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Immunological aspects of the ribosome

Ronald Vernell Loge
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IMMUNOLOGICAL ASPECTS OF THE RIBOSOME

By

Ronald V. Loge

B. S., University of Montana, 1971

Presented in partial fulfillment of
the requirements for the degree of
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**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Introductory Remarks</td>
<td>1</td>
</tr>
<tr>
<td>The Bacterial Ribosome</td>
<td>3</td>
</tr>
<tr>
<td>Delayed Hypersensitivity</td>
<td>12</td>
</tr>
<tr>
<td>Proposed Research</td>
<td>17</td>
</tr>
<tr>
<td>CHAPTER 2. MATERIALS AND METHODS</td>
<td>19</td>
</tr>
<tr>
<td>Animals</td>
<td>19</td>
</tr>
<tr>
<td>Bacterial Strains</td>
<td>19</td>
</tr>
<tr>
<td>Isolation and Purification of Ribosomes and Subunits</td>
<td>19</td>
</tr>
<tr>
<td>Preparation of Protein-deficient Particles</td>
<td>22</td>
</tr>
<tr>
<td>Extraction of Ribosomal Protein and RNA</td>
<td>23</td>
</tr>
<tr>
<td>Chemical and Physical Analyses</td>
<td>24</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>25</td>
</tr>
<tr>
<td>Induction of Delayed Hypersensitivity</td>
<td>26</td>
</tr>
<tr>
<td>Cutaneous Delayed Hypersensitivity Reactions</td>
<td>26</td>
</tr>
<tr>
<td>CHAPTER 3. RESULTS</td>
<td>28</td>
</tr>
<tr>
<td>Isolation of Ribosomes, Subunits, and Protein-deficient Particles</td>
<td>28</td>
</tr>
<tr>
<td>Chemical and Physical Analyses</td>
<td>31</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Composition and Sedimentation Coefficients of Various Ribosomal Particles</td>
<td>31</td>
</tr>
<tr>
<td>2.</td>
<td>Distribution of <em>M. smegmatis</em> Ribosomal Proteins in Polyacrylamide Gels</td>
<td>38</td>
</tr>
<tr>
<td>3.</td>
<td>Tabulated Results of the Skin Tests on Ten Groups of Guinea Pigs Tested with the Various Ribosomal-derived Antigens</td>
<td>42</td>
</tr>
<tr>
<td>4.</td>
<td>ST$_{50}$ Values and Potency and Specificity Indices for the Various Ribosomal Antigens</td>
<td>43</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Separation of 30S and 50S Ribosomal Subunits from <em>M. smegmatis</em> on a 10-30% Sucrose Gradient</td>
<td>30</td>
</tr>
<tr>
<td>2.</td>
<td>An Extrapolation to Infinite Dilution of the $S_{20,w}$ Values Obtained for the 50S and 30S Ribosomal Subunits and the 16S Core Particle</td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td>Densitometer Tracings from the Polyacrylamide Gel Electrophoresis Patterns for the 30S Proteins, Supernatant Proteins, and 16S Core Proteins</td>
<td>37</td>
</tr>
</tbody>
</table>
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Schlieren Patterns of <em>M. smegmatis</em> Ribosomes, Subunits, and Core Particles</td>
<td>29</td>
</tr>
<tr>
<td>II. Polyacrylamide Gel Electrophoresis of 16S Core Proteins, 30S Proteins, and Supernatant Proteins</td>
<td>36</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Introductory Remarks

The scientific revolution of the 17th century heralded new modes of scientific inquiry which allowed the isolation of phenomena and subsequent abstraction to theory. One of the consequences was the divergence of the basic sciences into hundreds of minor fields and sub-specialties. This is exemplified by a tour through any library housing a well-stocked collection of current scientific journals. Such branching into narrower fields has, no doubt, contributed greatly to the understanding of the basic mechanisms of natural phenomena, but, interestingly, discoveries in one area take on new values when workers in another field apply this "foreign" finding to their own investigative field.

In the field of immunology and especially one of its branches, the study of delayed hypersensitivity, the ribosome could be considered foreign to the subject. Briefly, the application of the ribosome to the field of delayed hypersensitivity has come about in the following way. As early as the 1890's Koch observed that experimental animals and men who had been infected with tubercle bacilli responded with a characteristic delayed inflammation in response to injection of an extract of media on which tubercle
bacilli had grown. This skin reaction found use as a
diagnostic test for tuberculosis. Research continued with
such mycobacteria culture extracts as skin test agents, but
workers were repeatedly frustrated with the fact that these
preparations were by no means specific; skin tests with
the culture extracts all responded alike regardless of what
species of mycobacteria happened to be the infecting agent.

After decades of research, another approach was
attempted and species specificity in skin tests was, at
least to a minor degree, attained. This method involved
using the protoplasmic material from the mycobacterium
cell. Since proteins had been implicated as the antigens
which elicited this delayed hypersensitivity, it was
thought that there might exist a set of characteristic
proteins in each mycobacterium species which could be
directed toward the delayed hypersensitivity response and
which may be responsible for the antigenicity of the
protoplasmic material.

There were some immunologists who thought that ribo­
somal proteins might be this set of proteins. Biochemists
had previously studied the ribosomes of *E. coli* and other
bacteria. Among other things, they found that each species
and to some extent each strain within a species of bacteria
carries its own distinct complement of ribosomal proteins.
While biochemists and biophysicists were deeply engaged in
studying more functional and structural details of the ribosome, two groups of immunologists decided to see if the species and strain specific ribosome would display specific antigenic behavior as a skin test antigen. The initial results were rewarding. A relatively specific reaction was observed and the ribosome received a new application.

Thus, application of biochemical and biophysical techniques and knowledge applied to the field of delayed hypersensitivity has resulted in a solid contribution to that field. The discussion to follow will describe some of the things presently known about the ribosome and, in more detail, how the ribosome has come to play a role in the field of delayed hypersensitivity.

**The Bacterial Ribosome**

The first evidence for the existence of ribosomes came in the early 1940's when Luria and co-workers (35) found that lysed bacteria release small particles which, in electron micrographs, appeared to be of uniform size and represented the bulk of the cytoplasm. Further studies revealed that these particles were similar in composition to ribonucleoprotein particles isolated from animal tissue (45), plants (69), and yeast (7). Zamecnik and co-workers (33) later established that these ribonucleoprotein particles were directly involved in protein synthesis.
Interest in these ribonucleoprotein particles grew greatly in the late 1950's since many workers felt that delineation of their structure would lead to a better understanding of how information from genes can ultimately result in the synthesis of proteins. Watson's group at Harvard (62) made the first thorough physical investigation of these bacterial particles, which by this time had been termed "ribosomes" by Roberts (51). Since up to 25% of the dry weight of *Escherichia coli* cells in the exponential growth phase is made up of ribosomes, exponential cultures of *E. coli* were used to provide the material necessary for Watson's classical investigations.

They found that ribosomes isolated and purified in the presence of 10mM Mg$^{+2}$ have a sedimentation coefficient of 70S, and that upon lowering the Mg$^{+2}$ concentration of the solution, the 70S particles dissociated into heavy and light subunits with sedimentation coefficients of 50S and 30S respectively. They also determined that these particles contain approximately 63% RNA and 37% protein.

Later physical studies by Hill and co-workers (19) gave respective molecular weights for the 70S, 50S and 30S particles of *E. coli* of $2.65(\pm0.2) \times 10^6$, $1.55(\pm0.05) \times 10^6$, and $0.90(\pm0.03) \times 10^6$ daltons. By using small angle X-ray scattering, Hill (20) estimated the dimensions of the 70S particle to be $135^\circ \times 200^\circ \times 400^\circ$, the 50S particle to
be 115° x 230° x 230°, and the 30S particle to be 55° x 220° x 220°. Somewhat smaller dimensions are obtained from electron micrographs (17, 23, 54) and probably represent an anhydrous state.

Although ribosomes may be multi-functional in vivo, their only well-established function is in protein synthesis. It appears that the ribosome is not simply the physical site of protein synthesis, but that it also plays an active and important chemical role in the actual synthesis of proteins, as will be described below. In addition, the ribosome plays a role in the accuracy of the codon-anticodon recognition reaction (4). In light of ribosomal complexities, it would be naive to assume that these are the only roles played by the ribosome. As more research is carried out, the ribosome may likely be found to have many more inherent functions.

The initiation step of protein synthesis is mediated by GTP and at least three initiation factors, \( F_1 \), \( F_2 \), and \( F_3 \), which are proteins loosely bound to the 30S ribosome (50, 70). An initiation complex is formed by the binding of messenger RNA (mRNA) to the 30S subunit and the binding of the initiating transfer RNA (N-formyl-methionyl-tRNA) to the P (peptidyl) site on the 30S subunit. The 50S subunit is then attached to form the 70S initiation complex.
In the next step the peptide chain is elongated by the sequential addition of new aminoacyl residues transferred from aminoacyl-tRNA esters bound to the A (aminoacyl) site. This binding to the A site also requires GTP plus two soluble factors, Ts and Tu (48). Each tRNA is specified by a codon on the mRNA. After formation of the peptide bond (catalyzed by peptidyl transferase which is located on the 50S subunit), the initiating tRNA is discharged from the P site, the peptidyl tRNA moves to the P site and the mRNA is translocated with respect to the ribosome to bring the next codon into position. The A site is then filled with the next coded aminoacyl tRNA. This translocation requires a soluble G factor and GTP (14).

