Delayed Hypersensitivity Reactions Provoked by Ribosomes from Acid-Fast Bacilli: Physical Characteristics and Immunological Aspects of Core Ribosomal Proteins from *Mycobacterium smegmatis*

R. V. LOGE, W. E. HILL, R. E. BAKER, AND C. L. LARSON

Departments of Chemistry and Microbiology, and the Stella Duncan Memorial Research Institute, University of Montana, Missoula, Montana 59801

Received for publication 5 December 1973

Ribosomal subunits from *Mycobacterium smegmatis* were analyzed by using sedimentation velocity, sedimentation equilibrium, and acrylamide gel electrophoresis experiments. These $s_{20,w}$ values for the subunits are 48.7S and 28.1S. The molecular weight of the 49S subunit is about $1.65 \times 10^6$, and that of the 28S subunit is $7.8 \times 10^5$. Both subunits contain about 37% protein and 63% ribonucleic acid. A protein-deficient particle having an $s_{20,w}$ value of 15.7S contains about 11% protein and 89% ribonucleic acid. Skin tests showed all subunits and proteins to be active as agents in provoking delayed hypersensitivity, but the 16S protein-deficient particle, as well as the proteins derived from it, was more specific than the subunits themselves.

Much study has been directed toward the isolation from mycobacteria of antigens capable of eliciting delayed skin reactions in sensitized hosts. The classical work was done with filtrates from cultures of mycobacteria. These filtrates contain a complex of antigens, including numerous proteins and polysaccharides, and provoke delayed hypersensitivity reactions in hosts sensitized with heterologous mycobacteria. More recently, protoplasmic extracts from mycobacteria have been shown to have skin-test activity equal to or greater than purified protein derivative (PPD), the accepted standard for tuberculin skin-test activity which is a protein precipitate derived from culture filtrates (3, 11-13). Current studies have also demonstrated that ribosomal material from acid-fast bacilli provokes delayed skin reactions in sensitized animals (1, 17). Furthermore, it has been shown that the 30S ribosomal subunit has skin-test potency and specificity greater than that of protoplasm (1).

In the process of preparing various ribosomal samples for this purpose, it becomes desirable to physically analyze the ribosome fractions at various stages of preparation. Since previous physical studies of bacterial ribosomes were made with material from organisms other than mycobacteria, this study was undertaken to provide some physical characteristics of ribosomes from acid-fast bacilli.

Techniques used for *Escherichia coli* ribosomal preparations were adapted for mycobacterial ribosomes. The resulting preparations were then analyzed by means of sedimentation velocity and sedimentation equilibrium experiments, polyacrylamide gel electrophoresis of the protein complements, and chemical tests for protein and ribonucleic acid (RNA). From these we have obtained the sedimentation coefficients, molecular weights, protein-to-RNA ratios, and gel patterns of proteins present on the large and small ribosomal subunits, as well as the protein-deficient 16S particle derived from the smaller subunits. These ribosomal preparations were used as skin-test antigens, and comparisons were made with protoplasm to determine the relative potency and specificity.

**MATERIALS AND METHODS**

**Animals.** Hartley strain albino female guinea pigs, obtained from a commercial source in Hamilton, Mont., and weighing between 300 and 500 g, were used.

**Bacterial strains.** The strains of *Mycobacterium smegmatis* and *M. bovis* (BCG) were obtained, maintained, grown, and harvested as previously described (1). After harvesting, the cells were either used immediately or frozen at -75°C until needed.

