20 gauge needle was filled with 1.5 ml of antigen solution (2 mg protein per ml) and an equal volume of Freund's adjuvant. The syringe was inverted, air was removed from the syringe and the needle removed. The double-gang assembly was then attached, inverted and the plunger was depressed until the solution filled the syringe and double-gang needle assembly. A second syringe was attached to the remaining needle of the assembly and the emulsion formed by alternately depressing each of the syringe plungers. The emulsions were stored in the cold room (4°) overnight to assure that they were indeed stable emulsions.

Cleaning of syringes

Following removal of excess emulsion, the plunger and barrel were soaked in Haemo-sol for several hours, washed thoroughly with water, and dried in an oven until use. This minimized bacterial contamination of the emulsions.

Comments

The above procedure proved very efficient for obtaining stable emulsions. The S-100 fractions were generally the easiest to emulsify, whereas the CR fractions were most resistant to emulsification. As the emulsions began to form, increased pressure was required to expell the mixture from the syringe.

Generally, passage between the syringes for 10-15 minutes was sufficient to obtain a stable emulsion. If a stable white emulsion failed to result, the sample was allowed to stand for 10-30 minutes after which the partial breakdown of the emulsion (detected by yellow discoloration of the mixture) could usually be corrected for by several quick passages through the double syringe assembly.

Prior to use of the double-gang assembly, the preparation of emulsions had been attempted by repeatedly passing the mixture through a 10 ml syringe into a small vial. This technique, in addition to being extremely ineffective, left the author with sore thumbs.

Immunization of Rabbits

Reagents and Equipment

2 ml Luer-Lok syringes

22 and 20 gauge Luer-Lok aluminum hub disposable needles

95% ethanol

Rabbits - Male, 3-4 lb.; New Zealand White; Kuiper's Rabbit Ranch; Gary, Ind.

Procedure

Each rabbit was removed from his cage and held at the edge of a table. One hand was placed on the rabbit's back and the other hand was used to gently grasp and extend the rabbit's hind legs. The hind legs were wiped with alcohol (care must be taken so as not to get alcohol near the rab-

bit's anus) and 1 ml of an antigen emulsion was injected intramuscularly into each hind leg of the rabbit. Although the injections were done with a 1.5 inch 22 gauge needle, the 2 ml syringe was most easily filled with a 1.5 inch 20 gauge needle. While refilling the syringe with the dose for the other hind leg, the rabbit's "excited state" was minimized if he was placed back in his cage. It is best to place a rabbit in his cage hind feet first, as he is less likely then to kick and possibly injure himself or the animal handler.

Each rabbit was immunized four times at weekly intervals with either S-100, CR or WR fractions from one of the organisms. The first dose was comprised of the cell fraction and Freund's complete adjuvant. The next three weekly injections contained the cell fraction plus Freund's incomplete adjuvant. Each rabbit was bled prior to the initial injection to obtain control serum samples (6-9 ml). One week after the fourth injection, approximately 15-20 ml of blood were again taken from each rabbit for preparation of the antiserum.

Isolation of Rabbit Whole Antiserums

Reagents

Xylene
95% ethanol
Vaseline

Procedure

Collection of the blood (12)

Food was withheld from the rabbits during the night preceding the collection of blood. This minimizes the amount of serum lipids which generally float as globules on the isolated serum. Blood was collected the following morning by ear bleeding. Each rabbit was removed from his cage, placed on the laboratory bench and the hind quarters extended and wrapped semi-loosely with a towel secured with a safety pin. A razor blade was used to remove the fine hairs from the outer edge of the rabbit's ear and the area of the marginal blood vessel. Pressing down on a rabbit's back will prevent the vertebral arching essential to his kicking and subsequent "get-away". The blood vessel was then dilated by rubbing it with xylene followed by rinsing with 95% ethanol or by several gentle snaps of a skilled technician's finger and thumb. (Ethanol and xylene must be removed before the actual bleeding.) A small amount of vaseline was applied to the shaved area of the ear and a small (0.5 cm) longitudinal slash was made in the marginal ear vessel. The ear was tilted so that a continual flow of blood dripped into a conical 15 ml centrifuge tube. After carefully removing the towel, the rabbit was carefully placed back in his cage.

A wood splint was placed in the centrifuge tube con-

taining the freshly-collected blood. The tube was covered with parafilm and allowed to stand at room temperature for 2 hours until clotting occurred. The tubes were then stored in the cold room overnight to facilitate clot contraction. The contracted clot was removed from the blood by withdrawing the wood splint. Next, the blood was centrifuged in the cold room at 5,000 RPM for 20 minutes (Sorvall GL-1 rotor). The supernatant whole serum (or whole antiserum) was decanted into 25 ml Wheaton bottles, covered with a rubber stopper and aluminum foil (for a secure seal, vial and stopper must be completely free from moisture) and stored at 4°. Both whole control serums and whole antiserums were obtained in this fashion.

Initial screening of antiserums (12)

A Pasteur pipet was dropped into the antiserum and a liquid column of about 1 cm was collected. Next, an equal volume of the corresponding antigen solution was drawn into the capillary pipet below the antiserum sample. While being held in a vertical position, the tip of the capillary pipet was sealed with melted paraffin. This may be easily accomplished by placing the tip of the capillary pipet into previously melted paraffin followed by quick cooling in a beaker of cold water.

A precipitate in the pipet tip indicates a positive antigen-antibody reaction. All the antiserums gave such

a positive test. In most of the samples the interface antigen-antibody precipitates had formed after 10-15 minutes and after 30-45 minutes had started to settle to the bottom of the capillary tube. The precipitates are more easily detected by means of a hand loupe and a high intensity lamp.