This process is repeated and chain elongation continues until a chain termination codon is reached whereupon the completed polypeptide chain is released from the ribosome. This release is mediated by a specific protein release factor which is bound to the ribosome (6). When the polypeptide chain is terminated, the ribosome is released from the mRNA strand by a heat stable releasing factor (RR) together with the G factor (21). Experimental evidence has accumulated which indicates that the ribosome is released as a free 70S which is subsequently dissociated into 50S and 30S subunits by a dissociation factor which appears to be identical to one of the initiation factors, F₃ (61).
This functional complexity of the ribosome is dependent on structural complexity. A single, symmetrical "box" with repeating units of RNA and protein could not carry out the numerous, intricate steps of protein synthesis. Thus, as the structural and functional complexities of the ribosomes are revealed, the structure-function inter-relationships become more deeply founded.

About two-thirds of the mass of the _E. coli_ ribosome is made up of RNA. The 30S subunit has one molecule of RNA, 16S, with a molecular weight of $5.5 \times 10^5$ daltons (28, 38), while the 50S subunit contains two RNA molecules, 23S and 5S, with respective molecular weights of $1.1 \times 10^6$ (28, 38) and $4.0 \times 10^4$ daltons (5). The RNA chain contains many regions of base pairing and resulting hairpin-loops of double-stranded helices (13).

The remaining third of the ribosome is predominantly made up of a set of basic proteins. Although the ribosomal proteins of _E. coli_ have been isolated and characterized by a number of laboratories, it has only recently been generally agreed that the 30S subunit contains 21 different proteins and that the 50S subunit contains another set of about 34 proteins (25, 66). Unlike the 30S particle, the 50S subunit contains multiple copies of several proteins, the precise composition of the particle varying according to the medium used for growing the cells (47, 59). It is
noteworthy that there are no proteins common to both the 50S and 30S subunits. This has been shown by both chemical analysis (47, 66) and immunological studies (47, 59, 66).

Although evidence suggests that the 50S subunit may likely contain at least one copy of each of its proteins, such is not the case with the 30S subunit (39). Based on molecular weight determinations, Kurland's group concluded that one copy of each protein cannot be accommodated by a single 30S particle (10, 18). They found three stoichiometric classes of 30S proteins: 1) those proteins present in amounts of one copy per subunit (unit proteins), 2) those proteins found in amounts less than 0.65 copy per subunit (fractional proteins) and 3) those proteins found in amounts intermediate between the unit and fractional proteins (marginal proteins). They, therefore, suggest that a heterogeneous population of 30S subunits may exist (10, 18).

In an effort to determine the structure of the ribosome, some workers began taking the ribosome apart, piece by piece, and, in this manner, began to get insights into some of the basic structural and functional designs. The conversion of ribosomes to subribosomal or "core" particles was first observed when ribosomes were centrifuged in a CsCl gradient (37). This treatment was found to detach part of the ribosomal protein ("split" protein), leaving particles of higher density and lower sedimentation
coefficients than the ribosomes from which they were derived. It was also found that direct treatment of ribosomes with high concentrations of LiCl, NaCl or KCl yielded similar results (1, 24, 36, 55). These efforts were culminated by the reconstitution of biologically active 30S and 50S ribosomes from the "core" particles and "split" proteins (41) and the reconstitution of active 30S ribosomes from 16S RNA and extracted 30S ribosomal proteins (64).

By deleting certain proteins in the total reconstitution of the 30S particles, it became possible to assign certain general functions to most of the ribosomal proteins (40). It was found that the proteins fell into groups depending upon functional class: 1) proteins required for assembly, 2) proteins required for normal protein synthesis, 3) proteins which stimulate protein synthesis but are dispensable and 4) two proteins to which no function could be assigned. It was also found that the physical assembly of the 30S ribosomal subunit was highly cooperative. That is, after initial binding of the first few assembly proteins, subsequent protein attachment was greatly enhanced. It was suggested that the assembly proteins have specific recognition sites on the RNA strand. Another interesting observation was that Kurland's unit proteins correspond exactly to those proteins required for the physical assembly of the 30S subunit (29). This would suggest that unit or assembly
proteins form a homologous population, and proteins required for function would vary within the heterogeneous population. Thus, in light of both the structural heterogeneity and functional groupings of the ribosomal proteins, it is possible that many of the proteins may have no single biological function, but rather they may function in a cooperative nature in maintaining the structural integrity of the ribosome as well as aiding in its function.

Not only are the ribosomal proteins of the 30S and 50S subunits found as unique sets, but ribosomal proteins also vary from species to species and to some extent among different strains of the same species of bacteria. Some antibiotic-resistant mutants have been found to have altered proteins on either 50S or 30S subunits (27, 65). A study of the differentiation of the ribosomal proteins of 60 strains of the bacterial genus *Escherichia* showed that most closely related strains had only one or two ribosomal protein components that differed (43). The different proteins were found on either the 30S or the 50S subunit. As the strains became more distantly related, many differences in the protein compositions in both subunits appeared. Different bacterial species have very few ribosomal protein homologies (44), and immunological analysis of ribosomal proteins from bacterial, yeast, animal and plant origin showed no cross reaction (52). Thus,
ribosomal proteins seem to distinctly reflect strain and species differentiation.

Ribosomes from mycobacteria have not been studied as extensively as those of *E. coli*, and only a limited number of physical studies have been carried out on a few species. Using *E. coli* $^{14}$C-ribosome markers, it was found that ribosomes from both *M. bovis* (BCG) and *M. tuberculosis* H37Ra sedimented in a sucrose gradient at a Mg$^{+2}$ concentration of 10mM with a main peak of 70S and minor peaks of 50S and 30S, the latter peaks being exclusive at lower Mg$^{+2}$ concentrations (68, 73). Similar sedimentation coefficients for ribosomal subunits from *M. smegmatis* were reported by Baker, *et al.* (2). Worcel (73) showed that RNA isolated from *M. tuberculosis* H37Ra exhibits a sedimentation profile similar to *E. coli* rRNA with two major peaks, 23S and 16S, and a minor peak, 4 to 5S. The structural integrity and functional capacity of the ribosomes depend on the age of the mycobacteria cell cultures and the method of preparation of the ribosomes (67, 73). In addition, the protein synthesizing activity of membrane-bound ribosomes of *M. smegmatis* 607 is higher than that of free ribosomes (15). With this limited information, it appears that mycobacterial ribosomes are at least similar to other bacterial ribosomes in gross structure and function.
Concluding this discussion of some of the pertinent facets of ribosomal structure and function, it is now necessary to define and discuss the state of delayed hypersensitivity, especially in relation to the classical example of delayed hypersensitivity, the tuberculin skin test, and the role that ribosomal antigens may play in this process.

**Delayed Hypersensitivity**

Delayed hypersensitivity (DH) is an allergic response which develops following certain infections or upon exposure to certain plants or chemicals. The allergic reaction develops many hours after a specific antigen is introduced into or onto a tissue of a sensitized host. Historically, DH was noted in certain chronic infections, the classical example being the response to proteins of the tubercle bacilli. Zinsser in 1921 described this "delayed" reactivity (22):

"...one in which there is no immediate effect, but within 4, 5, or more hours (after injection of tuberculoprotein), a swelling becomes apparent which in the course of 12 to 24 hours results in a swollen edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about 48 hours after the injection, and is accompanied by distinct signs of inflammation..."
This allergic response has been shown to be a cell-mediated process. Waksman (71) has characterized the basic DH reaction histologically by the following features: 1) the accumulation of hematogenous cells, lymphocytes and perhaps monocytes around vessels in close relation to the tissues which contain the antigen, 2) the increase in number of these cells either by further accumulation or proliferation, 3) the invasion of the antigen-containing parenchyma by histocytes, and 4) direct destruction of the antigen-containing elements, apparently by these histocytes (macrophages). Thus, the cells involved are primarily lymphocytes that have through previous contact become sensitized to the antigen, and macrophages that employ their phagocytic capacities to attack, to destroy and to remove foreign material.

Several soluble factors are elaborated in vitro, and presumably in vivo, by sensitized lymphocytes subsequent to stimulation by specific antigen, and some of these involve macrophages as well as normal lymphocytes. The best known of these soluble mediators of delayed hypersensitivity is migration inhibition factor (MIF). It was noted as early as 1932 that macrophage migration from sensitized lymphoid tissue explants could be inhibited by specific antigen. Methods have been since developed to quantitate the migration of macrophages packed in
capillary tubes (11, 16). If sensitized lymphocytes are also present in this collection of cells, incubation in the presence of specific antigen will inhibit the natural migration of macrophages out of the tube. This in vitro correlate to DH has been a valuable tool both in demonstrating the degree of DH in vitro and in showing the specificity of an antigen in relation to the DH response.

Pick and Turk (46) developed another in vitro correlate which has also found much use, the skin reactive factor test. When the supernatant fluid of a culture of sensitized lymphocytes exposed to specific antigen is injected into the skin of a normal guinea pig, a skin reactive factor (SRF), elaborated by the cultured lymphocytes in response to specific antigen, causes an erythematous, indurated reaction, having a histological picture closely related to that seen in the classical DH reaction. This reaction develops in a few hours and disappears by 10-12 hours after injection of SRF.

In 1891, Koch first described the effects of subcutaneous injection of tuberculin into sensitized humans and guinea pigs, and research in DH has since centered to a great extent about the antigens responsible for the tuberculin reaction. Koch's first preparation of "old tuberculin" (OT) consisted of a glycerin-broth culture of
human type *M. tuberculosis* maintained 6 to 8 weeks, concentrated by boiling and then filtered.

Siebert (53) later described another tuberculoprotein preparation which was an ammonium sulfate precipitate of unheated culture filtrates of synthetic media upon which bacilli had been grown. This extract, called PPD (purified protein derivative), was later shown to contain various protein and polysaccharide fractions. Since this is not a pure product, animals sensitized with any of the several species of mycobacteria may elicit a response to this antigenic substance.