**Antigens.** Protoplasm from BCG and *M. smegmatis* was prepared as previously described (1).
Ribosomes of *M. smegmatis* were obtained by using the following buffer solutions: wash buffer (0.01 M MgCl₂, 0.01 M tris(hydroxymethyl)amino-
methane (Tris-hydrochloride) [pH 7.4], 0.5 M NaCl); 30S buffer (0.001 M MgCl₂, 0.01 M Tris-
hydrochloride [pH 7.4], 0.07 M KCl); 30S buffer (0.002 M MgCl₂, 0.01 M Tris-hydrochloride [pH 7.4], 0.07 M KCl); and 16S buffer (0.001 M MgCl₂, 0.01 M Tris-hydrochloride (pH 7.4), 0.07 M KCl). During the entire procedure, all work was carried out at 4 C, and samples were either used immediately or stored at -75 C until needed.

*M. smegmatis* cells were initially disrupted by using either a Ribi cell fractionator (Sorvall) under conditions described previously (1) or by means of a Gifford-Wood mininimill. With this latter method, a slurry of approximately 60 g of cells in about 10 ml of wash buffer was mixed with about 30 ml of 0.12-mm glass beads and ground at 22.00 rpm for 45 min at 0 to 4 C with a 0.75-mm spacing between the rotor and stator in the mininimill. The resulting slurry was diluted to 250 ml with wash buffer and centrifuged twice at low speed (25,000 × g) for 10 and 45 min to remove the cellular debris and glass beads. The supernatant was then spun at 360,000 × g to pellet the ribosomes. These pellets were then resuspended in wash buffer and frozen at -75 C for further use.

The frozen, crude ribosomes were thawed, and 1% sodium deoxycholate (DOC) and 5% Brij-58 in wash buffer were stirred into the sample to bring the final concentration in the solution to 0.1% DOC and 0.5% Brij-58. This mixture was stirred vigorously for 5 min, diluted twofold, and centrifuged at 25,000 × g for 15 min to remove any released membranous material and insoluble DOC. The resulting supernatant was then centrifuged for 2.5 h at 360,000 × g, and the pellet was resuspended in 50S buffer in order to dissociate the subunits.

The ribosomal subunits were separated by zonal centrifugation as described by Eikenberry et al. (4). About 10,000 to 14,000 A₂₆₀ units of ribosomes were layered on a 10 to 30% linear sucrose gradient, with a 50% cushion, in a Spinco Ti-15 rotor, which was spun for 9 h at 35,000 rpm in a Beckman L2-65B ultracentrifuge. The effluent gradient was then monitored at 260 nm, and the resulting peak fractions were pooled to give pure subunit preparations. The samples were concentrated by precipitating the ribosomes with 2 volumes of cold ethanol, the precipitate then being pelleted by centrifugation at 25,000 × g for 15 min. The pellets were then resuspended in 50S or 30S buffer, respectively, and frozen at -75 C until needed.

16S core ribosomal particles were prepared by the method of Itoh et al. (8). A solution of 30S ribosomal subunits was allowed to stand for 12 h at 4 C in an equal volume of 7 M LiCl, 0.025 Tris (pH 8.8), and 0.005 M ethylenediaminetetraacetic acid (EDTA). The solution was then centrifuged at 400,000 × g for 3 h, the supernatant was labeled “supernatant proteins,” and the pellet was resuspended in 16S buffer and labeled “16S core particles” (Fig. 1).

Ribosomal proteins were extracted from the 30S ribosomal subunits and the 16S core particles by treatment with 67% acetic acid in the presence of 0.03 M Mg. The RNA precipitate was washed twice with the extracting solution for a more quantitative extraction, and the proteins were dialyzed for 48 h against 6 M urea, 0.02 M Tris-hydrochloride (pH 7.8). These were labeled “30S proteins” and “core proteins,” respectively (Fig. 1 and 2). Ribosomal RNA was extracted from the 30S ribosomes by the cold phenol method (21).

**Chemical analysis.** Protein determinations were made with the Folin reagent according to Lowry (14) with crystalline bovine serum albumin (Sigma Chemical Co.) used as the standard.

RNA concentration was determined by the orcinol method (15) by using *E. coli* ribosomal RNA (rRNA) as the standard.