Ouchterlony Double Immunodiffusion

Reagents and Equipment

Immunoframes (Gelman Instrument)
Glass microscope slides (Gelman Instrument)
Suction needle (Gelman Instrument)
Dies for cutting immunodiffusion patterns
Leveling board and level
Well cutters
12" x 20" x 6" air-tight plastic containers
Bacto-Agar (Difco)
NaCl (Anal. Reag., Mallenckrodt)
Sodium azide (Eastman Kodak)
High intensity lamp

Procedure

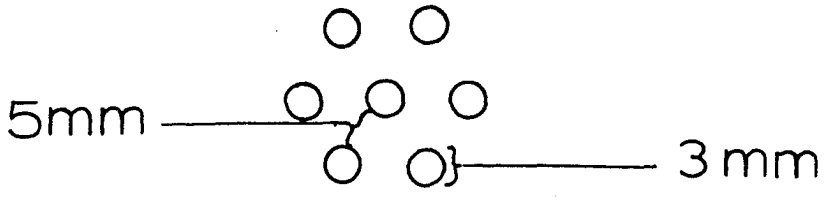
Preparation of immunodiffusion plates

An agar solution (100 ml, 1.25% bacto-agar and 0.85% NaCl) was heated to boiling and then kept at 65-75° on the steam bath while 0.05% sodium azide was added. The analytical grade NaCl was not pure and resulted in a slightly cloudy solution when dissolved in doubly distilled water. Therefore, it was necessary to filter the NaCl solution through a 0.45 mμ Millipore filter.

Six microscope slides (thoroughly cleaned and freed of glass particles by rubbing with a moist cloth) were

placed on the immunoframe which was then placed on the leveling board. The adjustable screw legs were turned until the frame was level. A rectangular dishpan was placed over the immunoframe to keep dust from the microscope slides. Since the immunodiffusion experimental results are permanently recorded by photography, it is essential to keep the immunodiffusion plates as dust-free as possible. A 10 ml large bore pipet was filled and emptied several times with the 65-75° agar solution. This prevents solidification of agar in the pipet. Three slides were then covered with 10 ml of the warm agar solution. The excess agar runs over the edges, leaving a solid agar strip of uniform thickness. The remaining half of the slides was immediately covered with agar in the same manner. The large dishpan was then placed over the immunoframe-leveling board apparatus for about 15 minutes. The plates were placed in the humid chamber (air-tight box containing several strips of moist sponge) and stored in the cold room until punching of the wells.

An immunodiffusion pattern containing a central well and six exterior wells was used throughout these experiments.



The immunodiffusion plates were removed from the humid chamber, allowed to warm up to room temperature and placed on the leveling board and illuminated from above with a high intensity lamp. The 7-well die was placed over two of the microscope slides on the immunoframe and the agar punch was carefully pressed through each hole in the die and twisted through a ¼ turn when the punch reached the microscope slide surface.

Since the original 3 mm agar punch prepared by R. Durbin was not hollow, it compressed the agar during the cutting, leaving very jagged, unsatisfactory well patterns. Therefore, an extremely effective well punch was fashioned from an empty ballpoint pen ink-barrel. The barrel tubing is slightly larger than 3 mm and unsharpened on both ends but can be turned down to the correct size and sharpened by placing the tubing in a drill press chuck and sharpening it with fine emery paper and a file. When sharpened, the tubing was very thin and thus caused a minimum of agar distortion during the punching procedure.

After withdrawing the agar punch carefully, with the 7-well die remaining in place, the agar plug was removed from each well by means of the suction needle. Extreme care was required in removing the previously punched agar plugs with the 2.6 mm suction needle. The suction needle was attached to a piece of tubing connected to a trap bottle and water aspirator. The tubing was pinched shut

with one hand and the needle was carefully inserted through a hole in the die pattern to the bottom of the well. The pinched tubing was then released and the suction needle was quickly withdrawn from the well. This method removes the agar plugs with a minimum of disturbance to the remaining agar on the microscope slide.

Immunodiffusion plates, prepared by the above technique, were placed in the humid chamber and stored in the cold room.

Addition of samples to wells

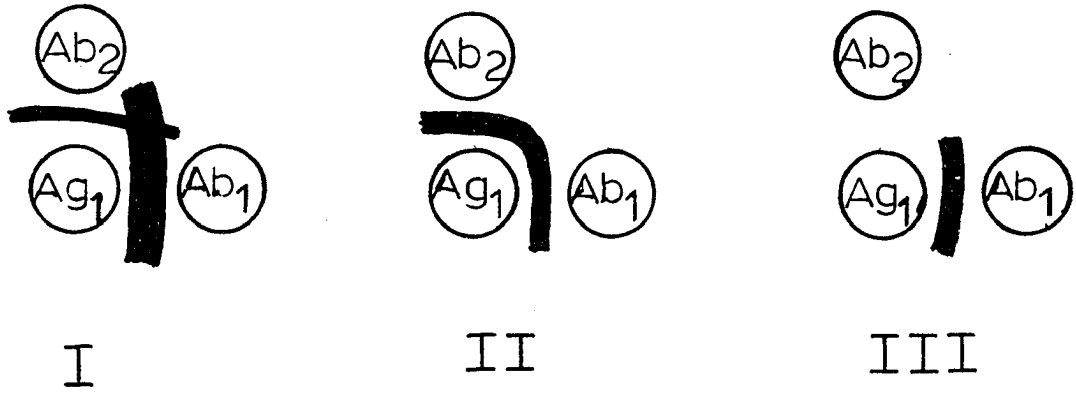
Addition of the antiserum and antigen samples to the wells requires practice and patience. Pasteur pipets, open-ended capillary tubes or Eppendorf pipets do not fill the wells effectively. All of the above instruments tend to release the sample in one aliquot. This results in overfilling or in splashing of the sample out of the wells and onto the surface of the agar. It was therefore necessary to design and construct the following special pipet. The bottom was sawed off from a 10 x 100 mm pyrex test tube and the test tube inserted into a 110 mm length of rubber tubing of appropriate diameter. An Eppendorf pipet tip (5-100 µl size) was inserted into the overlapping portion of the tubing and a 5 to 7 mm length of the pipet cut off. An intact Eppendorf pipet tip was then fitted onto the cut off tip. A piece of latex examination glove was stretched

over the other end of the test tube and secured with a rubber band. This diaphram allowed easy control of positive and negative pressure.