Since decades of research failed to greatly improve the specificity of the tuberculin skin test when culture filtrate antigens were used, Larson, et al. (30, 31, 32) disrupted mycobacteria cells and tested the protoplasmic fraction of the cell for skin test activity. Larson (30, 31, 32) and Counts and Kubica (8) indicated that equal or more specific skin reactions might be elicited in sensitized experimental animals by these protoplasmic extracts of homologous bacteria than by PPD.

Beam (3, 60) further refined mycobacteria protoplasm by subjecting it to high speed centrifugation to remove the particulate fractions. The resulting supernatant was reported to contain only a few polypeptide components which had molecular weights less than 10,000 daltons.
This "purified protoplasmic peptide" (PPP) fraction was claimed to have few antigenic components.

Earlier, Kanai and Youmans (26) prepared a cytoplasmic fraction from *M. tuberculosis* H37Ra which was similar to Beam's "PPP" and indicated that this fraction provoked delayed reactions in sensitized guinea pigs. They also described a particulate fraction consisting of intracellular particles which also had this capacity.

Using the rationale that Kanai and Youmans particulate fraction may include the ribosomal fraction and that ribosomal material may constitute a significant portion of the skin test active protoplasm of mycobacteria, Baker (2) proceeded to isolate ribosomes and ribosomal subunits from *M. bovis* (BCG) and *M. smegmatis* and employed them as skin test antigens. It was found that isolated 70S ribosomes did, indeed, elicit delayed skin reactions and that the potency and specificity of 70S ribosomes equaled that of homologous protoplasm. Furthermore, comparison of protoplasm, 50S, and 30S ribosomal subunits tested in heterologously and homologously sensitized guinea pigs showed that the 50S subunit was less potent and specific than protoplasm, while the 30S subunit was equally potent and more specific than protoplasm.

Ortiz-Ortiz, et al. (42) have since isolated ribosomal RNA and protein from BCG ribosomes and have found that
ribosomal protein provoked delayed skin reactions in homologously sensitized guinea pigs whereas the purified rRNA failed to cause any inflammatory reaction. Thus, the role of the ribosome in relation to this type of immunological response has been established. Further research may provide highly specific and potent skin test antigens derived from the mycobacterial ribosome.

Proposed Research

It is proposed in this study to extend the research described above, which has demonstrated that ribosomes and ribosomal material are active skin test agents in sensitized animals. Since it has thus far been established that a high relative potency and specificity resides with the 30S ribosomal subunit (2) and that ribosomal protein and not rRNA is the active antigenic factor (42), the next logical step was to do the following work: 1) isolation of the 30S ribosomal subunit from a mycobacterial strain \textit{M. smegmatis} (strain butyricum) was used in this study since it had already been shown that it is possible to isolate antigenically active 30S ribosomal subunits from this strain (2), 2) separation and purification of the RNA and protein fractions of the 30S subunit, 3) testing of the ribosomal RNA and protein components for skin test activity in homologously and heterologously sensitized guinea pigs
and, 4) separation and isolation of individual proteins or protein fractions which would elicit a specific DH response in specifically sensitized guinea pigs. It was hoped that such a study might not only yield an antigenic protein or protein fraction of high specificity, but it also would define more concisely the role that the ribosome plays in relation to the delayed hypersensitivity response to mycobacteria.
CHAPTER 2
MATERIALS AND METHODS

Animals

Hartley strain female guinea pigs weighing 300-500 gm were used for skin testing.

Bacterial Strains

The strains of M. bovis (BCG) and M. smegmatis (strain butyricum) were maintained, grown and harvested as described by Baker (2). To obtain maximal yield of cells and ribosomes, it was assumed that M. smegmatis cells should be harvested during their late log phase of growth. This time was determined by plotting a growth curve of packed cell wet weight and was found to fall at 4 to 4½ days. All cells were therefore harvested after 4 to 4½ days of growth. After harvesting, M. smegmatis cells were either immediately disrupted or frozen at -75°C until needed.

Isolation and Purification of Ribosomes and Subunits

The ribosomes of M. smegmatis were prepared as described below under conditions similar to those described by Baker (2) using the following buffers:

- wash buffer -- 0.01M MgCl$_2$, 0.01M Tris-HCl(pH 7.4), 0.5M NH$_4$Cl;
- 50S buffer -- 0.001M MgCl$_2$, 0.01M Tris-HCl(pH 7.4), 0.07M KCl;
30S buffer — 0.002M MgCl₂, 0.01M Tris-HCl (pH 7.4), 0.07M KCl; 16S buffer — 0.0001M MgCl₂, 0.01M Tris-HCl (pH 7.4), 0.07M KCl. During the entire preparatory procedure, all work was carried out at 4°C, and samples were either used immediately or frozen at -75°C until needed.

The M. smegmatis cells were disrupted using two different methods. One method employed a Sorvall-Ribi cell fractionator in which an approximately 20% slurry of fresh or frozen cells in wash buffer was disrupted at pressures of 30,000 pounds per square inch with the temperature maintained at 0-5°C. The viscous effluent was stirred for five minutes and whole cells, cell walls, and cellular debris were removed by two low-speed centrifugations, 12,100 x g (10,000 rpm — Beckman JA20 rotor) for 15 minutes and 27,200 x g (15,000 rpm — Beckman JA20 rotor) for 45 minutes. The supernatant was then spun at 361,000 x g (60,000 rpm — Beckman Ti60 rotor) for 2.5 hours in a Beckman model L2-65B ultracentrifuge to pellet the ribosomal fraction. The pellet was rinsed 3 times with wash buffer, suspended in wash buffer and washed at least 5 hours with stirring. The solution was centrifuged at 27,000 x g for 20 minutes to remove any membranous or denatured protein material. The resulting supernatant was centrifuged at 361,000 x g for 2 hours to pellet the ribosomes. The pellet was rinsed, resuspended in wash buffer and centrifuged.
at low speed to remove more insoluble material. The resulting crude ribosome fraction was then frozen at -75°C until needed for further purification.

The second method of cell disruption employed a Gifford-Wood Mini-Mill. A slurry of approximately 60 gm of cells in about 10 ml of wash buffer was mixed with about 30 ml of 0.12 mm glass beads and ground at 22,000 rev/min. for 45 minutes at 0-5°C with the Mini-Mill spacing set at 0.03 inches. The resulting slurry was diluted to 250 ml with wash buffer and stirred for 15 minutes. Glass beads and cellular debris were removed by centrifugation and the subsequent work-up of the ribosomes was the same as described in the first method.

The frozen, crude ribosomes were thawed, and to the sample was added with stirring 1% sodium deoxycholate (DOC) -5% Brij-58 in wash buffer to a total concentration of 0.1% DOC-0.5% Brij-58. This mixture was stirred vigorously for 5 minutes, diluted 2-fold and centrifuged at 27,000 x g for 15 minutes to remove any released membranous material and insoluble DOC. The supernatant was centrifuged for 2.5 hours at 361,000 x g. The resulting pellet was resuspended in 50S buffer to dissociate the ribosomes into 50S and 30S subunits.
The resulting 30S and 50S particles were separated by layering about 70 ml of a linear gradient of the 50S-30S pool containing from 10,000 to 14,000 O.D.\textsubscript{260} units in 50S buffer on a radially linear 10-30% sucrose gradient with a 50% sucrose cushion in a Spinco Ti-15 rotor which was spun at 4 °C for 9 hours at 35,000 rpm in a Spinco model L2-65B preparative ultracentrifuge. Ten ml fractions from this zonal centrifugation were collected using a Gilson Escargot Fraction Collector. A sample from every third tube was analyzed at 260 nm, and the absorbancy was recorded by a Cary Model 15 Recording Spectrophotometer. Fractions under the 50S peak and under the 30S peak were combined and the ribosomal subunits were precipitated with 2 volumes of cold ethanol. The precipitated 50S and 30S subunits were pelleted by centrifugation for 15 minutes at 27,000 x g and resuspended in 50S and 30S buffer, respectively.

Ribosomes and subunits were monitored for purity after each step of the preparation procedure using sedimentation velocity centrifugation in a Spinco Model E analytical centrifuge as described below.

**Preparation of Protein Deficient Ribosomal Particles**

Proteins were detached from 30S ribosomes by a method similar to that of Spitnik-Elson and Atsmon (55). Ribosomes (1 mg/ml) were left overnight on ice in a medium containing
0.002M MgCl$_2$, 0.010M Tris-HCl(pH 7.4) and 1.0 M NaCl. After at least 12 hours, the solution was centrifuged at 65,000 rpm in a Beckman 65 rotor for 3 hours. The supernatant was decanted and labeled "split proteins" and the pellet was suspended in 30S buffer and labeled "split ribosomes."

Core ribosomal particles were prepared by the method of Itoh (24). A sample of 30S ribosomes was incubated 12 hours at 4°C with an equal volume of 7M LiCl, 0.025M Tris-HCl(pH 7.8) and 0.005M EDTA. After incubation, the mixture was centrifuged for 3 hours at 65,000 rpm to pellet the core particles. The supernatant was decanted and labeled "supernatant proteins." The pellet was suspended in 16S buffer and labeled "16S ribosomes."

**Extraction of Ribosomal Protein and RNA**

Proteins were extracted from 30S ribosomes and 16S core particles by treatment with 67% acetic acid in the presence of 30mM Mg$^{2+}$ (18). The RNA precipitate was washed twice with the extracting solution for a more quantitative extraction of the proteins. The extracted proteins were then dialyzed 48 hours against a solution of 6M urea, 0.02M Tris-HCl (pH 7.8).

RNA was extracted from the 30S ribosomes by the cold phenol method (57).
Chemical and Physical Analyses

Protein determinations were done with the Folin reagent according to Lowry (34) with crystalline bovine serum albumin (Sigma Chemical Company) as the standard. RNA was determined by the orcinol method (12) using E. coli rRNA as the standard.

