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Isolation and Partial Characterization of an Immunogenic Moiety Obtained from *Salmonella typhimurium*

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Ribosomal preparations obtained from *Salmonella typhimurium* by differential centrifugation and sodium dodecyl sulfate (SDS) treatment of the bacillary lysate were found to be immunogenic in F_1 hybrid (C_3H/HeJ × DBA/2J) and albino Swiss mice, as determined by progressive host survival. The immunity obtained was independent of the need for adjuvant and dependent on the dosage of immunogen given. Immunizations with the ribosomal preparations induced an immune response comparable to that obtained by vaccination with living organisms and significantly greater than that obtained by immunization with heat-killed salmonellae, purified lipopolysaccharide, or crude and SDS-treated endotoxin preparations. No effect on the immunogenicity of the ribosomal fraction was observed by enzymatic treatment with trypsin, Pronase, deoxyribonuclease, and pancreatic ribonuclease. Linear sucrose density gradient resolution of the preparations showed that the immunogenicity of the ribosomal fraction was not unique to any one of its subcomponents. Ethyl alcohol-precipitated, crude ribonucleic acid preparations obtained from the ribosomal and sucrose density-resolved ribosomal preparations were found to induce an immune response comparable to that obtained by immunization with the entire ribosomal fraction. Dialysis in doubly distilled demineralized water slightly reduced the immunogenicity of the preparation; however, comparable dialysis in 10^{-4} M $MgCl_2$-phosphate buffer did not. Chemical assays of the preparations found to be immunogenic were performed.

It is well documented that living vaccines produce a more effective protective immunity in mice to challenge infection with the homologous organism than that obtained by immunization with heat-killed or chemically altered salmonellae (5-9, 13, 14). The resultant immune response has been attributed to a unique bacteriostatic property(s) inherent in the cellular elements of the immune host and is devoid of classical antibody intervention (2, 12, 17, 22, 27). However, much controversy still exists over whether some molecular component of the virulent salmonellae has the potential to induce a protective immune response comparable to that obtained by immunization with the living virulent or attenuated organisms.

Auzins and Rowley (1) reported the presence of a heat-labile surface antigen of virulent *Salmonella typhimurium* which was capable of provoking an immune response in mice, demonstrable as an increased rate of phagocytosis by mouse peritoneal macrophages. Subsequently, Jenkin and Rowley (18) isolated a crude immunogenic moiety from three strains of salmonellae by sodium dodecyl sulfate (SDS) treatment and ethyl alcohol precipitations of the bacillary mass. The heat-labile "antigen" consisted of approximately 40% protein and appeared to be associated with the polysaccharide portion of the "O" somatic antigen. In contrast, Collins and Milne (9) were unable to find any significant protection after immunization with SDS- or sodium deoxycholate-prepared cell wall extracts of virulent *S. enteritidis* when inhibition of bacterial growth in vivo was used as the criteria for "protective" immunity. Similarly, when host survival was employed as the criterion for protection, the extracts of *S. enteritidis* were only slightly, if at all, immunogenic and not nearly as effective as were immunizations with the living attenuated or virulent organisms.

In the present investigation, subcellular extracts

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of *S. typhimurium*, prepared by chemical treatment and differential centrifugation of the bacillary lysate, were examined for their ability to stimulate protective immunity in mice to challenge infection by the homologous bacilli. This study clearly shows the ability of ribosomal extracts and crude ribonucleic acid (RNA) preparations of *S. typhimurium* to be effective as protective immunogens as measured by host survival.

MATERIALS AND METHODS

Organisms. *S. typhimurium* strains 208-050 (from the Ohio State University culture collection), RIA, and Sr-11 (the latter two kindly supplied by L. Joe Berry, Bryn Mawr College, Bryn Mawr, Pa.) were used throughout this investigation. Cultures in logarithmic growth, determined spectrophotometrically at optical density $(OD)_{550}$ (20), were used in all experiments unless otherwise indicated.

Experimental animals. F_1 hybrid [C_3H/HeJ × DBA/2J (C_3H/DBA)] mice were purchased from Jackson Laboratories, Bar Harbor, Me., and albino Swiss mice were purchased from Harlan Industries, Cumberland, Ind. Young adults weighing from 18 to 25 g were used throughout this investigation.

At 30 days postinfection (the time beyond which no more deaths, through 3 months of observation, were recorded), the LD_{50} values for SR-11, 208-050, and RIA, determined intraperitoneally (ip) in Swiss mice, were 7×10^2, 10^4, and 5×10^7 bacteria, respectively, as determined by the method of Reed and Muench (26). The LD_{50} value for the 208-050 strain in the C_3H/DBA hybrid mice at 30 days postinfection was 10^5 bacteria. The survival data are reported at 30 days postchallenge. Percentage fractions were calculated as significant to the nearest whole per cent. The significance of differences in the survival data was determined by the chi square test (3).

