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

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Ribosomal subunits from *Mycobacterium smegmatis* were analyzed by using sedimentation velocity, sedimentation equilibrium, and acrylamide gel electrophoresis experiments. These $s^0_{20,w}$ values for the subunits are 48.7S and 28.1S. The molecular weight of the 49S subunit is about 1.65×10^6, and that of the 28S subunit is 7.8×10^5. Both subunits contain about 37% protein and 63% ribonucleic acid. A protein-deficient particle having an $s^0_{20,w}$ 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 $MgCl_2$, 0.01 M tris(hydroxymethyl)aminomethane (Tris-hydrochloride) [pH 7.4], 0.5 M NH_4Cl); 50S buffer (0.001 M $MgCl_2$, 0.01 M Tris-hydrochloride [pH 7.4], 0.07 M KCl); 30S buffer (0.002 M $MgCl_2$, 0.01 M Tris-hydrochloride [pH 7.4], 0.07 M KCl); and 16S buffer (0.001 M $MgCl_2$, 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 minimill. 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 minimill. 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 A_{260} 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 $E_{260}^{1\%} = 145$ (6). Concentrations of the 16S core particles were determined by using an extinction coefficient of $E_{260}^{1\%} = 208$. Concentrations of rRNA solutions were determined spectrophotometrically by using $E_{260}^{1\%} = 223$ (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 $s_{20, w}^0$ 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.
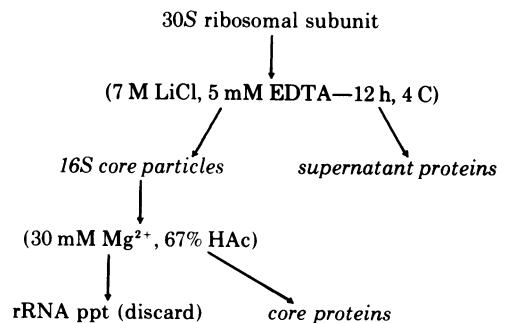
30S ribosomal subunit
↓
(7 M LiCl, 5 mM EDTA—12 h, 4 C)
↙ ↘
16S core particles   *supernatant proteins*
↓
(30 mM Mg^{2+}, 67% HAc)
↓ ↘
rRNA ppt (discard)   *core proteins*

FIG. 1. *Flow diagram for isolation of 16S core particles, supernatant proteins and core proteins. Abbreviations: HAc, acetic acid; ppt, precipitate.*
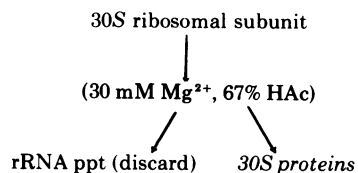
30S ribosomal subunit
↓
(30 mM Mg^{2+}, 67% HAc)
↙ ↘
rRNA ppt (discard)   *30S proteins*

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 µg/ml) in a volume of 100 to 300 µliters were layered onto the stacking gel and run at 1.5 mA/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 destained 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% (ST_{50}) was calculated from the results of skin tests obtained with various antigens. A ST_{50} value is defined as that 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* protoplasm. 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 minimill 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
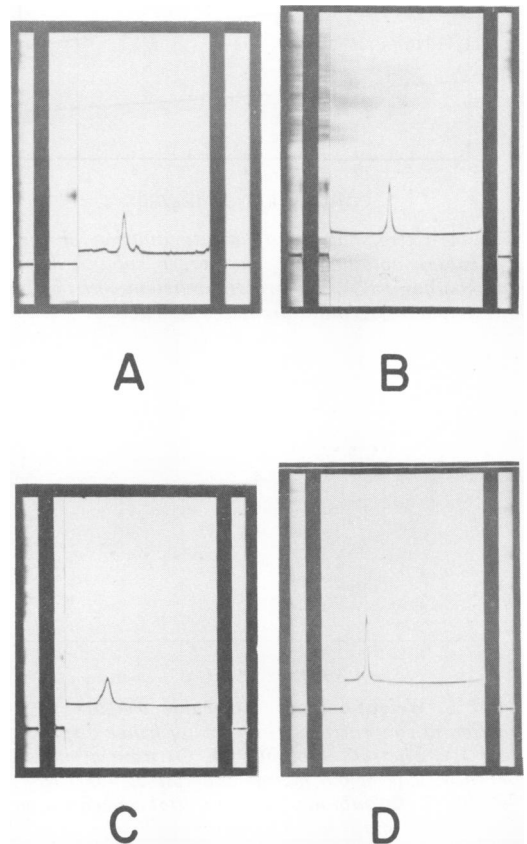