Concentration of the ribosomes and ribosomal subunits was determined spectrophotometrically by using *E₅₄₀ = 145* (6). Concentrations of the 16S core particles were determined by using an extinction coefficient of *E₅₄₀ = 208*. Concentrations of rRNA solutions were determined spectrophotometrically by using *E₅₄₀ = 223* (21).

**Physical methods.** Sedimentation velocity experiments were made in a Spinco model E analytical ultracentrifuge using an AND rotor. All runs were made at 60,000 rpm and at 4 C using schlieren optics with a 75° phase-plate angle. Sedimentation coefficients were corrected for temperature, salt concentration, and radial dilution effects and extrapolated to infinite dilution giving *S₅₀.₀* values.

Sedimentation equilibrium experiments were carried out under conditions outlined previously (6), except that only the weight-average molecular weights were obtained in this study. Speeds used were 4,000 and 6,000 rpm for the 50S and 30S subunits, respectively.

**Fig. 1.** Flow diagram for isolation of 16S core particles, supernatant proteins and core proteins. Abbreviations: HAc, acetic acid; ppt, precipitate.

**Fig. 2.** Flow diagram for isolation of 30S proteins. Abbreviations are as in Fig. 1.
**Electrophoresis.** The procedure used for the polyacrylamide gel electrophoresis was that of Reisfeld et al. (19) as modified by Traub and Nomura (22). Protein samples (30 to 200 mg/ml) in a volume of 10 to 300 l were layered onto the stacking gel and run at 1.5 A/cm tube until the tracking dye, pyronin red, reached the bottom of the running gel (approximately 4 h). The gels were stained for 1 h in a 0.25% solution of amido black in 7% acetic acid and de-stained electrophoretically in 7% acetic acid.

**Cutaneous delayed hypersensitivity reactions.** Sensitization of groups of guinea pigs with either *M. smegmatis* or BCG and subsequent skin tests were performed as previously described (1). The skin lesions were measured at 24 h, and the volumes of the skin lesions were calculated (25). Those skin lesions having volume equal to or greater than 15 mm³ were recorded as being positive. A skin test 50% (ST₅₀) was calculated from the results of skin tests obtained with various antigens. A ST₅₀ value is defined as the amount of antigen required to elicit a positive skin test in one-half of the animals tested.

**Histological studies.** Guinea pigs sensitized in the hind footpads with frozen *M. smegmatis* cells in incomplete Freund adjuvant were injected intradermally with 1.0 µg of either *M. smegmatis*, 16S core ribosomal particles, or *M. smegmatis* protoplas. These animals were examined 6 h after injection of antigen to determine whether reactions were present. They were sacrificed 24 h after testing, and the lesions were excised and fixed in Formalin. Paraffin sections (5 µm) were stained with hematoxylin and eosin and examined microscopically.

**RESULTS**

**Chemical and physical studies.** Disruption of the mycobacteria using either the Ribi cell fractionator or the minifluid yielded fluids which had schlieren patterns typical for crude 70S ribosomal preparations, as observed by sedimentation velocity experiments (Fig. 3A). The isolated ribosomal subunits and the 16S protein-deficient particle were sedimented in the analytical centrifuge, giving single, homogenous peaks (Fig. 3B–D). Sedimentation coefficients of the subunits and the core particle were obtained by determining the sedimentation coefficients of a dilution series and extrapolating to infinite dilution (Fig. 4).

Sedimentation equilibrium experiments were used to obtain the molecular weight of both subunits (Fig. 5). It is apparent that the 30S sample was somewhat heterogeneous since the molecular weight increased across the cell, but the molecular weight of the subunit appeared to be approximately 780,000, as shown near the bottom of the cell. The molecular weight of the 50S subunit appeared slightly larger than that obtained for the *E. coli* 50S subunit (6), but this can probably be attributed, in at least one sample, to slight contamination with dimer which was apparent in the early schlieren patterns of sedimentation velocity experiments. It is also true that the 50S subunit is membrane bound, and some membranous material may still have been bound to the 50S subunit, which may also account for the slight amber color of the 50S ribosomal subunits.