For the immunodiffusion experiments, the immunodiffusion plates were removed from the humid chamber, allowed to warm up to room temperature and the antigen and antiserum solutions added as described above. The plates were then incubated in the humid chamber at room temperature for about 48 hours and the immunodiffusion patterns photographed.

Appearance of precipitin bands

Double immunodiffusion experiments (i.e. both antigens and antibodies diffuse through the agar) were performed in this study. In this technique, opposing wells are filled with the antigen and antiserum samples. These diffuse towards each other through the agar medium until the concentration of each reactant is such that the antigen-antibody complexes precipitate out of solution and appear as bands in the agar medium. Three types of precipitin bands are generally encountered and are shown below.



Type I: Band of partial identity (PI). Antiserum sample 2 contains some antibodies specific for some of the antigens in antigen sample 1. Therefore, antigen sample 2 contains only some of the proteins (or very similar proteins) found in antigen sample 1. Theoretically, the length of the spur is a quantitative measure of the amount of similar proteins, but on a micro-immunodiffusion scale, the spur length cannot be measured accurately. Bands of type I or III were most often found in the present study.

Type II: Band of complete identity (CI). Antiserum samples 1 and 2 both contain identical antibodies specific for antigen 1. Therefore, antigen 1 and 2 must be identical.

Type III: Band of non-identity (NI). Antiserum 2 does not react with antigen 1. Therefore, antigen 1 must contain different proteins.

Photography of immunodiffusion patterns

Attempts were first made to photograph the immunodiffusion patterns using a Simon-Omega enlarger, Kodak Panatomic-X or Kodak Super-Ortho sheet film and a high intensity illumination source (GE no. 93 bulb). The film was placed in a film holder about 2 feet above the immunodiffusion frame which was illuminated from below. This "Rube Goldberg" set-up was unsatisfactory as it involved too many variable parameters such as exposure time, devel-

oper concentration and light source problems. Photographs obtained by this method were not reproducible from one day to the next.

Consequently, the following technique was used which yielded consistently reproducible photographs of acceptable quality. The set-up involved a Polaroid MP-3 Land Camera with black and white Polapax 200 speed, type 42 film. Two bricks were stood on end about one foot apart. The immunoframe was placed on the bricks and illuminated from below by two 75 watt Sylvania photo flood lamps held at a 45° angle via a ring stand and clamps. With the polaroid system, good photographs were obtained using a 4.5 lens setting and a 1/8 second exposure time.

Spectrophotometric Assay of the Precipitin Reaction

Reagents

0.01 M Sodium chloride - 0.01 M potassium phosphate buffer (pH 8)

Antigen solutions- diluted with buffer I (also 0.006 M in spermidine and 0.006 M in 2-mercaptoethanol, pH 7.4)

Procedure

The basis of this method (13, 14) is the fact that as the antigen-antibody complexes form, the reaction mixture becomes turbid. The degree of turbidity is directly proportional to the amount of antigen-antibody complex formed in the reaction mixture. The amount of light scattering is measured in a spectrophotometer at 380 nm in terms of absorbance.

One hundred fifty microliters of whole antiserum were mixed with 2.8 ml of the phosphate buffer followed by the addition of 50 µl of the antigen solution. The absorbance (380 nm) increase beyond that due to the sum of the antigen and antiserum absorbances was measured in a Zeiss PMQ II spectrophotometer and used as a quantitative measure of the extent of the antigen-antibody reaction.

Much difficulty was encountered with this method during the initial experiments since the results using the same antiserum-antigen combinations under identical conditions were not reproducible. After much experimentation,

it was discovered that the problem was one of mixing the reactants reproducibly after the final addition of the antigen sample so as not to effect the kinetics of the reaction.

The following method was found to be reproducible and was used for all the spectrophotometric assays. The phosphate buffer (2.8 ml) was pipetted into the cuvette followed by 150 μl of the whole antiserum sample. After the addition of a 50 μl antigen sample, the cuvette was immediately covered with a small piece of parafilm and mixed by inverting it 10 times over approximately a 20-second time interval. The absorbance increase at 380 nm was followed in the spectrophotometer. Time zero was taken as the point of addition of the antigen sample.

The antigen and antiserum absorbance values were determined as follows: 50 μl of the antigen solution (2 mg protein per ml) were pipetted into a cuvette containing 2.95 ml of the phosphate buffer. The cuvette was covered with parafilm and the solution was mixed by inversion (10 times). The absorbance was read in the spectrophotometer against a phosphate buffer blank. The absorbance of the antiserum was obtained by mixing 150 μl of antiserum with 2.8 ml of phosphate buffer (see below), measuring the absorbance versus a buffer blank and multiplying this value by the factor 295/300 to allow for the dilution upon addition of the antigen. To the 2.95 ml of diluted antiserum were then added 50 μl of antigen solution (see below).

A 1:3 ratio of antigen to antiserum solution was used throughout these experiments since earlier immunodiffusion experiments had shown that, for most of the reactant combinations, the 1:3 ratio of antigen to antiserum was near the equivalence point (see below). The absorbance values were reproducible over a 10-15 minute interval.

RESULTS AND DISCUSSION

Ouchterlony Double Immunodiffusion

Antigen-antibody ratio

Each antibody molecule is known to be bivalent (capable of binding to two antigen sites (15). Since each antigen molecule has several sites (antigenic determinants) available for antibody binding, large networks of antigen-antibody complexes result and, if of appropriate concentration, precipitate out of the agar medium. Therefore, one has to perform a series of experiments to determine the concentration ratio of antibody to antigen that results in maximal precipitation (equivalence point).