By using the extinction coefficient for E. coli ribosomes and subunits, $E_{260}^{1%} = 145$, as determined by Hill (19), concentrations of ribosomal solutions were determined by measuring the optical density at 260nm. Concentrations of rRNA solutions were also determined at 260nm using an extinction coefficient of $E_{260}^{1%} = 223$ (57). Concentrations of protein deficient 30S particles were determined at 260nm using extinction coefficients intermediate between 145 and 223 depending on the percentage of protein remaining in the particle.

Sedimentation velocity studies were made in order to obtain sedimentation coefficients for the various ribosomal particles. All runs were made at 60,000 rpm and at 4.0°C using schlieren optics and a 75° bar angle. After reaching speed, five pictures were taken at 4 minute intervals on Kodak Metallographic Plates. The peaks were measured using a Nikon Model 6C microcomparator. The data were analyzed by a computer program based on the following formulae:
\[ S = \frac{\ln r_b(t) - \ln r_b(t_o)}{\omega^2 (t - t_o)} \]

where \( r_b(t) \) is the position of the peak at time \( t \), and \( \omega \) is the angular velocity (radians per second), and

\[ S_{20,w} = \frac{(1-\bar{\nu} \rho)_{20,w} \eta_{T,b}}{(1-\bar{\nu} \rho)_{T,b} \eta_{20,w}} S_{T,b} \]

the partial specific volume, density and viscosity respectively of the water of buffer, and the subscripts \( w, b, 20, T \) refer to water, buffer, \( 20^\circ C \) and the temperature of the buffer during the experiment. This calculation gives sedimentation coefficients corrected to standard conditions of water at \( 20^\circ C \). The partial specific volumes, \( \bar{\nu} \), were assumed to be the same as \textit{E. coli} ribosomal subunits.

Determination of the \( S^0 \) values for the subunits was made by extrapolation to infinite dilution of the sedimentation coefficients \( (S_{20,w}) \) obtained at a series of concentrations (corrected for radial dilution).

**Electrophoresis**

The procedure used for polyacrylamide disc electrophoresis was that of Reisfeld, \textit{et al.} (49) as modified by Traub and Nomura (63). The electrophoresis was carried out at pH 4.5 in a Polyanalyst Disc Electrophoresis Apparatus (Buchler Instruments, Inc.). Usually the protein sample (30 \( \mu \)g/ml to 200 \( \mu \)g/ml) in a volume of 100 to 300 \( \mu \)l was layered onto the upper gel and was electrophoresed at 1.5 ma/tube until the tracking dye, pyronin red, reached the
bottom of the gel (approximately 4 hours). The gels were stained for one hour in a 0.25% solution of amido black in 7% acetic acid. Destaining was accomplished electrophoretically in 7% acetic acid. Densitometer tracings of the stained gels were obtained using a linear transport adaptor on a Gilford Spectrophotometer at a wavelength of 660nm.

**Induction of Delayed Hypersensitivity**

Hartley strain guinea pigs were sensitized by subcutaneous injection of 100 μg of viable *M. smegmatis* cells contained in 0.2 ml of incomplete Freund's adjuvant into each hind footpad. Other groups of guinea pigs were sensitized by intraperitoneal injection of 4 x 10^6 viable units of *M. bovis* (BCG).

**Cutaneous Delayed Hypersensitivity Reactions**

Skin tests were done on guinea pigs sensitized at least two weeks before testing. The reactions were provoked by intracutaneous injections of serial two-fold dilutions of antigen contained in 0.1 ml of sterile physiological saline (0.85% NaCl w/v). The antigens used were BCG and *M. smegmatis* protoplasm as prepared by Baker (2), 50S and 30S ribosomal subunits, "split" ribosomes and "split" proteins, 16S ribosomes and "supernatant" proteins, proteins extracted from the 30S subunit and from the 16S core particle, and rRNA from the 30S subunit. The animals were checked at various times
after testing. After 24 hours the lesions were measured by reading two right angle diameters and the thickness then was measured with a caliper (Schnelltaster). Volumes were determined from this information from a computer generated table based on the following formula (72):

\[ V = (W \times L \times \frac{1}{3}T)^0.75 \]

where \( V \) is the volume of lesion in \( \text{mm}^3 \), and \( W, L \) and \( T \) are, respectively, the width, length and thickness of the lesion in mm. Only those lesions whose volumes were at least \( 15 \text{ mm}^3 \) were taken as positive skin tests. \( ST_{50} \) values were calculated from the tabulated results of the skin tests. An \( ST_{50} \) value is that amount of antigen required to elicit a positive skin test in one-half of the animals tested. In the cases where exactly half of the animals tested did not respond to a particular antigen concentration tested, the \( ST_{50} \) value was obtained by interpolation between the dose tested which fell above the 50\% mark and the dose below.
CHAPTER 3
RESULTS

Isolation of Ribosomes, Subunits and Protein-Deficient Particles

Disruption of cells with the Sorvall-Ribi cell fractionator as discussed in the previous section gave average yields of crude ribosomes of 0.3 to 0.5% of the packed cell wet weight. Disruption with the Gifford-Wood Mini-Mill gave essentially the same yields as with the Ribi fractionator but was the preferred method since disruption could be carried out more easily and much more quickly. Disruption of the mycobacteria by either method gave sedimentation velocity schlieren patterns typical for crude 70S ribosome preparations (see Plate I (a)).

The 30S and 50S ribosomal subunits were isolated by zonal centrifugation (see previous section). After centrifugation, fractionation and monitoring the absorbancy of the fractions, pure subunit samples were obtained from those fractions of the gradient showing no overlap. Figure 1 shows the results of a typical separation of 30S and 50S ribosomal subunits. The isolated subunits were homogeneously pure as determined by sedimentation velocity centrifugation (Plate I (c,d)).

Treatment of 30S ribosomes with 1M NaCl resulted in a protein-deficient particle which sedimented in an analytical centrifuge as a single, homogeneous peak. A homogeneous, single peak was also obtained after treatment of 30S ribosomes with 3.5M LiCl and EDTA (Plate I (e)).
Plate I.  Schlieren patterns of *M. smegmatis* ribosomes, subunits and core particles at various stages of purification obtained from sedimentation velocity studies of these particles: (a) crude 70S ribosomes, (b) 30S-50S ribosomal subunit pool, (c) 50S ribosomal subunits, (d) 30S ribosomal subunits and (e) 16S core ribosomes all in their respective buffers. Sedimentation is from left to right. All patterns were taken 12 minutes after reaching speed using a 75° phase plate angle. Pattern (a) was run at a speed of 52,000 rev./min., and patterns (b) - (e) were run at 60,000 rev./min. Temperature was 4°C.
Figure 1. Separation of 50S and 30S ribosomal subunits from *M. smegmatis* on a 10-30% sucrose gradient. 14,000 O.D. 260 units of a 30S-50S ribosomal pool were centrifuged 9 hours at 4 °C in a Spinco Ti-15 rotor. Ten ml fractions were collected. Absorbancies of 1:100 dilutions of every third tube were read at 260nm. The shaded areas represent the fractions used for further work.
Chemical and Physical Analyses

The RNA and protein compositions of the ribosomal subunits and the two protein deficient particles studied are listed in Table 1.

A series of sedimentation velocity studies at increasing dilution was made on each particle, and the $S_{20,w}^0$ values were extrapolated to infinite dilution as shown in Figure 2. The resultant $S_{20,w}^0$ values for the 50S subunit, 30S subunit and the 16S core particle are recorded in Table 1.

![Table 1](image)

* Although a $S_{20,w}^0$ value was not determined for the split ribosome prepared by 1M NaCl treatment, a $S_{20,w}^0$ value was obtained and was found to be 20.0 at a concentration of 1.8 mg/ml. Assuming a concentration dependence similar to the 16S core particles or the 30S ribosomal subunits, this would then correspond to a $S_{20,w}^0$ value of approximately 21 to 22S.
Figure 2. An extrapolation to infinite dilution of the $S_{20,w}$ values obtained for the 50S ribosomal subunit (Δ), the 30S ribosomal subunit (o), and the 16S core particle (□).
Assuming that *M. smegmatis* ribosomal subunits have the same frictional coefficient as *E. coli* ribosomal subunits, the molecular weights of *M. smegmatis* ribosomal subunits can be approximated based on molecular weights and the $S_{20,\text{w}}^0$ values of *E. coli* ribosomal subunits. These are 1.55 x $10^6$ daltons and 50.8S for the *E. coli* 50S subunit and 0.90 x $10^6$ daltons and 31.8S for the 30S subunit (19). Using these values, the *M. smegmatis* 50S and 30S subunits therefore have respective molecular weights of approximately 1.50 x $10^6$ and 0.80 x $10^6$ daltons. The lower $S_{20,\text{w}}^0$ values and resultant molecular weights may be reflections of several factors: 1) fewer proteins on the ribosomal subunits, 2) lower average molecular weights of the proteins or 3) smaller RNA molecules. It is possible that the assumption that *E. coli* and *M. smegmatis* ribosomal subunits have similar frictional coefficients is not valid. The lower $S_{20,\text{w}}^0$ values of the *M. smegmatis* ribosomal subunits would then suggest a looser, less compact structure than found with the *E. coli* ribosomal subunits.

Throughout the entire preparation scheme, extreme care was taken to insure that the resulting ribosomal subunits and especially the 30S subunit would be free of contaminating, extraneous material. Routine washing of the crude ribosome with $\text{NH}_4\text{Cl}$ buffers to remove non-ribosomal protein material plus treatment with sodium deoxycholate to remove any membranous
material which might be bound to the ribosome were initial steps necessary to obtain pure ribosomal subunits from *M. smegmatis* ribosomes. Zonal centrifugation of the subunits further eliminates the possibility of the presence of any additional non-ribosomal material with the exception of any material bound directly to the ribosome or material which would sediment at exactly the same rate as the subunits. This latter possibility seems unlikely.