The RNA and protein composition of the ribosomal subunits and the 16S core particle are listed in Table 1 along with their sedimentation coefficients and molecular weights.

**Electrophoresis.** Disc electrophoresis patterns were obtained from the extracted 30S ribosomal protein, the supernatant proteins, and the proteins extracted from the 16S core particles (core protein) (Fig. 6). It can be seen...
TABLE 1. Composition and physical characteristics of ribosomal particles from M. smegmatis

<table>
<thead>
<tr>
<th>Particle</th>
<th>$s_{20,w}$ (Svedberg units)</th>
<th>Mol wt</th>
<th>Protein (%)</th>
<th>RNA (%)</th>
<th>Protein/RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large subunit</td>
<td>$48.7 \pm 0.5$</td>
<td>$1.65 \times 10^{26}$</td>
<td>$36 \pm 2$</td>
<td>$64 \pm 2$</td>
<td>$0.56$</td>
</tr>
<tr>
<td>Small subunit</td>
<td>$28.1 \pm 0.5$</td>
<td>$7.8 \times 10^{5}$</td>
<td>$37 \pm 2$</td>
<td>$62 \pm 2$</td>
<td>$0.60$</td>
</tr>
<tr>
<td>16S core particle</td>
<td>$15.7 \pm 0.7$</td>
<td>$11 \pm 2$</td>
<td>$89 \pm 2$</td>
<td>$0.12$</td>
<td></td>
</tr>
</tbody>
</table>

*a* This value may be slightly high due to some dimerization and possibly some membranous contamination.

---

**FIG. 4.** Extrapolation to infinite dilution of the $s_{20,w}$ values obtained for the larger subunit (Δ), smaller subunit (O), and protein-deficient core particle (□). The $s_{20,w}$ values are noted in Table 1.

**FIG. 5.** Weight-average molecular weights as a function of concentration in terms of fringe displacement (j). The 49S subunits (■, ○) were at initial concentrations of 0.5 mg/ml and run at 4,000 rpm. The 28S (Δ, ◢) subunits were at initial concentration of 0.5 mg/ml and run at 6,000 rpm.

from this figure that the distribution of proteins between the core- and supernatant-protein fractions shows the expected complementarity: all of the 30S proteins, as well as an additional band designated 15a, were present. The presence of this additional band is yet unexplained. It should be noted that, of the five bands present in the core protein fraction, only two were also found in the supernatant protein fraction. Proteins 4, 11, and 17 were unique core proteins.

**Delayed hypersensitivity reactions.** The results of all skin tests performed on four groups of guinea pigs sensitized with M. smegmatis and three groups of guinea pigs sensitized with BCG and tested with serial dilutions of the various antigens derived from ribosomes are tabulated in Table 2. 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 spe-
specific as culture filtrate antigens (PPD) in eliciting skin reactions. BCG protoplasm was also used as a control. The tabulated results from Table 2 were converted into ST₅₀ values (Table 3).

Experiment 1 (Tables 2 and 3) confirms our previous findings that the 30S ribosomal subunit rather than the 50S subunit possessed relatively specific skin-test reactivity. Selective removal of increasing amounts of protein from the 30S subunit resulted not only in a concomitant increase in skin-test potency of the resulting particles when compared with the remainder of the preparation left after protein removal, but also a significant increase in relative specificity (experiments 2 and 3). Thus, a nearly fourfold increase in specificity resulted after the removal of 70% of the ribosomal protein from the 30S subunit by treatment with 3.5M LiCl, EDTA. Interestingly, the proteins removed by treatment with either NaCl or LiCl (split proteins and supernatant proteins) failed to display appreciable skin-test reactivity. Some delayed reactivity was noted in some of the animals tested with the highest concentrations of these antigens, but the lesions were not large enough to satisfy the criteria for a positive reaction.