Preparation of bacterial cultures for extraction. Five-liter culture bottles containing 400 ml of Brain Heart Infusion (BHI) agar were inoculated with 10 ml of a logarithmic-phase broth culture of *S. typhimurium* (SR-11 or 208-050) and incubated at 37 C for 8 to 10 hr. The bacteria were harvested and washed three times in 40 ml of cold 0.01 M phosphate buffer, *p*H 7.1 at 4 C, and finally suspended in 3 volumes (v/w) of cold sucrose buffer (30) containing 0.44 M sucrose, 3×10^{-2} M $MgCl_2$, 0.25% (w/v) SDS, and 2×10^{-4} M sodium-phosphate buffer, *p*H 7.1 at 4 C. The resultant suspension was immediately used or stored at −70 C and thawed by overnight refrigeration.

Preparation of the ribosomal fraction. The procedure followed was basically that of Youmans and Youmans (34, 35) with slight modification. The bacterial suspension was homogenized for 30 sec in an ice-jacketed micro-Waring Blendor. The cellular homogenate was ruptured in 40-ml samples in a cold French pressure cell at 10,000 through 14,000 psi. The ruptured mass was collected in 25 ml of a sucrose buffer ice slush and centrifuged for 10 min at 27,000 × *g* in a Sorvall (model RC-2B) refrigerated centrifuge to remove intact bacterial cells and cellular debris. The supernatant fluid was decanted and recentrifuged at 45,000 × *g* to remove any residual cellular debris remaining from the first centrifugation. The upper four-fifths of the supernatant fluid was carefully decanted and saved for ultracentrifugation.

Gram stains and plate cultures were made after the 45,000 × *g* centrifugation to determine the presence of bacterial contaminants in the supernatant fluid. No viable *S. typhimurium* was noticeable either by microscopic observation or by the plate culture technique.

The supernatant fluid from the 45,000 × *g* centrifugation was centrifuged at 105,000 × *g* for 2 to 3 hr in a Beckman (model L) ultracentrifuge. The yellow-orange pellet obtained from this centrifugation, designated the particulate fraction (33), was then suspended in chloride buffer containing 10^{-4} M $MgCl_2$ and 0.01 M phosphate buffer, *p*H 7.1 at 4 C, with a Teflon pestle and adjusted to a concentration of 20 mg/ml (wet weight). Samples of this suspension were set aside for immunizations and sucrose density gradient centrifugation for eventual separation of the ribosomal subfractions (32). To the remainder of the suspension we added an equal volume of 1% (w/v) SDS in 10^{-4} M $MgCl_2$, 0.01 M phosphate buffer, and the mixture was kept at room temperature for 1 hr under constant agitation, followed by refrigeration overnight at 4 C. The precipitated SDS (35) was subsequently sedimented out of solution by centrifugation for 15 min at 37,000 × *g*. The upper two-thirds of the supernatant fluid was carefully decanted and centrifuged at 105,000 × *g* for 2 to 4 hr. The clear, transparent pellet obtained from this centrifugation, presumed to be the ribosomal fraction, was suspended in chloride buffer to the desired concentration.

To insure both homogeneity and sterility, the preparation was gently homogenized in a Dounce ball-type hand homogenizer and subsequently filtered through a membrane filter (Millipore Corp., Bedford, Mass.; Swinny adapter, type HA, 0.45 μm grid). Filtering the preparation in this way did not interfere with either the immunogenicity of, or the ribosomal concentration in, the colloidal suspension. Ribosomal preparations prepared in this manner were either used immediately or stored at 4 C until use. No decrease in the immunizing potential of the preparations, either by freezing at −20 C and thawing one time in 4 days or by refrigeration for 3 days, was observed.

Bacterial endotoxin and lipopolysaccharide (LPS) preparations. Purified LPS was obtained from Difco and was suspended in pyrogen-free, isotonic saline. Crude endotoxin preparations from *S. typhimurium* SR-11 and 208-050 were obtained by steaming for 15 min a pyrogen-free, saline-washed, 18-hr BHI broth culture of each strain. Endotoxin preparations were also obtained by pasteurization of the virulent organism as described elsewhere (24, 25). To obtain detergent-treated preparations, the crude endotoxin was treated with 0.5% SDS for 1 hr at room temperature, stored overnight at 4 C, and centrifuged at 23,000 × *g* for 10 min to sediment out the precipitated SDS. The supernatant fluid from this centrifuga-

tion was employed in the immunizations as the sham-treated endotoxin. Since there was no significant difference in the results obtained by immunization with the autoclaved or pasteurized endotoxin preparations, the results were combined.