The protein concentration of all antigen samples had been adjusted to 2 mg/ml. Protein antibody concentration in the antiserums is difficult to determine and hence an empirical approach was used to determine the optimum antigen-antibody ratio. A fixed volume of antigen (4 µl) was placed in the center well and varying volumes (4 to 12 µl) of antiserum were placed in the external wells. Representative photographs of the patterns obtained are shown below (Fig. 1)

Fig.1 ANTIGEN - ANTISERUM RATIO (B. 2184)

		WELL	CONTENT
S-100 SYSTEM			
	1	* 1	4 µl Ab
	2	2	8 µl Ab
	3	3	12 µl Ab
		center	4 µl Ag
CR SYSTEM			
	1	1	4 µl Ab
	2	2	8 µl Ab
	3	3	12 µl Ab
		center	4 µl Ag
WR SYSTEM			
	1	1	4 µl Ab
	2	2	8 µl Ab
	3	3	12 µl Ab
		center	4 µl Ag

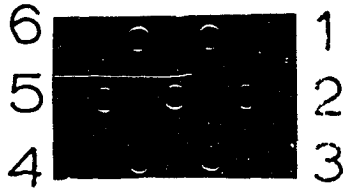
* "R ube Goldberg" photo set-up results

As can be seen from Fig. 1, the 1:3 antigen-antiserum solution ratio resulted in maximal precipitation. Experiments analogous to the above were performed with all the S-100, crude ribosome and washed ribosome fractions. In all cases the 1:3 ratio was at or near the equivalence point and hence this ratio was used for all subsequent immunodiffusion experiments. Specifically, the antigen wells contained about 4 µl of the antigen solution, whereas the wells containing antiserum were filled with three 4 µl aliquots in the following manner: After the addition of the first 4 µl aliquot, about 10 minutes were required for the well to empty. The subsequent 4 µl aliquots were added to the wells in the same manner. During this time, several strips of moist sponge were placed near the immunoframe and the frame was covered with an inverted dishpan. This precaution minimized the loss of water from the agar medium and prevented dust from contaminating the immunodiffusion plates.

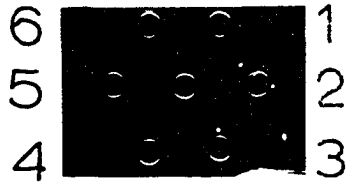
Ribosome purification

In order to assess the degree of ribosome purification, cross-reactions were carried out between the various antigen and antibody preparations. Representative photographs are shown in Fig. 2. These results are tabulated in tables 1 and 2. Extent of relative precipitin band formation is indicated by a number of plus signs (arrived at by visual inspection) and, where applicable, the type of precip-

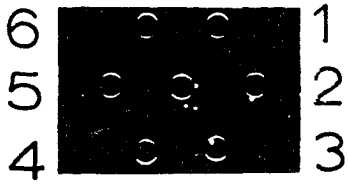
Fig. 2 RIBOSOME PURIFICATION
(B.10 Ag and Ab)



WELL	CONTENT
1	S-100 Ab
2	CR Ab
3	WR Ab
4	WR control serum
5	CR control serum
6	S-100 control serum
center	S-100 antigen



1	S-100 Ab
2	CR Ab
3	WR Ab
4	WR control serum
5	CR control serum
6	S-100 control serum
center	CR antigen



1	S-100 Ab
2	CR Ab
3	WR Ab
4	WR control serum
5	CR control serum
6	S-100 control serum
center	WR antigen

Table 1. RIBOSOME PURIFICATION
(Mesophiles)

ORGANISM	EXPERIMENT	ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE
B. lich	1-A	S-100	S-100	+8	--
		S-100	CR	+4	PI
		S-100	WR	+1	PI
	1-B	CR	S-100	+3	PI
		CR	CR	+8	--
		CR	WR	+6	PI
	1-C	WR	S-100	+1	PI
		WR	CR	+5	--
		WR	WR	+5	--
B. pum	2-A	S-100	S-100	+4	--
		S-100	CR	+2	PI
		S-100	WR	+1	--
	2-B	CR	S-100	+3	PI
		CR	CR	+6	--
		CR	WR	+5	PI
	2-C	WR	S-100	+1	--
		WR	CR	+4	--
		WR	WR	+6	--
B. X-1	3-A	S-100	S-100	+7	--
		S-100	CR	+3	PI
		S-100	WR	+1	PI
	3-B	CR	S-100	+3	PI
		CR	CR	+7	--
		CR	WR	+7	PI
	3-C	WR	S-100	+2	--
		WR	CR	+8	--
		WR	WR	+8	--

Table 2. RIBOSOME PURIFICATION (Thermophiles)

ORGANISM	EXPERIMENT	ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE
B. 10	4-A	S-100	S-100	+8	--
		S-100	CR	+4	PI
		S-100	WR	+1	PI
	4-B	CR	S-100	+1	PI
		CR	CR	+6	--
		CR	WR	+5	PI
	4-C	WR	S-100	+1	PI
		WR	CR	+8	--
		WR	WR	+7	--
B. 2184	5-A	S-100	S-100	+8	--
		S-100	CR	+3	PI
		S-100	WR	+1	--
	5-B	CR	S-100	+2	PI
		CR	CR	+7	--
		CR	WR	+6	PI
	5-C	WR	S-100	NR	--
		WR	CR	+7	--
		WR	WR	+5	--
B. FJW	6-A	S-100	S-100	+7	--
		S-100	CR	+3	PI
		S-100	WR	+1	PI
	6-B	CR	S-100	+1	PI
		CR	CR	+6	--
		CR	WR	+6	PI
	6-C	WR	S-100	+1	--
		WR	CR	+7	--
		WR	WR	+7	--

itin band is also indicated. N.R. indicates no reaction.

It can be seen from tables 1 and 2 that in experiments 1-A through 6-A the amount of cross-reaction between S-100 antigens and washed ribosome antiserums was considerably less than that involving crude ribosome antiserums. These findings indicate that the washed ribosomes were only very slightly contaminated with S-100 proteins.

The extent of reaction between crude ribosome antigens and either crude ribosome or washed ribosome antiserum was about the same (experiments 1-B through 6-B). However, the crude ribosome antigens cross-reacted with the S-100 antiserums, further indicating that the crude ribosomes were contaminated with S-100 proteins.