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 (experiment 3). Core proteins extracted from the 16S core particle were more potent and specific than protoplasm. Thus, 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 greater specificity than does protoplasm.

Determination of the relative potency of four active ribosomal antigens (experiment 4) showed that the extracted core proteins were about twice as potent as either the 16S core particles themselves or the 30S ribosomal proteins which were, in turn, more potent than the 30S subunits. This order of potency implies that the core proteins are, in fact, a major factor responsible for the specific skin-test activity of the 30S ribosomal subunit.

**Histological studies.** None of the antigens used for skin tests produced Arthus reactions. Examination of the hematoxylin-and-eosin-

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Antigen</th>
<th>M. smegmatis</th>
<th>M. bovis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>0.25</td>
</tr>
<tr>
<td>1</td>
<td>30S ribosomes</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50S ribosomes</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protoplast</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16S core particle</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supernatant proteins</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protoplast</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ribosomal RNA</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S core particles</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S core proteins</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protoplast</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCG protoplasm</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30S ribosomes</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30S proteins</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S core particles</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S core proteins</td>
<td>5/5</td>
<td></td>
</tr>
</tbody>
</table>

*See Materials and Methods for preparative methods of various fractions. All antigens were from *M. smegmatis* unless otherwise noted.

* Numerator, number of animals positive; denominator, number of animals tested.

ND, Not done.

NR, No reaction.
stained paraffin sections of tissue taken 24 h after injection of antigens failed to show marked differences in the histology of the skin lesions provoked by either protoplasm or the ribosomal antigens. No generalized tissue necrosis was apparent, nor were significant numbers of polymorphonuclear cells demonstrable. Mononuclear cells were predominantly found around the vessels of the deep dermis, subcutaneous fat, and connective tissue, and fewer were found in the septa and underlying muscle. These data are in agreement with the data previously reported for the crude ribosome and the 50S and 30S ribosomal subunits (1).

**DISCUSSION**

Some physical aspects of ribosomes from mycobacteria have previously been studied by Trnka and Smith (24) and Worcel et al. (26). In neither case did the workers attempt to physically characterize their material, but rather they illustrated sample purity by electron micrographs and sedimentation coefficients estimated from sucrose gradient centrifugation. In this study, sedimentation velocity studies were made on a concentration series of 50S, 30S, and 16S samples, from which the $s_{20,w}$ values were obtained. These values are 48.7S and 28.1S for the large and small subunit, respectively, as compared with 50.2S and 31.8S for the large and small subunit, respectively, from *E. coli* (7). The $s_{20,w}$ values for the core particle was found to be 15.7S.

Since the sedimentation coefficients were somewhat lower than those obtained from *E. coli* subunits, sedimentation equilibrium studies were made to determine the molecular weight of the mycobacteria subunits. Due to limited amounts, samples with some contamination were used. Nonetheless, the 780,000-dalton value obtained for the 28S subunit was exactly that which would be expected if the decrease in the sedimentation coefficient as compared with the *E. coli* subunit was entirely due to decrease in the molecular weight, assuming the partial specific volumes and the frictional coefficients were the same for both subunits.

In the case of the larger subunit, the case is not altogether clear. The molecular weight expected from the sedimentation coefficient decrease, using the above assumptions, would be about $1.5 \times 10^{6}$ rather than $1.65 \times 10^{6}$ as observed. This difference is probably due, at least in part, to the presence of dimer, which was noticeable in the schlieren pattern of one of the samples used. However, it is also possible that a portion of the membrane to which the 50S subunit was bound was still present on this subunit. This might also account for the amber color these subunits retained in spite of repeated washings.

Therefore, using these values as criteria, we would conclude that the molecular weight of the 28S subunit is 780,000, whereas the actual molecular weight of the 49S subunit is unknown but somewhat less than $1.65 \times 10^{6}$.