A   B

C   D

FIG. 3. *Schlieren patterns of M. smegmatis ribosomes, subunits, and core particles obtained from sedimentation velocity studies of these particles: (A) crude 70S ribosomes, (B) 50S ribosomal subunits, (C) 30S ribosomal subunits, and (D) 16S core particles, all in their respective buffers. Sedimentation is from left to right. All patterns were taken 12 min after reaching speed by using a 75° phase-plate angle. Pattern A was run at a speed of 52,000 rpm, and the rest were run at 60,000 rpm. Temperature was 4 C.*

TABLE 1. *Composition and physical characteristics of ribosomal particles from M. smegmatis*

Particle	$s_{20, w}^0$ (Svedberg units)	Mol wt	Protein (%)	RNA (%)	Protein/ RNA
Large subunit	48.7 ± 0.5	1.65×10^{6a}	36 ± 2	64 ± 2	0.56
Small subunit	28.1 ± 0.5	7.8×10^5	37 ± 2	62 ± 2	0.60
16S core particle	15.7 ± 0.7		11 ± 2	89 ± 2	0.12

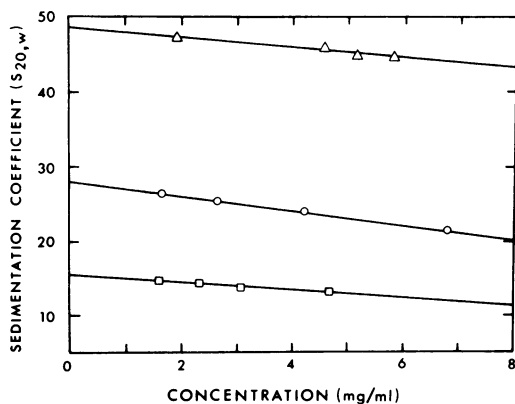[a] This value may be slightly high due to some dimerization and possibly some membranous contamination.

FIG. 4. *Extrapolation to infinite dilution of the $s_{20, w}^0$ values obtained for the larger subunit (△), smaller subunit (○), and protein-deficient core particle (□). The $s_{20, w}^0$ values are noted in Table 1.*

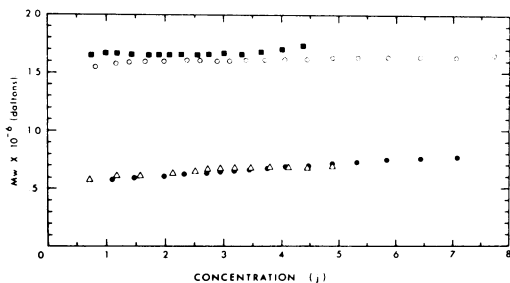FIG. 5. *Weight-average molecular weights as a function of concentration in terms of fringe displacement (j). The 49S subunits (■, ○) were at initial concentrations of 0.5 mg/ml and run at 4,000 rpm. The 28S (△, ●) subunits were at initial concentration of 0.5 mg/ml and run at 6,000 rpm.*

FIG. 6. *Polyacrylamide gel electrophoresis patterns of the 28S subunit proteins (center), the supernatant proteins removed from the 16S core particle (right), and the core proteins associated with the 16S core particle (left). Origin of protein 15a is undetermined.*

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
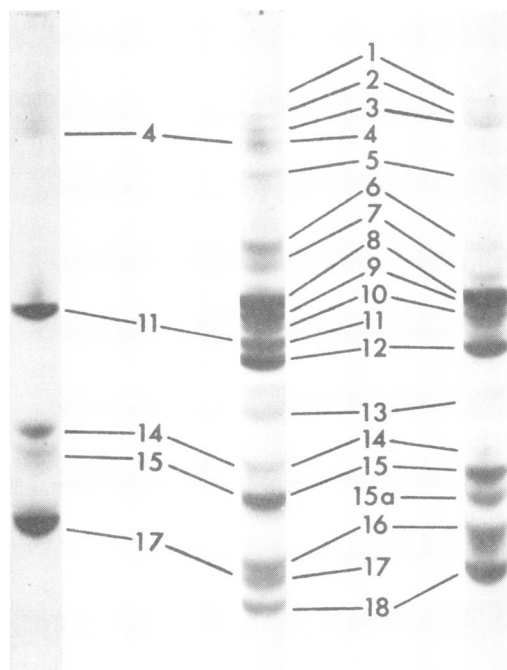
were also found in the supernatant protein fraction. Proteins 4, 11, and 17 were unique core proteins.