Experiments 1-C through 6-C show that the washed ribosom antigens elicited only a slight reaction when challenged with the S-100 antiserums. This confirms the above conclusion that washed ribosomes were only slightly contaminated with S-100 proteins. Thus, all 3 groups of cross-reactions were internally consistent.

The above experiments also showed that the extent of purification was greater for the thermophile ribosomes than for the mesophile ribosomes. Analytical ultracentrifugation experiments performed by Dr. Stenesh are in agreement with these findings since these patterns indicated a smaller amount of low molecular weight material in the case of the thermophile washed ribosomes as compared to the mesophile washed ribosomes.

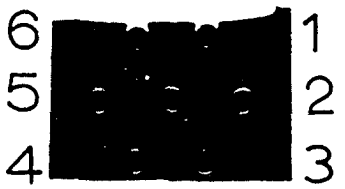
Washed ribosome cross-reactions

The washed ribosome preparations (antigens and antiserums) were used to assess the degree of similarity between ribosomes from the different organisms. To do this, washed ribosome antigens and washed ribosome antiserums were cross-reacted and the results analyzed as in the previous section. Representative photographs are shown in Fig. 3 and the data are tabulated in Tables 3 and 4.

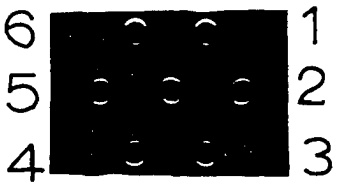
First, comparisons were carried out among the mesophiles The results in Table 6 show that, of the mesophilic species, B. lich and B. pum are more closely related to each other than they are to B. X-1, at least at the level of the ribosomal proteins. With respect to the thermophiles, all 3 organisms appear to be very closely related by the same criterion. This finding was expected since the thermophiles represent strains of one species.

Next, comparisons were carried out between mesophiles and thermophiles. These results are summarized in Table 4. It can be seen from this table that none of the mesophile washed ribosome antigens cross-reacted with any of the three thermophile washed ribosome antiserums. However, the three mesophile washed ribosome antiserums did cross-react with the thermophile washed ribosome antigens. This cross-reaction was most pronounced with the B.lich washed ribosome antiserum. These results may be interpreted as follows: The antigenic site (or several such sites) which

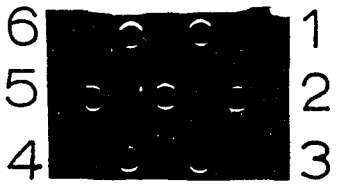
Fig. 3 WASHED RIBOSOME CROSS-REACTIONS



WELL	CONTENT
1	B. FJW Ab
2	B. 2184 Ab
3	B. 10 Ab
4	B. FJW Ab
center	B. FJW Ag



1	B. FJW Ab
2	B. lich Ab
3	B. FJW Ab
4	B. X-1 Ab
5	B. FJW Ab
6	B. pum Ab
center	B. FJW Ag



1	B. pum Ab
2	B. 10 Ab
3	B. pum Ab
4	B. FJW Ab
5	B. pum Ab
6	B. 2184 Ab
center	B. pum Ag

Table 3. WASHED RIBOSOME CROSS-REACTIONS

ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE	ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE
B. lich	B. lich	+5	--	B. 10	B. 2184	+4	PI
B. lich	B. pum	+2	PI	B. 10	B. 10	+6	--
B. lich	B. X-1	+1	PI	B. 10	B. FJW	+5	PI
B. pum	B. lich	+3	PI	B. 2184	B. 2184	+6	--
B. pum	B. pum	+6	--	B. 2184	B. 10	+5	PI
B. pum	B. X-1	+1	PI	B. 2184	B. FJW	+4	PI
B. X-1	B. lich	+2	PI	B. FJW	B. 2184	+5	PI
B. X-1	B. pum	+1	--	B. FJW	B. 10	+5	PI
B. X-1	B. X-1	+4	--	B. FJW	B. FJW	+6	--

Table 4. WASHED RIBOSOME CROSS-REACTIONS

ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE	ANTIGEN	ANTISERUM	REACTION AMOUNT	REACTION TYPE
B. lich	B. 2184	NR	NI	B. 10	B. lich	+3	PI
B. lich	B. 10	NR	NI	B. 10	B. pum	+2	--
B. lich	B. FJW	NR	NI	B. 10	B. X-1	+1	--
B. lich	B. lich	+5	--	B. 10	B. 10	+5	--
B. pum	B. 2184	NR	NI	B. 2184	B. lich	+4	PI
B. pum	B. 10	NR	NI	B. 2184	B. pum	+2	--
B. pum	B. FJW	NR	NI	B. 2184	B. X-1	+1	--
B. pum	B. pum	+6	--	B. 2184	B. 2184	+6	--
B. X-1	B. 2184	NR	NI	B. FJW	B. FJW	+4	PI
B. X-1	B. 10	NR	NI	B. FJW	B. pum	+2	--
B. X-1	B. FJW	NR	NI	B. FJW	B. X-1	+1	--
B. X-1	B. X-1	+4	--	B. FJW	B. FJW	+6	--

is readily accessible to antibody interaction in ribosomes of the thermophiles is not readily accessible in ribosomes of the mesophiles. If this is the case, it follows that this particular antigenic site must be near the surface of the thermophilic ribosomes, while it must be either modified, blocked or occurring in the interior of the mesophilic ribosomes. Thus, B. lich would appear to be genetically more closely related to the thermophiles than to either B. pum or B. X-1. The immunoinhibition experiments discussed later in this thesis support this interpretation.

Lastly, the washed ribosome antigens were cross-reacted with ribosome antiserums from different organisms. These antiserums were kindly provided by Dr. S.R. Suskind, McCollum-Pratt Institute. These antiserums (listed below) were challenged with all six of the mesophile and thermophile washed ribosome antigens using the usual 1:3 antigen-antiserum ratio.