It is difficult to assess the efficacy of the DOC treatment for the removal of attached membranous fractions. Although there are currently no good methods for quantitating lipid bound to the ribosome, it is doubtful that this presents a problem with the 30S subunit. The large ribosomal sub-unit has been shown to be membrane bound in eukaryotic systems (53), and a similar situation may be true for mycobacteria. Solutions of the isolated 50S subunits did have a characteristic yellow-brown color which may be caused by chromophores such as cytochromes bound to residual membranous material, but the solutions of 30S subunits were colorless. Further refinement of the 30S subunits to protein-deficient particles, and eventually to isolated proteins, would further insure the purity of the final products. Both the homogeneity of the schlieren patterns obtained for the various ribosomal particles and the consistent RNA and protein determinations
of the particles argue strongly that the particles isolated are only ribosomal in nature. Were these ribosomal preparations contaminated with membraneous material, spurious results would have been evident in both the protein determinations due to membraneous proteins, and in the orcinol test due to membraneous polysaccharides. Only bound lipids could have gone undetected by the above tests.

**Electrophoresis**

Total 30S ribosomal proteins, supernatant proteins and 16S core proteins from the 3,5M LiCl, EDTA treatment were analyzed by polyacrylamide gel electrophoresis. The disc electrophoresis patterns obtained are shown in Plate II. Densitometer scans of the gels are shown in Figure 3. Gel (b) in Plate II was used for photographic clarity. It is not the same gel represented in Figure 3. Each protein band in the 30S protein pattern was designated by a number corresponding to its electrophoretic mobility (see Plate II and Figure 3).

A total of 18 separate protein bands are discernible for the 30S ribosomal proteins. The possibility of multiple components in a single band would have to be resolved by either two-dimensional gel electrophoresis or by column chromatography. Electrophoresis of the core proteins reveals three major protein bands and two minor bands.
Plate II. Polyacrylamide gel electrophoresis: (a) extracted 16S core proteins, (b) total extracted 30S proteins, (c) supernatant proteins. Protein samples were prepared and electrophoresed as described in Materials and Methods. Electrophoresis is from top to bottom.
Figure 3. Densitometer tracings from the polyacrylamide gel electrophoresis patterns obtained for total 30S proteins (——), supernatant proteins (---), and 16S core proteins (....). Electrophoresis is from left to right.
while the supernatant proteins are resolved into 16 bands.

Since the gels were electrophoresed until the tracking
dye reached the bottom of the gels, it was possible to compare
the bands present in each of the gels (Plate II and Figure 3).
The comparative data from the electrophoresis studies are
summarized in Table 2. The distribution of proteins shows
a striking complementarity between the core proteins and the
supernatant proteins. When added together, they represent
all the bands found in total 30S proteins plus an extra
band, 15a. The reason for the presence of this extra band
is not certain; it may be an altered form of a normal
protein. A very minor band in the supernatant protein gel
visible by direct observation and also detected in the densi­
tometer scan is found just below band number 5. Although this
band is common only to the supernatant protein fraction, little
significance can be assigned to it since it appears in such
low quantities.

### Table 2

| Band Number: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 15a | 16 | 17 | 18 |
|--------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|
| 30S Proteins:| + | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | -  | +  | +  |
| Supernatant Proteins:| + | + | - | + | + | + | + | + | + | +  | (++) | +  | +  | -  | +  | +  | -  | +  |
| Core Proteins:   | - | - | + | - | - | - | - | - | - | -  | -  | +  | (++) | -  | -  | +  | -  | -  |

*(++) -- protein present in reduced amounts.*
Proteins 4, 11, and 17 are absent from the supernatant fraction but are all found in the core protein fraction. Proteins 4, 11, and 17 therefore represent three protein components common only to the 16S particle. Protein 15 is present in the supernatant fraction and also in small amounts in the core protein fraction. Likewise, protein 14 found in the core protein fraction is present also in the supernatant fraction, but in reduced amounts. The presence of these two protein components in both the supernatant and core fractions can be explained in the following way: Since the reproducible removal of specific fractions of ribosomal proteins by defined salt concentrations is caused by the disruption of the electrostatic interactions between the protein and RNA, it is evident that 3.5M LiCl in the absence of Mg^{+2} disrupts the binding interactions between the M. smegmatis ribosomal RNA and at least 15 30S ribosomal proteins. This leaves a core ribosomal particle made up of the original RNA and at least four protein components. Another protein, 15, appears to be present, but apparently treatment with high salt does not suffice to remove this protein component from all core particles. Hence it is present in the supernatant fraction and in small amounts in the core protein fraction. The protein represented by band 14 appears to be only slightly labile to dissociation from the core particle by high salt since it is present in the core particle in high
amounts while found in the supernatant fraction only in reduced amounts.

The protein content of the 16S core particle is 11%. This represents 30% of the original protein content of the 30S subunit. Based on this percentage and the assumption that *M. smegmatis* 30S ribosomal subunit contains 18 and possibly as many as 20 different proteins, one would expect 5 or 6 proteins in the core particle. Three major protein bands and two minor bands fall a bit short of the expected number; however, based on Kurland's theory of a heterogeneous 30S ribosomal subunit population (28), each 30S subunit contains only about 250,000 daltons of protein rather than 410,000 daltons which is the sum of the molecular weights of all the 30S ribosomal proteins. A molecular weight of 250,000 corresponds to approximately 12 ribosomal proteins. Thirty percent of this figure gives an expected 3-4 proteins. Thus, the heterogeneous 30S population may also be the case with mycobacterial ribosomes. It is also possible that any of the three major bands of the core proteins may represent more than just a single protein.

**Cutaneous Delayed Hypersensitivity Reactions**

The results of all skin tests performed on five groups of guinea pigs sensitized with *M. smegmatis* and five groups of BCG-sensitized guinea pigs tested with serial dilutions of
the various antigens derived from ribosomes are tabulated in Table 3. To evaluate the potency and specificity of the antigens, the animals were also tested with serial dilutions of *M. smegmatis* protoplasm to provide a base-line since protoplasm is at least as potent and specific as culture filtrate antigens in eliciting skin reactions. BCG protoplasm was also used as a control. The tabulated results from Table 3 have been, in turn, converted into ST<sub>50</sub> values (Table 4) as described in Materials and Methods. A potency index was determined for each antigen. This is the potency of a given antigen compared to that of homologous protoplasm as determined in homologously sensitized guinea pigs. The specificity of the antigens was evaluated by the specificity index. This is the ratio of the activity of the antigen in guinea pigs sensitized with homologous organisms versus its activity in guinea pigs sensitized with heterologous organisms in comparison to the same activity of homologous protoplasm. The potency and specificity indices are also recorded in Table 3.

The following is a sample calculation of the potency and specificity indices: In Experiment I (Table 3) 4 of 6 animals sensitized with *M. smegmatis* responded with a positive skin reaction to 0.125 µg (4 units where 1 unit = 0.031 µg) of 30S ribosomes while only 1 of 6 were positive at a dose of 0.062 µg (2 units). Interpolation gives a
TABLE 3
Tabulated Results of the Skin Tests on Ten Groups of Guinea Pigs Tested with the Various M. smegmatis Ribosomal-derived Antigens

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Amount of antigen (µg, dry weight) provoking a 24 hour reaction in guinea pigs sensitized with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. smegmatis</td>
</tr>
<tr>
<td>I</td>
<td>30S ribosomes</td>
</tr>
<tr>
<td></td>
<td>50S ribosomes</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
</tr>
<tr>
<td>II</td>
<td>Split ribosomes</td>
</tr>
<tr>
<td></td>
<td>Split proteins</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
</tr>
<tr>
<td>III</td>
<td>16S ribosomes</td>
</tr>
<tr>
<td></td>
<td>Supernatant proteins</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
</tr>
<tr>
<td>IV</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td></td>
<td>16S ribosomes</td>
</tr>
<tr>
<td></td>
<td>Supernatant proteins</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
</tr>
<tr>
<td>V</td>
<td>30S ribosomes</td>
</tr>
<tr>
<td></td>
<td>30S proteins</td>
</tr>
<tr>
<td></td>
<td>16S ribosomes</td>
</tr>
<tr>
<td></td>
<td>16S proteins</td>
</tr>
</tbody>
</table>

a = numerator = no. of animals positive, denominator = no. of animals tested.
b = 1M NaCl treatment.
c = 3.5M LiCl, EDTA treatment.
d = Extracted from 30S ribosomes.
e = Extracted from 16S ribosomes.
f = Extracted from 30S ribosomes.
### TABLE 4