Enzymatic treatments. Bovine pancreatic trypsin (salt-free, A grade) and Pronase (B grade) were obtained from Calbiochem. Both enzymes were added in crystalline form in concentration ratios of 0.05 mg of enzyme per mg of ribosomal preparation suspended in chloride buffer. Deoxyribonuclease (2× crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) was added in crystalline form in the ratio of 0.01 mg per mg (wet weight) of ribosomal material in chloride buffer. Bovine pancreatic ribonuclease (5× crystallized, Nutritional Biochemicals Corp.) was added in crystalline form to the ribosomal material suspended in 0.01 M phosphate buffer, in the ratio of 0.05 mg of enzyme per mg of preparation. All preparations were kept for 12 hr at 4 C, followed by 30 min of incubation at 37 C.

Rate-zonal and analytical ultracentrifugations. Ribosomes were resolved into their subcomponent parts on 5 to 20% linear sucrose density gradients containing 10^{-4} M $MgCl_2$ in 0.01 M phosphate buffer, *p*H 7.1 at 4 C, for 6.5 hr at 25,000 rev/min in a Beckman (model L_2, rotor 25:3) temperature-controlled ultracentrifuge (32). Fractions were collected dropwise from the bottom of the tube in 0.4-ml quantities, and the OD_{260} of the fractions was determined on a Beckman DU spectrophotometer. Since known markers or radio-labeled ribosomes were not employed in the gradients to minimize contamination of the resolved subfractions, analytical ultracentrifugations (Beckman model E analytical ultracentrifuge, Schlieren optics, 48,000 rev/min) were carried out on the preparations employed in the charging of the density gradients, and the mean *S* values from three trials were determined by the slope method of Schachman (28). *S* values are reported at 20 C in 0.01 M phosphate buffer, *p*H 7.17 at 24 C, and are approximations of $S_{20,w}$. By mathematical comparison (23), OD_{260} peaks obtained in the rate-zonal centrifugation of the particulate preparation from which the ribosomal subunits were obtained (32) were designated with their respective *S* values.

Immunizations. All mice were immunized subcutaneously (sc) with 0.1 ml of the vaccine preparations and were challenged ip 15 days postimmunization with the homologous bacilli suspended in 0.1 ml of sterile saline, unless otherwise specified. Where indicated, the subcellular fractions were incorporated into Freund's incomplete adjuvant (Difco) in a 3 to 1 ratio (three parts adjuvant to one part preparation) and injected into the mice in 0.4-ml quantities.

Chemical assay. Total nucleic acid (NA) concentrations were estimated by the procedure of Warburg and Christian (31). The adsorption of all preparations at 235, 260, and 280 nm was determined from wavelength spectra (210 to 300 nm) of the fractions on a Shimadzu MPS-501 recording spectrophotometer and was reported as 260:235 and 260:280 ratios (15, 19). Protein concentration was determined by the procedure of Lowry et al. (21), employing bovine serum albumin (Fraction V; Pentex Inc., Kankakee, Ill., in the preparation of a standard curve. RNA concentrations were determined by the Orcinol method (29), and deoxyribonucleic acid (DNA) concentrations were determined by the diphenylamine assay (10). Liver extract, soluble RNA (Nutritional Biochemicals), and pancreatic DNA (Nutritional Biochemicals) were employed as standards.

RESULTS

Definition of subcellular immunogen. In three separate attempts to immunize F_1 hybrid mice with prefiltered portions of the particulate preparations obtained from the first 105,000 × *g* centrifugation, the preparations proved lethal for 95% of the mice within 48 hr postinjection. Lethality was attributed to endotoxin associated with the membranous elements in the preparation.

To obtain a suitable immunizing preparation from the subcellular components, the particulate fraction was treated with 0.5% SDS for 1 hr at 25 C. Portions (0.1 ml) of the resultant ribosomal preparation incorporated into 0.3 ml of Freund's incomplete adjuvant were injected into hybrid mice either sc or ip. Control mice were similarly injected sc or ip with 0.3 ml of adjuvant mixed in 0.1 ml of chloride buffer. At 15 days postimmunization, representative numbers of each group of treated animals were infected with 0.5 LD_{50} of the moderately virulent *S. typhimurium* 208-050. Mice immunized ip were challenged intravenously (iv) and mice immunized sc were challenged ip to avoid introducing the challenge organism into an area previously irritated by injection. The results of two such experiments are pooled and presented in Table 1. Protective immunity, as indicated by survival at day 30 ($P < 0.001$), was obtained by immunization with the ribosomal preparation. Freund's incomplete adjuvant appeared to have little significant effect on the normal animals when injected either sc or ip ($P > 0.05$).