The protein-to-RNA ratios of both the 49S and 28S subunits are in excellent agreement with those values obtained for *E. coli* ribosomal subunits (5). These results, when coupled with the molecular weights, would imply that the subunits from *M. smegmatis* contain less protein and RNA than do their *E. coli* counterparts. However, gel electrophoresis showed 18 bands for the 28S subunit proteins, which is approximately the number obtained from *E. coli* 30S subunits if it is assumed that some of

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Antigen*</th>
<th>ST$_{50}$ values for the various ribosomal antigens</th>
<th>M. smegmatis</th>
<th>BCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ST$_{50}$ units* in guinea pigs sensitized with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30S ribosomes</td>
<td>3.3*</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50S ribosomes</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Protoplasm</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>BCG protoplasm</td>
<td>ND*</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16S core particles</td>
<td>3.3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Supernatant proteins</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Protoplasm</td>
<td>3.2</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BCG protoplasm</td>
<td>14.0</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ribosomal RNA</td>
<td>no reaction</td>
<td>no reaction</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16S core particles</td>
<td>4.0</td>
<td>&gt;16</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16S core proteins</td>
<td>2.5</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Protoplasm</td>
<td>3.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BCG protoplasm</td>
<td>12.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30S ribosomes</td>
<td>12.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30S proteins</td>
<td>10.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16S core particles</td>
<td>10.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16S core proteins</td>
<td>5.0</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* See Materials and Methods for preparative methods of various fractions. All antigens were from *M. smegmatis* unless otherwise noted.

* One unit = 0.031 µg of antigen.

* 3.3 × 0.031 or 0.1 µg of antigen required to provoke a positive skin test in one-half of the animals sensitized with *M. smegmatis* treated with this dose. This is an interpolated value from the tabulated results.

* ND, No data.
the bands represent two or more proteins, as is
the case with E. coli 30S subunit proteins. Our
results suggest that either the mycobacterial
ribosomal proteins are somewhat smaller than
their E. coli counterparts, or that the average
28S subunit actually contains fewer proteins
than the average 30S subunit. In either case, it
would appear likely that the 28S subunit is
heterogeneous, as is the E. coli 30S subunit (5).
It should be noted that the protein-to-RNA
ratios of the 16S core particles from M.
smeagmatis are similar to those reported by Itoh
e et al. (8) for the 16S-stripped E. coli subunits.

Electrophoresis patterns of the proteins of E.
coli and M. smeagmatis cannot be adequately
compared at this time until further studies have
been made. Although the various ribosomal
proteins from E. coli have been characterized
quite completely, there is presently no way to
determine similarity between the proteins of the
two strains unless extensive characterization of
the proteins from the mycobacterial ribosomes
is performed.

The results of this study demonstrate that
proteins isolated from the smaller subunit of M.
smeagmatis ribosomes are potent and specific
agents for eliciting delayed hypersensitivity
reactions in specifically sensitized hosts. This is
in marked contrast to the classical and more
recently studied skin-test antigens, namely old
tuberculin (OT), PPD, and protoplasmic ex-
tacts, which are not well-defined substances.
Although they are potent antigens, they show
varying degrees of nonspecificity. Other workers
have obtained purified polypeptides (2) or tu-
berculin-active peptides (16) which have de-

dined purity and specificity. However, both the
high degree of specificity and the clear defini-
tion of the source and nature of the core proteins
are novel aspects in the study of delayed hyper-
sensitivity due to mycobacteria.