**ST Values and Potency & Specificity Indices for the Various Ribosomal Antigens**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Antigen</th>
<th>ST&lt;sub&gt;50&lt;/sub&gt; units&lt;sup&gt;a&lt;/sup&gt;(1 unit - 0.031 μg antigen)</th>
<th>Potency Index&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specificity Index&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Guinea pigs sensitized with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M. smegmatis</td>
<td>M. bovis (BCG)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>30S ribosomes</td>
<td>3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>50S ribosomes</td>
<td>4.0</td>
<td>4.0</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
<td>2.0</td>
<td>4.0</td>
<td>1.0</td>
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<td></td>
<td>BCG protoplasm</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5</td>
<td>ND</td>
</tr>
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<td>II</td>
<td>Split ribosomes</td>
<td>4.0</td>
<td>14.0</td>
<td>1.0</td>
</tr>
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<td>Split proteins</td>
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<td>&gt;16</td>
<td>&lt;0.25</td>
</tr>
<tr>
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<td>M. smeg. protoplasm</td>
<td>4.0</td>
<td>7.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>16.0</td>
<td>2.5</td>
<td>0.33</td>
</tr>
<tr>
<td>III</td>
<td>16S ribosomes</td>
<td>3.3</td>
<td>16</td>
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</tr>
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<td>Supernatant proteins</td>
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<td>&gt;16</td>
<td>&lt;0.20</td>
</tr>
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<td>M. smeg. protoplasm</td>
<td>3.2</td>
<td>3.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>14.0</td>
<td>1.6</td>
<td>0.23</td>
</tr>
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<td>IV</td>
<td>Ribosomal RNA</td>
<td>no reaction</td>
<td>no reaction</td>
<td>ND</td>
</tr>
<tr>
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<td>&gt;16</td>
<td>0.75</td>
</tr>
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<td></td>
<td>16S proteins</td>
<td>2.5</td>
<td>16.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
<td>3.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>12.0</td>
<td>2.0</td>
<td>0.25</td>
</tr>
<tr>
<td>V</td>
<td>30S ribosomes</td>
<td>12.0</td>
<td>ND</td>
<td>0.42&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
<td>10.0</td>
<td>ND</td>
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<td></td>
<td>16S ribosomes</td>
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<td>ND</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>M. smeg. protoplasm</td>
<td>5.0</td>
<td>ND</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Notes:**

- **ST<sub>50</sub> unit** = the amount of antigen necessary to provoke a positive 24 hr. skin reaction in one-half of the animals tested with that dose.
- **Potency Index** = \( \frac{ST_{50} \text{ units M. smegmatis protoplasm}}{ST_{50} \text{ units antigen}} \)
- **Specificity Index** = \( \frac{ST_{50} \text{ units antigen in BCG animal}}{ST_{50} \text{ units antigen in M. smegmatis animal}} \)
- **ND** = No data.
- **NC** = Cannot be calculated.
- **NC** = Potency Index relative to 16S proteins.

<sup>a</sup> ST<sub>50</sub> unit = the amount of antigen necessary to provoke a positive 24 hr. skin reaction in one-half of the animals tested with that dose.

<sup>b</sup> = 3.3 × 0.031 μg antigen or 100 μg antigen required to provoke a positive skin test in one-half the animals sensitized with M. smegmatis tested with this dose. This is an interpolated value from the tabulated results.

<sup>c</sup> Potency Index = ST<sub>50</sub> units M. smegmatis protoplasm / ST<sub>50</sub> units antigen

<sup>d</sup> Specificity Index = ST<sub>50</sub> units antigen in BCG animal / ST<sub>50</sub> units antigen in M. smegmatis animal

<sup>e</sup> ND = No data.

<sup>f</sup> NC = Cannot be calculated.

<sup>g</sup> = Potency Index relative to 16S proteins.
hypothetical dose of approximately 100 μg or 3.3 units of 30S ribosome which would cause a positive skin reaction in one-half (3 of 6) of the animals tested. Three of six of the guinea pigs responded positively to 0.062 μg (2.0 units) of M. smegmatis protoplasm. Therefore, the potency index for the 30S ribosomes is 2.0/3.3 or 0.60. In BCG-sensitized guinea pigs it took 8.0 units of the 30S ribosomes and 4.0 units of M. smegmatis protoplasm to elicit positive skin reactions. The specificity index is then calculated by dividing the ST50 units for 30S ribosomes in BCG animals (8.0) by the ST50 units for 30S ribosomes in M. smegmatis animals (3.3). This is then divided by the same ratio obtained for M. smegmatis protoplasm. Therefore, the specificity index for 30S ribosomes is \[ \frac{8.0}{4.0} \div \frac{3.3}{2.0} = 1.2. \]

Experiment I: Based on previous studies which showed that relatively high skin test potency and specificity resides in the 30S ribosomal subunit (2), it was decided to pursue further studies with the 30S subunit. Both 30S and 50S subunits were tested in groups of guinea pigs sensitized with either M. smegmatis or M. bovis (BCG). The results of this experiment confirmed the previous findings (2). The 30S subunit was 60% as potent as M. smegmatis protoplasm whereas the 50S subunit was only 50% as potent. Furthermore, the 30S subunit was 1.2 times more specific than protoplasm while the 50S subunit showed even less specificity (one-half) than protoplasm.
Experiment II: In the next experiment the 30S subunits were treated with 1M NaCl yielding split ribosomes and split proteins. The split ribosomes showed a marked increase in potency compared to the intact 30S subunit. The potency increased to a value equivalent to protoplasm. Following this removal of some of the ribosomal proteins there was also an increase in relative specificity, from 1.2 for the 30S subunit to 1.9 for the split ribosome. The split proteins, however, displayed very little skin test reactivity in either sets of guinea pigs.

Experiment III: Treatment of 30S ribosomal subunits with 3.5M LiCl and EDTA resulted in a further loss of ribosomal proteins. The residual 16S core ribosomal particles were now equal in potency to homologous protoplasm but had markedly increased specificity, being 4.4 times that of protoplasm. Thus, a nearly four-fold increase in specificity resulted following the removal of 70% of the ribosomal proteins from the 30S subunit. The supernatant proteins which were removed by this treatment were virtually inactive in all animals tested. Some delayed skin reactivity was noted in some of the animals tested with the highest concentration of antigen, but the lesions were not large enough to satisfy the criteria for a positive reaction.
Experiment IV: When isolated rRNA from the 30S subunit was tested for skin test activity, there was essentially no inflammatory reaction at any of the injection sites. Protein extracted from the 16S ribosomal particle was 1.2 times more potent than protoplasm and 4.9 times as specific. Removal of both the RNA and a majority of the proteins from the 30S subunit yields a set of proteins which display both higher potency and a greater specificity than protoplasm.

Experiment V: In order to determine the relative potency of four active ribosomal antigens, a set of guinea pigs was tested with serial dilutions of 30S ribosomal subunits, proteins extracted from the 30S subunit, 16S core particles and the proteins extracted from the 16S core particles. The 16S proteins were twice as potent as both the total 30S ribosomal proteins and the 16S particle and nearly 2.5 times as active as the 30S subunit itself.

It is noteworthy that polyacrylamide gel electrophoresis of the 16S protein showed 3 major bands and 2 minor bands. Of these 5 proteins, 2 are also found in the supernatant fraction. Since the supernatant fraction was quite inactive as a skin test antigen, it could be concluded that the skin test activity of the 16S proteins can be attributed to, at the most, only three proteins.
CHAPTER 4

DISCUSSION

The results of this study show unequivocally that specific proteins isolated from the smaller subunit of *M. smegmatis* are very potent and quite specific agents in eliciting delayed hypersensitivity reactions in specifically sensitized hosts. This is a marked difference from the classical and more recently studied skin test antigens, namely, OT, PPD, and protoplasmic extracts, which are potent but relatively non-specific and are not well-defined substances. In order to realize the magnitude of this difference, it is necessary to review the method by which these active core proteins were obtained in pure form and characterized and then to compare these methods to those used in obtaining the other antigens (OT, PPD, and protoplasm).

To attain the high degree of purity in the ribosomal preparations necessary for study in a biological system, methods of isolation and purification were followed which have been shown consistently to give pure, homogeneous ribosomal preparations from other bacterial species. Using the methods of Hill, et al. (20), ribosomes and ribosomal subunits were isolated from *M. smegmatis* cells by cell disruption, differential centrifugation and zonal centrifugation as outlined previously. After each preparative step, the ribosomal
solutions were analyzed by sedimentation velocity centrifugation and were found not only to be homogeneously pure, but they also displayed sedimentation patterns similar to the well defined *E. coli* ribosomal system.

This is in direct contrast to the earlier work of Kanai and Youmans (26) who simply isolated the microsomal fraction of ruptured mycobacterial cells without further refinement or analysis. Although they demonstrated that this "ribosomal" fraction was active as a skin test agent, their methods of preparation could in no manner guarantee that the active antigenic component was ribosomal in nature. This question of purity was taken up by Worcel (73) who showed such "ribosomal" preparations to be variable mixtures of ribosomes, cytoplasmic membrane fragments and cell walls. These latter components are, in fact, active, but non-specific, skin test antigens.

The later work of Baker (2) established that skin test activity is found with purified ribosomal particles. The basic steps of isolation and purification of the *M. smegmatis* ribosomes and subunits used in his study were also similar to the methods of Hill (20). Whereas Baker stressed the 260/280nm absorbancy ratio as an estimate of the purity of his ribosomal preparations, such a criterion of purity was not felt to be justifiable in this study. Although ribosomes do, in
general, have a 260/280 ratio of about 2.0, adsorption onto the ribosomes of any non-chromophoric material such as membraneous lipids would not affect this ratio. Thus it was felt that a stronger standard of purity could be based on scrutiny of each particle and protein fraction by careful physical and chemical characterization. Resultant biological activity could then be assigned to a well-defined macromolecular system.

As a frame of reference, the following is known about the well-studied *E. coli* ribosome: It is made up of a large and a small subunit having respective sedimentation coefficients \( S_{20,w}^{0} \) of 50.8 and 31.8. Both subunits are made up of approximately 63% RNA and 37% protein. Treatment of either subunit with increasing concentration of monovalent salts results in the successive loss of proteins leaving protein-deficient particles characterized by lower sedimentation coefficients and loss of biological activity. All but a few of the ribosomal proteins can be removed by such methods, and the resultant particles have been termed "core" ribosomal particles. All of the 21 proteins of the 30S subunit have been shown to be different from all of the 34 50S proteins. In addition, species differentiation has been based on the finding that each species of organism carries its own specific complement of ribosomal proteins. Thus, any given species of bacteria may have from just a few to
as many as 55 ribosomal proteins which differ from any
other bacterial species. With these few points in mind,
we can now discuss the physical and chemical characteristics
which were found for the *M. smegmatis* ribosomal subunits,
protein-deficient particles, and specific protein fractions.