ANTISERUM	TYPE OF ORGANISM
Neurospora ribosomes	mold
Neurospora ribosomal protein	mold
Xenopus ribosomes	bacterium
Sheep ribosomes	animal
E. coli ribosomal protein	bacterium

In no case did an antiserum cross-react with any of the thermophile or mesophile antigens. These findings indicate the presence of intergeneric differences at the level of the ribosome. However, in vitro experiments have shown that t-RNAs, ribosomes and supernatants from various organisms are interchangeable in the reactions of protein synthesis (16, 17). It follows that those proteins which differ in the various organisms are probably not located at the sites on the ribosomes which are involved in protein synthesis.

Immunoinhibition of Protein Synthesis

In view of the findings discussed in the previous section, it was of interest to determine the effect of washed ribosome antiserum in a cell-free amino acid incorporating system. These experiments were performed by N. Schechter using the incorporating systems from B. lich and B. 10.

Because of drastic alterations in ionic strength, the addition of control serum to the incubation mixture decreased the incorporation of phenylalanine by about 50% in the cell-free systems. Nevertheless, an additional effect due to the antibodies could be observed. The addition of antiserum to B. lich WR led to an inhibition of about 20% at either 37° or 55° in the B. 10 system but had no effect in the B. lich system. Likewise, addition of antiserum to B. 10 WR led to an inhibition of about 30% at

either 37° or 55° in the B. 10 system but had no effect in the B. lich system.

These data are in accordance with the immunodiffusion results and suggest further that the particular antigenic sites involved are critically located with respect to phenylalanine incorporation in B. 10 but are not so located in B. lich. Thus the surface environment in which protein synthesis occurs in the two organisms is not identical. This fact may be of significance in conferring upon the thermophilic system its unique heat stability.

Quantitive Assay of the Precipitin Reaction

While the double immunodiffusion method was extremely sensitive, it was not quantitative, and hence, a means of measuring the extent of the precipitin reaction was required. The single radial immunodiffusion method was employed first. When antigen is allowed to diffuse radially from a well in a uniformly thin layer of agar containing antibody, the final area of the precipitate zone is directly proportional to the amount of antigen employed. Ouchterlony (18) was the first to use this method for quantitative purposes. In studies of the toxin produced by Corynebacterium diptheriae, he observed that the width of the precipitate zone was directly proportional to the concentration of antigen and inversely proportional to the concentration of antiserum in the agar.

Later workers (20) confirmed Ouchterlony's findings but showed that the diffusion process must be allowed to proceed until all antigen is combined with antibody. The method was also found to be temperature-independent provided the reaction completion requirement was fulfilled.

Experiments of Mancini, et. al. (21) showed that the single radial immunodiffusion method was extremely sensitive and quantitative. An antigen concentration of 0.0025 µg could be measured by transcribing the precipitate zone to paper and weighing the latter.

In order to test this method with the preparations under study here, S-100 and washed ribosome fractions of B. 10 and B. 2184 were used. The antiserum was diluted 1:10 and 1:20 with barbituric acid buffer (pH 8.6, ionic strength 0.1) containing 3% agar and used to prepare immunodiffusion plates. The agar-antiserum plates were punched with wells and charged with the homologous antigen samples. Original antigen concentrations (2 mg ribosomal protein per ml) and 3-fold dilutions of this were used for these experiments. The plates were observed after a 4-day incubation period in a humid chamber at room temperature.

The results were unsatisfactory because an antigen-antiserum concentration that permitted precipitation to occur not only formed a precipitate around the well but several concentric bands of precipitate as well. Hence,

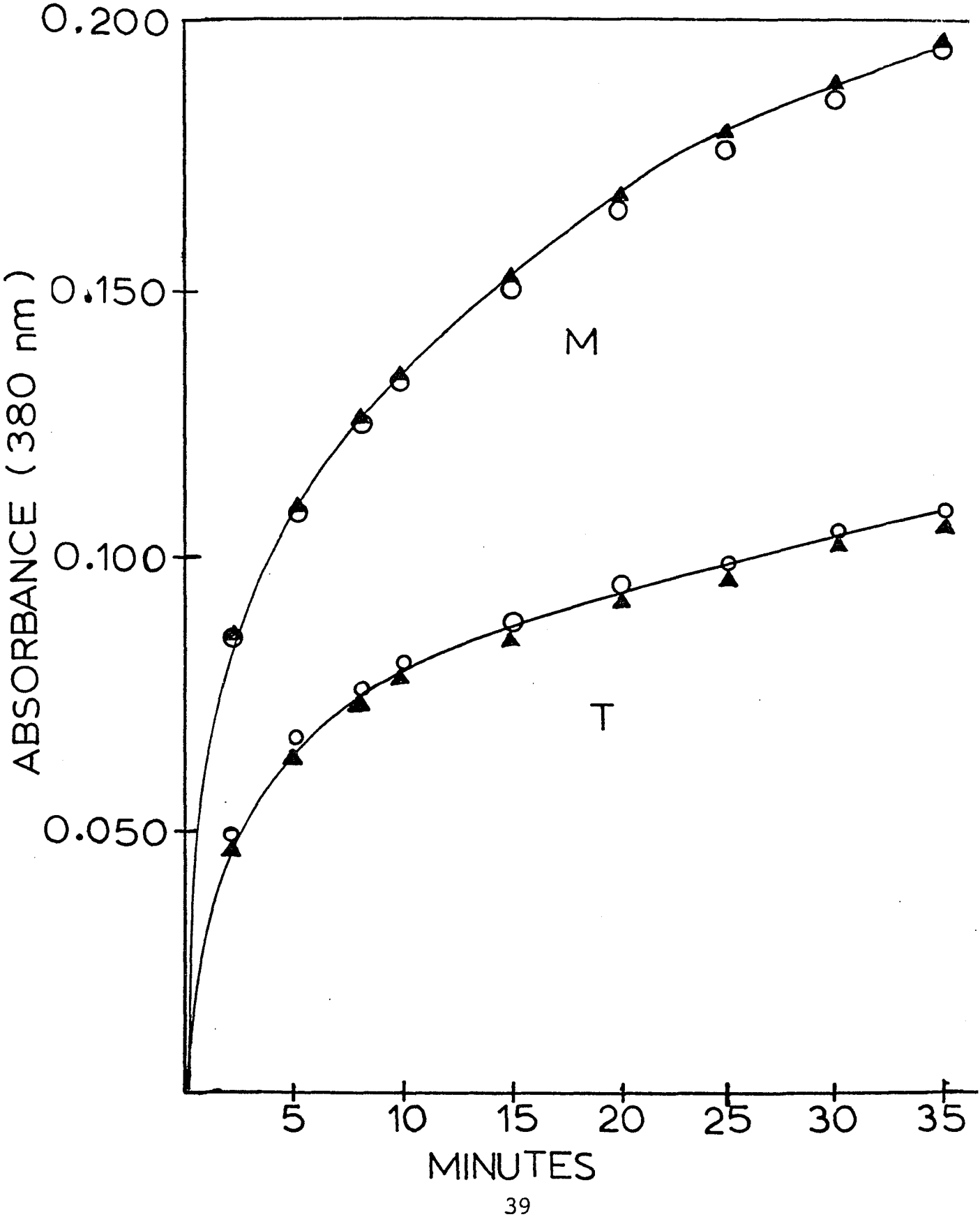
it appeared that the single radial immunodiffusion method is probably not applicable to complex antigen systems, such as ribosomes, which contain several different proteins and result, not only in one, but several concentric precipitin bands.