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-

cific 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 ST_{50} 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 2. *Tabulation of results of skin tests on seven groups of guinea pigs tested with the various ribosome-derived antigens*

Expt no.	Antigen[a]	Amount of antigen (μg; dry weight) provoking a 24-h reaction in guinea pigs sensitized with:									
		M. smegmatis					*M. bovis*				
		0.50	0.25	0.125	0.062	0.031	0.50	0.25	0.125	0.062	0.031
1	30S ribosomes	6/6[b]	6/6	4/6	1/6	0/6	4/6	3/6	2/6	0/6	0/6
	50S ribosomes	6/6	6/6	3/6	0/6	0/6	6/6	4/6	4/6	1/6	0/6
	Protoplasm	6/6	6/6	6/6	3/6	0/6	6/6	6/6	6/6	4/6	0/6
	BCG protoplasm	ND[c]	ND	ND	ND	ND	6/6	6/6	6/6	4/6	1/6
2	16S core particle	6/6	6/6	5/6	1/6	0/6	3/6	1/6	0/6	0/6	0/6
	Supernatant proteins	2/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	Protoplasm	6/6	6/6	5/6	0/6	0/6	6/6	6/6	4/6	0/6	0/6
	BCG protoplasm	4/6	0/6	0/6	0/6	0/6	6/6	6/6	6/6	5/6	0/6
3	Ribosomal RNA	NR[d]	NR	NR	NR	NR	NR	NR	NR	NR	NR
	16S core particles	6/6	6/6	3/6	1/6	0/6	2/6	0/6	0/6	0/6	0/6
	16S core proteins	6/6	6/6	6/6	2/6	0/6	3/6	1/6	0/6	0/6	0/6
	Protoplasm	6/6	6/6	5/6	1/6	1/6	4/6	4/6	3/6	1/6	0/6
	BCG protoplasm	4/6	2/6	0/6	0/6	0/6	6/6	6/6	4/6	3/6	2/6
4	30S ribosomes	4/5	1/5	0/5	0/5	0/5	ND	ND	ND	ND	ND
	30S proteins	4/5	2/5	0/5	0/5	0/5	ND	ND	ND	ND	ND
	16S core particles	4/5	2/5	0/5	0/5	0/5	ND	ND	ND	ND	ND
	16S core proteins	5/5	4/5	2/5	1/5	0/5	ND	ND	ND	ND	ND

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

[b] Numerator, number of animals positive; denominator, number of animals tested.

[c] ND, Not done.

[d] NR, No reaction.

TABLE 3. ST_{50} values for the various ribosomal antigens

Expt no.	Antigen[a]	ST_{50} units[b] in guinea pigs sensitized with:	
		M. smegmatis	BCG
1	30S ribosomes	3.3[c]	8.0
	50S ribosomes	4.0	4.0
	Protoplasm	2.0	4.0
	BCG protoplasm	ND[d]	1.5
2	16S core particles	3.3	16
	Supernatant proteins	>16	>16
	Protoplasm	3.2	3.5
	BCG protoplasm	14.0	1.6
3	Ribosomal RNA	no reaction	no reaction
	16S core particles	4.0	>16
	16S core proteins	2.5	16.0
	Protoplasm	3.0	4.0
	BCG protoplasm	12.0	2.0
4	30S ribosomes	12.0	ND
	30S proteins	10.0	ND
	16S core particles	10.0	ND
	16S core proteins	5.0	ND

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

[b] One unit = 0.031 µg of antigen.

[c] 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 teated with this dose. This is an interpolated value from the tabulated results.

[d] ND, No data.

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 physi-cally 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 $s^o_{20,w}$ 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 $s^o_{20,w}$ 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 1.5×10^6 rather than 1.65×10^6 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 1.65×10^6.

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

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. smegmatis* are similar to those reported by Itoh et al. (8) for the 16S-stripped *E. coli* subunits.

Electrophoresis patterns of the proteins of *E. coli* and *M. smegmatis* 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. smegmatis* 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 extracts, 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 tuberculin-active peptides (16) which have defined purity and specificity. However, both the high degree of specificity and the clear definition of the source and nature of the core proteins are novel aspects in the study of delayed hypersensitivity due to mycobacteria.

Protoplasm, described by Larson et al. (11-13) and Counts and Kubica (3), is capable of provoking skin reactions in sensitized experimental animals of equal or greater specificity than homologous PPD. Baker et al. (1) further demonstrated that the 30S subunits from mycobacterial ribosomes were capable of provoking delayed skin reactions with specificity somewhat 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 increased 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 antigens 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. smegmatis* 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 species. 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 serological studies are needed to confirm this observation.

Since protein removal is effected by disruption of the electrostatic binding interactions between the ribosomal protein and RNA, it has been suggested that the protein-RNA interaction 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 environment.

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 ribosomal 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.

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