The $S_{20,w}^0$ values, 48.7 and 28.1, respectively, obtained
for the *M. smegmatis* ribosomal subunits are somewhat lower
than the sedimentation coefficients which have been reported
for other mycobacterial ribosomal subunits (68, 73). Since
these latter sedimentation coefficient determinations were
made employing *E. coli* $^{14}$C-ribosomal markers in a sucrose
gradient, the $S_{20,w}^0$ values reported herein represent a more
accurate determination. The protein and RNA compositions
for *M. smegmatis* ribosomal subunits were respectively
found to be 36 percent and 64 percent for the 50S
ribosomal subunit, and 37 percent and 62 percent for the
30S ribosomal subunit. Within the limits of error in
the protein and the RNA determinations, these values fall
in close agreement with those of Baker, et al. (2) who
found a protein to RNA ratio of 38/62 for *M. smegmatis*
and *M. bovis* (BCG) ribosomal subunits. These figures
are quite close to the 37/63 protein to RNA ratio reported
for *E. coli* ribosomes.
If the mycobacterial protein-deficient particles and *E. coli* protein-deficient particles obtained by the same treatment are compared, they are found to be very similar. Spitnik-Elson, *et al.* (1, 55) reported that an *E. coli* protein-deficient particle obtained by the treatment of 30S subunits with 1M NaCl or 2M LiCl contained 25% protein and had a $S_{20}^o$ value of 18.5 at a concentration of 2.5 mg/ml. Itoh, *et al.* (24) described a particle isolated after treatment of 30S subunits with 2M LiCl as having 22% protein and a sedimentation coefficient of 21 as determined in a sucrose gradient. The particle obtained from *M. smegmatis* 30S ribosomal subunits by 1M NaCl treatment containing 19% protein and having a $S_{20, w}^o = 21-22$ fits quite closely the description of the corresponding *E. coli* subribosomal particle.

The sedimentation coefficient, $S_{20, w}^o = 15.7$ of the *M. smegmatis* ribosomal core particle corresponds well with Itoh's reported value of 16S for the *E. coli* 3.5M LiCl, EDTA-core particle (24), but again it should be noted that Itoh's value was determined in a sucrose gradient with 16S rRNA as a marker. Itoh found 9% protein in the *E. coli* core particle whereas the *M. smegmatis* core particle contained
11% protein. The fact that both *M. smegmatis* and *E. coli* 30S ribosomal subunits can be manipulated to yield essentially the same type of protein-deficient particles suggests that there may be basic structural similarities between 30S ribosomal subunits of both species.

Itoh (24) proposes that treatment of ribosomes with increasing concentrations of salts causes a stepwise dissociation of protein which may consist of more or less cooperative losses of groups of proteins, i.e., detachment of specific groups of proteins as a result of, perhaps, similar or associative RNA-protein binding interactions. These losses are specific to each step and are considered to occur in the reverse order of the assembly process. He found the following transformation:

\[
\begin{align*}
30S & \rightarrow 25-23S \rightarrow 21-19S \rightarrow 16S \\
30S & \rightarrow 25-23S \rightarrow 21-19S \rightarrow 16S
\end{align*}
\]

(37% protein) \quad (27-23% protein) \quad (22-19% protein) \quad (9% protein)

The *M. smegmatis* protein-deficient particles obtained by treatment with 1M NaCl and 3.5M LiCl, EDTA probably represent the last two steps in the above transformation and therefore are, perhaps, the first two steps in the in vivo assembly of the mycobacterial ribosome. Itoh maintains that the 16S particle represents the true core particle since the remaining core proteins cannot be removed even with very high salt concentrations.
Itoh claims that upon treatment of ribosomes with monovalent salts, the decrease in sedimentation coefficient is due mainly to release of proteins of the ribosomal particles. Atsmon, et al. (1) add that, especially in the case of Mg$^{+2}$ depletion, the sedimentation coefficient is a function of loss of protein and unfolding. According to this group, Mg$^{+2}$ depletion causes the normally compact, folded ribosomal structure to open up, and after unfolding, the various salts have ready access to most parts of the ribosome and effectively disrupt the electrostatic binding forces between the proteins and RNA.

The proteins remaining on the core particle are apparently not bound directly to RNA by electrostatic forces (Mg$^{+2}$ participation) since removal of Mg$^{+2}$ by EDTA does not result in salt-mediated release of these proteins from the rRNA molecule. There is experimental evidence to support the idea that in the case of 2M LiCl core particles (similar to 1M NaCl treated 30S ribosomes) the majority of the proteins in the core particle itself are bound to the 16S RNA by non-electrostatic interactions (56). Since many of these proteins contain numerous hydrophobic amino acid residues, it has been suggested that the protein-RNA interaction in the 2M LiCl core particle may be largely hydrophobic in nature (56). These findings can be extrapolated to the 16S core particle to explain its protein-RNA binding interactions.
in the presence of high concentrations of monovalent salts. One implication of hydrophobic interactions in the core particles is that the RNA molecule cannot be completely unfolded but is rather in such a conformation as to protect the remaining proteins from the aqueous environment.

It is also interesting to note that upon treatment with increasing salt concentration, the first proteins to be split from the *E. coli* 30S ribosomal subunit are, in general, those classified as fractional (10, 18), i.e., those found in less than one copy per subunit. The proteins of the resulting core particle are mainly unit proteins. These proteins are known to be required for the physical assembly of the 30S subunit. Five of the 30S proteins have been shown to have specific binding sites on the 16S rRNA (58). Of these, four are found in the 2M LiCl particle, and all five are classified topographically as internal (9) and enter the physical assembly sequence early.

It is not clear how many and which proteins are found on the *E. coli* 16S core particle. Itoh's group (24) is the only one which has studied this particle. They analyzed the ribosomal proteins on a carboxymethyl cellulose column. Using this method, the 21 ribosomal proteins of the *E. coli* 30S subunit were divided into 11 components. The 16S core particles were found to contain only one plus reduced amounts
of another of these 11 protein components. Thus, it is not possible to compare the proteins found in the *M. smegmatis* protein-deficient ribosomal particle with those obtained from *E. coli* because 1) the correlation between Itoh's column chromatography and conventional electrophoretic analysis of *E. coli* ribosomal proteins is not known, and 2) it has been shown in our lab (unpublished results) that there are extensive dissimilarities between the polyacrylamide gel electrophoresis patterns of the *M. smegmatis* and *E. coli* ribosomal proteins.

In spite of lack of correlation to any known core protein fractions, it is of great significance that the core proteins themselves isolated from *M. smegmatis* cells are a well-defined, reproducible antigenic fraction. Since by gel electrophoresis they have been unambiguously shown to be a specific fraction of the 30S protein complement, both their number and direct source is known. This is certainly novel to the field of delayed hypersensitivity.

In direct contrast, culture filtrate antigens (OT and PPD) represent products of cell lysis after extended culture. Granted, many cellular components may be subject to catabolic degradation upon aging and death of the mycobacterial cells, but this yet leaves innumerable potential antigenic components in the culture filtrate. PPD has indeed been shown to contain
numerous protein and polysaccharide fractions. Likewise, protoplasm represents an ill-defined mixture of cytoplasmic antigens. Isolation of a single antigenic fraction from either preparation nearly defies determination of source. Neither has isolation of a single antigenic fraction provided specificity in delayed skin reactions. It appears to be critical that a species specific antigen must be isolated in order to attain specific skin reactions. Again, we must consider the ribosome.

Although strain and species specific protein complements have been demonstrated for the 30S and 50S ribosomal subunits of a number of different bacteria (65, 27, 43), no work has yet been done to show this to hold for mycobacterial strains. Judging from the results of this study, however, it is probably safe to assume that at least some species specificity is exhibited by mycobacterial ribosomal proteins. It can be asked: Are mycobacterial species specific ribosomal proteins found in the 30S or 50S subunit or both, and is it possible that the 16S core proteins (or at least one of them) are species specific? Only a rigorous comparative analysis of the ribosomal proteins of many mycobacteria will fully answer these questions. We are presently left with only crude inferences from studies of other bacteria and the results of this study.
The only evidence which would suggest species specificity in the core protein fraction comes from the work of Osawa's group (43). While investigating the differences in the composition of ribosomal proteins in various bacteria, they found that the major protein component found in 16S core particles was the only protein component which differed in the 30S ribosomal proteins of *Salmonella heidelberg* and *Arizona arizonae*. One specific component was also found in the 50S subunit. They found, however, that different strains or species of the genus *Escherichia* differed in a number of specific 30S and 50S ribosomal proteins - one of these being the protein component present in reduced amounts in their 16S core particle. It is therefore possible that a protein component of mycobacterial core ribosomal particles may be strain or species specific, but without experimental evidence from different mycobacteria, one must accept any suggestion of such specificity based on other bacteria as conjecture.

In an attempt to evaluate the possibility that mycobacterial ribosomal proteins might contain specific antigens capable of evoking delayed hypersensitivity reaction in animals sensitized with mycobacteria, a series of experiments were carried out which resulted in the isolation of a protein fraction from the 16S core particle which displayed both
specificity and sensitivity as a skin test antigen. The intermediate steps which ultimately resulted in the isolation of these proteins also yielded interesting results relating to the role of the ribosome in eliciting potent and specific delayed skin reactions.

Since it was originally decided to investigate the skin test activity of antigens which could be derived from the 30S ribosomal subunit, it was necessary to confirm that the 30S subunit was indeed antigenically active. The results obtained were in accord with the findings of Baker (2). The 30S subunit did display a potency approaching that of homologous protoplasm and also showed a greater specificity, while the 50S subunit had a lesser degree of both skin test activity than either homologous protoplasm or the 30S subunit.