The method of choice for quantitative evaluation of the precipitin reaction in these ribosomal preparations was the spectrophotometric method developed by Gordon (13, 14). Typical graphs showing the increase in absorbance due to the formation of the antigen-antibody complexes are given in Fig. 4. Spectrophotometric data for the cross-reactions involving CR and WR antigens and antiserums are given in tables 5 and 6. Tables 7 and 8 give results for the WR antigen and antibody cross-reactions. Compilation of data corresponds to tables 1,2,3 and 4 in the immunodiffusion section. Corrected absorbance increases are listed for 10 and 15 minute reaction times. Since S-100, CR or WR antiserum (even at elevated concentrations) resulted in only minimal absorbance changes when reacted with the S-100 antigens, spectrophotometric experiments were carried out using only the CR and WR antibody and antigen preparations.

Ribosome purification

In the B. lich systems (table 5) the WR antibodies reacted equally well with both the CR and WR antigens. This indicates that the adsorbed impurities on the CRs

Fig. 4 Absorbance at 380 nm, corrected for Ag and Ab rdgs. Duplicates, M = B. lich WR Ag-Ab, T = B. 10 WR Ag-Ab systems.



probably did not "mask" many ribosomal antigen determinant sites. However, the CR antibodies reacted about 20% better with the CR antigens than with the WR antigens, indicating that non-ribosomal impurities were present on the CRs but not on the WRs.

For the B. X-1 system, both the WR and CR antibodies reacted equally well with either the CR or WR antigens. Thus, nothing can be said about ribosomal purity from these findings.

In the B. pum system, the reaction of WR antigens with WR antibodies was about 60% as large as that with CR antigens. The CR antibody reacted 60% as well with WR antigens as with CR antigens. These results indicate that the impurity on the B. pum CRs might be ribosomal in nature (acquired during the isolation procedure).

Results in Table 6 show that the thermophilic ribosomes had been purified to a greater extent (greatest for B. FJW and least for B. 10). For both B. 2184 and B. FJW, the same type of impurity "masking" (30-50% for B. FJW CRs and 70% for B. 2184 CRs) was found as for the mesophilic systems. For the B. 10 system, the WR antibodies reacted equally with CR or WR antigens, whereas the CR antibodies reacted 70% as well with CR antigens as with WR antigens, indicating an "unmasking" of antigenic sites during the purification of the CRs.

Analytical ultracentrifugation experiments using CRs

and WRs were in agreement with the above findings.

Washed ribosome cross-reactions

The data for the ribosome cross-reactions, using WR preparations are shown in tables 7 and 8 (table 7 summarizes the mesophile-mesophile and thermophile-thermophile data). It can be seen from table 7, part I (A,B, and C) that there was significant cross-reaction between the different mesophilic preparations. When a particular antiserum was reacted under identical conditions with antigen samples (all of the same ribosomal protein concentration) from different species, the extent of cross-reaction was essentially the same. An exception to the above results was found with the B. lich antigen-antiserum system which gave a higher value. This may be due to a larger number of antigenic determinant sites on the B. lich ribosomes compared to those on ribosomes of B. pum or B. X-1. This interpretation is suggested since absorbance increase is directly related to the amount of antigen-antibody complexes formed in the immunochemical reaction. If the number of antigenic determinant sites on particular ribosomes is greater, then larger "networks" of antigen-antibody complexes could be formed and, hence, an increase in absorbance would result.

With respect to the thermophiles (table 7, part II-A, B, and C) similar conclusions can be reached except that here, both B. 2184 and B. FJW antigen-antiserum systems

Table 5. RIBOSOME PURIFICATION (Mesophiles)

B. lich	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
	WR	WR	0.134	0.151
	WR	CR	0.141	0.154
	CR	CR	0.060	0.063
	CR	WR	0.048	0.050

B. X-1	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
	WR	WR	0.039	0.042
	WR	CR	0.035	0.037
	CR	CR	0.042	0.046
	CR	WR	0.047	0.052

B. pum	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
	WR	WR	0.047	0.058
	WR	CR	0.077	0.080
	CR	CR	0.065	0.069
	CR	WR	0.073	0.084

Table 6. RIBOSOME PURIFICATION (Thermophiles)

B. 10 ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
WR	WR	0.078	0.088
WR	CR	0.080	0.088
CR	CR	0.105	0.119
CR	WR	0.134	0.154

B. 2184 ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
WR	WR	0.186	0.198
WR	CR	0.064	0.081
CR	CR	0.050	0.057
CR	WR	0.186	0.200

B. FJW ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
WR	WR	0.078	0.085
WR	CR	0.055	0.048
CR	CR	0.040	0.044
CR	WR	0.089	0.097

Table 7. ABSORBANCE OF ANTIBODY-ANTIGEN SYSTEMS (WASHED RIBOSOME CROSS-REACTIONS)

Part I.