Protoplasm, described by Larson et al.
(11-13) and Counts and Kubica (3), is capable of
provoking skin reactions in sensitized experi-
mental animals of equal or greater specificity
than homologous PPD. Baker et al. (1) further
demonstrated that the 30S subunits from myco-

bacterial ribosomes were capable of provoking
delayed skin reactions with specificity some-
what greater than that of protoplasm. The
results reported here show that, as proteins are
sequentially removed from the 30S ribosomal
subunit, the residual particles have both in-
creased skin test-potency and specificity. When
inactive rRNA is removed from the proteins of
the 16S core ribosomal particle, the protein
fraction is about two times more specific than
the parent 30S ribosomal subunit and about

four times as specific as homologous protoplasm
in its ability to elicit delayed skin reactions.

The degree of cross-reactivity observed with
both PPD and protoplasm when tested in
heterologously sensitized animals suggests that
these materials contain antigens common to the
heterologous organism. Conversely, the anti-
gen in these materials which elicit skin-test
reactions at the terminal dilutions may well
represent species-specific proteins. This implies
that the high degree of skin-test specificity
displayed by the core protein fraction is a
function of its uniqueness to the M. smeagmatis
species. Although strain and species-specific
protein complements have been demonstrated
for the 30S and 50S ribosomal subunits of a
number of bacteria (10, 18), no work has yet
been done to prove this for mycobacterial spe-
cies. Our results indicate that at least some
species specificity is exhibited by mycobacterial
ribosomal proteins. A rigorous comparative
analysis of the ribosomal proteins of many
mycobacteria plus further physical and serolog-
ical studies are needed to confirm this observa-
tion.

Since protein removal is effected by disrup-
tion of the electrostatic binding interactions
between the ribosomal protein and RNA, it has
been suggested that the protein-RNA interac-
tion in the core ribosomal particle may be
largely nonelectrostatic or hydrophobic in
nature (20). In addition, a number of known E.
coli proteins have been shown to have specific
binding sites on the 16S rRNA molecule (28).
One implication of hydrophobic interactions of
the core proteins is that the ribonucleoprotein
particle cannot be completely unfolded but is
rather in such a conformation as to protect the
remaining proteins from the aqueous environ-
ment.

With respect to this last point, it should be
emphasized that the supernatant proteins were
relatively inactive as skin-test antigens, and the
16S core particles and core proteins were both
very active. This would suggest a model of
"hydrophobic protection" wherein 30S ribo-

somal subunits upon injection (or release from
lysed infecting cells) are immediately partially
degraded, leaving protein in a hydrophobic core
particle relatively protected from extracellular
proteases and which can then be effectively
processed, resulting in a delayed reaction. If this
rationale is correct, then this procedure could be
used to produce potent and specific skin-test
antigens for other organisms.

It should be emphasized that this method of
isolating specific agents for provoking delayed
hypersensitivity is somewhat novel, since most
techniques involve the isolation of specific agents from rather heterogeneous mixtures. In this case, we have isolated well-defined agents and then altered them to increase the specificity of the delayed hypersensitivity reaction. It would appear that this type of procedure may be of great worth in many areas of immunology.

It is interesting to note in this respect that Youmans and Youmans (27) have also found a 16S RNA particle in their preparations which is reported to confer specific immunity to H37Rv challenge upon mice inoculated with this antigen. Whether or not these two 16S preparations are analogous has yet to be determined. Current studies are underway to determine the degree to which the five ribosomal proteins contribute to the delayed hypersensitivity activity of the 16S ribosomal subunit and the biological properties associated with this ribosomal subunit.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants AI05370-10 and GM17436-01A1 from the National Institutes of Allergy and Infectious Diseases and General Medical Sciences, respectively, and by National Science Foundation grant GB-17497. C. L. L. was supported by Public Health Service research career award 5-K06-AI1165-02-08-NCR from the National Institute of Allergy and Infectious Diseases. W. E. H. was supported by Public Health Service research career development grant 3-KO4-GM19692-01A1SBBCA from the National Institute of General Medical Sciences.

We wish to acknowledge the excellent work of Charlene Loge in preparing the mycobacteria, and the technical assistance obtained from the Stella Duncan Research Institute for the immunological aspects of this study.

LITERATURE CITED