Subsequent studies with protein-deficient particles derived from the 30S ribosomal subunit demonstrated that successive removal of protein resulted in particles of increasing skin test potency and specificity. The proteins removed from the 30S ribosome were inactive as skin test antigens which suggested that the active portion of the 30S ribosomes lay not with the proteins near the surface of the ribosomal subunit as might be expected, but rather in a region of the ribosome where the proteins may be bound.
to the rRNA through hydrophobic interactions. This was, in part, borne out by the finding that the core proteins themselves displayed an even greater skin test activity than the 16S core particle and that the ribosomal RNA was entirely antigenically inactive.

This finding relates to the studies of the tuberculin activity of BCG ribosomes by Ortiz-Ortiz, et al. (42). They reported that purified BCG ribosomal RNA failed to elicit any inflammatory reaction in BCG-sensitized guinea pigs while the total extracted ribosomal protein did elicit skin reactions in sensitized guinea pigs. Although they found that the skin test reactivity of ribosomal proteins was somewhat greater than PPD, no studies were made to test the specificity of their ribosomal protein preparation. In contrast, the present study demonstrates the potency of 30S protein and 16S core protein preparations as being somewhat greater than that of protoplasm which is, in turn, at least as potent as PPD in causing delayed reactions in sensitized guinea pigs (30). Of greater significance, however, is the demonstration of heightened specificity when ribosomal proteins are physically separated to produce the pure, active 16S core fraction.

The demonstration that the potency of the various ribosomal antigens in evoking delayed hypersensitivity skin
reactions follows the order of 16S core proteins > 16S core particles ≈ 30S proteins > 30S ribosomal subunits shows that much of the antigenic activity of the 30S subunit has been narrowed down to the core protein fraction. This order of potency would be indicative of a single, highly active protein fraction of the 30S ribosome. Removal of either inactive RNA and/or inactive proteins from the 30S subunit would naturally yield a fraction of high antigenicity on the dry weight basis.

As pointed out earlier, the gel electrophoresis analysis gave 5 protein bands for the core protein fraction. Two of the 5 bands were common to both the inactive supernatant fraction and the core fraction implying that the high antigenic activity of the core fraction was due to, at the most, only three core ribosomal proteins. As stated before, this represents the first reproducible isolation of a protein fraction of high tuberculin activity which can be definitively assigned to a subcellular component where the number and nature of the component is clearly defined.

This method of obtaining a specific, potent antigen is eminently more reproducible and exact than the numerous attempts which have been made to physically or chemically separate tubercle protein components of culture filtrate antigens in hopes of isolating such a potent, species
specific skin test antigen. The large number of protein components contaminated with polysaccharide and cell wall material which are found in culture filtrates have made such studies quite difficult and generally unsuccessful. Attempts have also been made to refine protoplasmic material. Beam, et al. (3) subjected protoplasm to differential centrifugation followed by extraction of lipid and polysaccharide. The resulting fraction was termed purified protoplasmic peptide (PPP) and was reported to have relatively few protein components. The pellet from the high-speed centrifugation of the crude protoplasm was reported to contain "ribosomes" which were inactive in gel diffusion analysis and in skin tests in sensitized guinea pigs. Since protoplasm is prepared by disruption of mycobacterial cells in distilled water, it is unlikely that intact, undegraded ribosomes were present in their "ribosomal pellet." Rather, PPP may contain antigenically active ribosomal proteins released from the breakdown of ribosomes.

From the cytological point of view, it is apparent that protoplasm, which is merely the cytoplasmic contents of the cell, contains, among other things, degraded ribosomal material - the amount depending on the state of the cells at the time of disruption. It is possible that PPD may also contain a few ribosomal antigens released from the
cells upon lysis. The specificity of reaction of either of these preparations is dependent upon the presence of species specific antigens. It is most probable that different mycobacteria have many antigenically similar enzymes, structural proteins, and polysaccharides. Therefore, while it has been possible to isolate a pure, potent and specific antigen, this antigen is not necessarily unique, but rather it may represent one specific agent that will elicit delayed hypersensitivity.

The degree of cross-reactivity observed with both PPD and protoplasm when tested in heterologously sensitized animals suggests that they contain antigens common to the heterologous organism. Conversely, antigens displaying skin test specificity may well represent species specific proteins. This argues strongly that the high degree of skin test specificity displayed by the core protein fraction is a function of its uniqueness to the \textit{M. smegmatis} species. Further physical and serological studies are needed to confirm whether or not one or more of the core proteins are truly strain specific antigenic proteins. If study of either the 16S core particles or the core proteins themselves from various mycobacteria could show that these fractions do contain active, species specific antigens, it would then be possible to isolate a diagnostic skin test antigen of unequaled specificity.
In conclusion, the results of this study confirm and extend previous work which demonstrated that mycobacterial ribosomes are both potent and specific antigens for eliciting skin test reactions in sensitized guinea pigs. The significant finding of this study was the demonstration that it is possible to isolate from mycobacterial ribosomes an antigenic fraction of high skin test potency and specificity, containing relatively few protein components by isolation of the 30S ribosomal subunit followed by selected removal of most of the ribosomal proteins and subsequent isolation of the residual proteins.
CHAPTER 5

SUMMARY

Ribosomal subunits were isolated from late log phase *M. smegmatis* cells by conventional techniques of disruption, differential centrifugation and sucrose gradient centrifugation. Sedimentation coefficients for the large and small subunits were found to be 48.7 and 28.1, respectively. The protein and RNA compositions of these subunits are consistent with values reported for other mycobacteria.

Two different protein-deficient ribosomal particles were prepared from the 30S subunit by treatment with 1M NaCl and 3.5M LiCl-EDTA. The particles obtained correspond closely to particles prepared by the same methods using *E. coli* ribosomal subunits. Sedimentation coefficients obtained for the NaCl split particle and the LiCl 16S core particle were 21-22 and 15.7, respectively. Protein analysis revealed that approximately one-half of the protein was removed from the 30S particle by the NaCl treatment and about 70% was removed by the LiCl-EDTA treatment.

Polyacrylamide gel electrophoretic analysis of the proteins extracted from the core particle revealed three major and two minor protein bands. Of these 5 bands, two were also common to the set of proteins which were removed from the 30S ribosome by the LiCl treatment. This implies
that only three of the protein bands are unique to the core protein fraction.

Studies were made on the ability of these various ribosomal-derived antigens to produce cutaneous delayed hypersensitivity in homologously and heterologously sensitized guinea pigs. As had been previously demonstrated, the *M. smegmatis* 30S ribosomal subunit was found to be nearly as potent as and somewhat more specific than *M. smegmatis* protoplasm in eliciting delayed skin reactions. The 50S ribosomal subunits meanwhile displayed both lesser potency and specificity than homologous protoplasm.

The split ribosomal particles prepared by 1M NaCl treatment were found to be equal in potency to protoplasm while displaying specificity twice that of protoplasm. The proteins which had been split off the 30S subunit were essentially inactive as skin test antigens. The 16S core particles were found to be as potent as the split ribosomes but with twice the specificity. The proteins released from the 30S subunit by the LiCl treatment were also essentially antigenically inactive.

Skin test studies of the proteins extracted from the 16S core particle showed that these few proteins were more potent and slightly more specific than even the 16S core particles. On the other hand, rRNA isolated from the 30S
subunit failed to elicit any inflammatory reaction. A comparative study of the active ribosomal derived antigens all in one set of guinea pigs revealed the following order of potency in eliciting delayed skin reactions: 16S core proteins > 16S core particles ≥ 30S proteins ≥ 30S ribosomal subunits. This order of potency demonstrates that by sequential removal of RNA and/or proteins from the 30S subunit, a set of proteins free from less specifically reactive material has been attained. The resultant set of proteins exhibits both increased potency and specificity relative to the original 30S ribosomal subunit and homologous protoplasm.
LIST OF REFERENCES


Viviparus ater (Ottaviani et al., 1993). Observation of a TNF-α-like molecule in B. glabrata came from hemocyte monolayers incubated in their own plasma. The increase in TNF-α level following exposure of the hemocytes to LPS was not at a level of statistical significance to state that it is the hemocytes that are induced by LPS into producing this cytokine.

Some researchers believe that the results showing evidence for cytokines in invertebrates are caused by non-specific binding of plasma lectins to the antibodies used in ELISA assays. Our research has shown that hemocytes from 13-16-R1 snails are capable of significantly higher LPS-induced production of an IL-1-like molecule and also the production of TNF-α-like molecule. This indicates that snail hemocytes can be activated in a manner similar to mammalian macrophages that have been shown to produce IL-1 and TNF-α following exposure to LPS. This evidence, combined with a number of experiments showing that mammalian cytokines can upregulate the immune response in invertebrates, argues against a lectin giving false results. Although it is possible that hemocytes could produce a plasma protein capable of false positives in ELISA’s for the presence of cytokines, this thesis has also shown that snail plasma does not non-specifically bind to a B. burgdorferi OspC antibody. Given this body of evidence it is likely that hemocytes are producing a signaling molecule with functional similarities to cytokines.
Isolation of the genes responsible for production of these compounds should be possible with further experimentation.

Determining the mechanisms responsible for resistance to schistosomes in *B. glabrata* snails may play an important part in controlling or eradicating schistosomiasis as well as increasing our understanding of invertebrate immunity. If future research shows that a cytokine-like molecule plays a pivotal role in resistance to schistosome infection in its intermediate host then it may be possible to select for this trait in natural populations in endemic areas. Even partial resistance to the schistosome may lower the incidence of this disease in the human population in these endemic areas thereby improving the health of people burdened by schistosomiasis.
LITERATURE CITED