	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	ABSORBANCE 15 min.
	B. lich	B. lich	0.184	0.208
(A)	B. lich	B. pum	0.079	0.085
	B. lich	B. X-1	0.085	0.095
	B. pum	B. lich	0.064	0.064
(B)	B. pum	B. pum	0.047	0.058
	B. pum	B. X-1	0.051	0.055
	B. X-1	B. lich	0.046	0.050
(C)	B. X-1	B. pum	0.038	0.044
	B. X-1	B. X-1	0.039	0.042

Part II.

	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	ABSORBANCE 15 min.
	B. 10	B. 2184	0.121	0.128
(A)	B. 10	B. 10	0.078	0.088
	B. 10	B. FJW	0.082	0.090
	B. 2184	B. 2184	0.186	0.198
(B)	B. 2184	B. 10	0.061	0.071
	B. 2184	B. FJW	0.101	0.110
	B. FJW	B. 2184	0.117	0.126
(C)	B. FJW	B. 10	0.058	0.065
	B. FJW	B. FJW	0.181	0.195

led to significantly higher absorbance values. Hence, one might conclude that ribosomes from these strains contain a larger number of antigenic determinant sites than the ribosomes of B. 10. Furthermore, it is apparent that ribosomes B. 2184 and B. FJW are structurally more closely related to each other than to B. 10 ribosomes.

The suggestion that the number of antigenic determinant sites on B. 2184 ribosomes is greater than that on B. 10 ribosomes can also account for the findings (table 7, part II-A) that the reaction between B. 10 antiserum and B. 2184 WR antigen was greater than that of the B. 10 antiserum-antigen homologous system.

The data for ribosome cross-reactions using WR preparations for mesophilic-thermophilic and thermophilic-mesophilic antigen-antibody systems are given in table 8. It can be seen from part I (A,B and C) of this table that all three mesophilic WR antiserums cross-reacted with all three thermophilic WR antigens. The magnitude of the absorbance increase can be correlated well with the differences in the number of antigenic determinant sites deduced above. The positive result indicates that some of the antigenic sites on the thermophile ribosomes are identical with or similar to sites on the mesophilic ribosomes. On the other hand, (table 8, part II - A,B and C) none of the thermophile WR antiserums cross-reacted when challenged with any of the mesophile WR antigens. Since the ribosomes

Table 8. ABSORBANCE OF ANTIBODY-ANTIGEN SYSTEMS (WASHED RIBOSOME CROSS-REACTIONS)

Part I.

	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
(A)	B. lich	B. 2184	0.239	0.259
	B. lich	B. 10	0.130	0.142
	B. lich	B. FJW	0.234	0.245
	B. lich	B. lich	0.184	0.208
(B)	B. pum	B. 2184	0.058	0.060
	B. pum	B. 10	0.024	0.025
	B. pum	B. FJW	0.065	0.068
	B. pum	B. pum	0.047	0.058
(C)	B. X-1	B. 2184	0.149	0.198
	B. X-1	B. 10	0.024	0.024
	B. X-1	B. FJW	0.040	0.042
	B. X-1	B. X-1	0.039	0.042

Part II.

	ANTISERUM	ANTIGEN	ABSORBANCE 10 min.	15 min.
(A)	B. 10	B. lich	0.001	0.001
	B. 10	B. lich	0.000	0.000
	B. 10	B. X-1	0.002	0.003
	B. 10	B. 10	0.078	0.088
(B)	B. 2184	B. lich	0.002	0.002
	B. 2184	B. pum	0.002	0.002
	B. 2184	B. X-1	0.004	0.004
	B. 2184	B. 2184	0.186	0.198
(C)	B. FJW	B. lich	0.003	0.002
	B. FJW	B. pum	0.001	0.000
	B. FJW	B. X-1	0.000	0.000
	B. FJW	B. FJW	0.181	0.195

were emulsified (and presumably degraded) prior to injection into the rabbits, ribosomal antiserums include antibodies specific for either surface or internal proteins. This, together with the above findings, suggests that the surfaces of the mesophilic and thermophilic ribosomes differ. Specifically, that certain antigenic sites available on the surface of thermophilic ribosomes are either modified, blocked or occur within the interior of the mesophilic ribosomes.

This conclusion is in agreement with findings reached earlier from the immunoinhibition and immunodiffusion studies.

SUMMARY

S-100 supernatants, crude and washed ribosomes were isolated from three thermophilic and three mesophilic bacteria of the genus Bacillus. Antibodies to these isolated fractions were prepared in rabbits. Several different immunochemical techniques were used for the various antibody-antigen systems in order to assess the purity of the ribosomes and to measure the similarities between these ribosomes. The Gordon spectrophotometric method yielded quantitative results, whereas the Ouchterlony double immunodiffusion method provided semiquantitative data. The thermophile washed ribosomes were shown to have been significantly purified in comparison to the crude ribosomes.

Cross-reactions were demonstrated for all WR mesophile-mesophile and thermophile-thermophile systems. It was concluded that some ribosomes (those of B. lich, B. FJW and B. 2184) contained a larger number of antigenic sites than did the remaining ribosomes.

Cross-reactions between mesophile-thermophile and thermophile-mesophile systems showed that, for WRs, thermophilic antibodies did not cross-react with mesophilic antigens, whereas mesophilic antibodies reacted with thermophilic antigens. It was concluded that certain antigenic sites available on the surface of thermophile ribosomes

are either modified, blocked or occur within the interior of the ribosomes from the mesophiles. This conclusion was corroborated by both immunodiffusion studies and immuno-inhibition experiments using *in vitro* phenylalanine incorporating systems from *B.* lich and *B.* 10.

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VITA

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