TABLE 1. *Ribosomal immunization[a] to challenge with 0.5 LD_{50} of S. typhimurium 208-050*

Conditions	Per cent survival (no./total) 30 days postchallenge
Immunization ip; iv challenge...	100 (20/20)[b]
Immunization sc; ip challenge...	100 (20/20)[b]
Adjuvant controls[c].....	22 (4/18)[d]
Uninoculated controls.....	17 (4/24)

[a] Immunization dose: 0.2 mg (dry weight).
[b] $P < 0.001$.
[c] Combined results obtained from mice immunized ip and sc and challenged iv and ip, respectively.
[d] $P < 0.05$.

Since there was no significant difference ($P > 0.2$) in the degree of survival obtained when mice were immunized sc or ip and challenged ip or iv, immunization and challenge routes, unless otherwise indicated, were standardized to sc and ip, respectively.

To assay the degree of immunity obtainable by immunization with the ribosomal preparations, F_1 hybrid and Swiss mice were immunized with 0.1, 0.01, and 0.001 mg (dry weight) of the preparation incorporated into adjuvant. Mice immunized sc with 2.5×10^5 heat-killed RIA (prepared by boiling for 30 min, washing two times, and resuspending in isotonic saline) and both ip and sc with 10^2 live 208-050 were included in determinations of the efficacy of the ribosomal immunogen in comparison to heat-killed and living vaccines. At 15 days postimmunization, each group of treated animals was challenged with 10^4 LD_{50} of virulent SR-11. Since the results obtained from both the hybrid and Swiss mice were comparable, they were combined and the data from four such experiments are pooled and presented in Table 2. The degree of immunity obtained with 0.001 mg of the ribosomal material was comparable to that obtained by immunization with the live vaccine ($P > 0.2$) and far greater than that obtained by immunization with the heat-killed bacteria.

Adjuvant independence and dose response of the ribosomal immunogen. To examine the possibility that the ribosomal preparations are immunogenic when not incorporated into adjuvant, Swiss mice were immunized with various quantities of the ribosomal material incorporated into either Freund's incomplete adjuvant or in 0.2 ml of 10^{-4} M $MgCl_2$-phosphate buffer. At 15 days postimmunization, all mice were challenged with 10^4 LD_{50} of virulent SR-11. The data from three such experiments are pooled and presented in Table 3. The immunity obtained was directly dependent on the dosage of immunogen administered and was independent, with 0.001-mg (dry weight) immunizing doses, of the need for adjuvant ($P < 0.05 > 0.01$). Adjuvant, however, appeared to increase the efficacy of the immunizing preparation slightly, as is seen by the difference between the 0.001-mg dose without adjuvant and the 0.0001-mg dose incorporated in adjuvant.

Challenge dependency of ribosomal immunizations. Swiss mice immunized with 1.0-, 0.1-, 0.01-, and 0.001-mg (dry weight) portions of the ribosomal preparation were challenged 15 days postimmunization with 10, 10^3, 10^4, 10^5, and 10^6 LD_{50} of virulent SR-11. Mice immunized with a living vaccine, 4×10^5 RIA, were included at 10, 10^3, and 10^4 LD_{50} challenge dose levels for means of comparison. The survival at 30 days for each of the challenge doses greater than and including 10^4 LD_{50} is presented in Table 4. Since there were no significant differences observed at 10 and 10^3 LD_{50} challenge doses among any of the immunizing preparations, these values are not included in

TABLE 2. *Comparative immunogenicity of heat-killed and living S. typhimurium to ribosomal immunizations in F_1 hybrid and Swiss mice*[a]

Prepn	Amt injected (mg. dry wt)	Per cent survival (no./total) 30 days postchallenge
Live immunization (208-050)		
Immunization sc; ip challenge	10^2 [b]	100 (20/20)
Immunization ip; iv challenge	10^2 [b]	100 (17/17)
Heat-killed RIA[c]	2.5×10^5 [b]	30 (6/20)[d]
Ribosomal prepn + adjuvant	0.1	100 (11/11)
	0.01	100 (21/21)
	0.001	100 (15/15)[e]
		33 (4/12)[d]
Adjuvant controls[f]		
Unimmunized controls		17 (5/30)

[a] Challenge dose: 10,000 LD_{50} of SR-11.
[b] Bacteria.
[c] Immunized sc; challenged ip.
[d] $P > 0.05$.
[e] $P < 0.001$.
[f] Immunized sc with 0.3 ml of Freund's incomplete adjuvant + 0.1 ml of 10^{-4} M $MgCl_2$ buffer.

TABLE 3. *Adjuvant independence and dose response exhibited by ribosomal immunizations in Swiss mice*[a]

Treatment	Amt injected (mg, dry wt)	Per cent survival (no./total) 30 days postchallenge
Ribosomal prepn + adjuvant	5.0	100 (20/20)
	0.1	100 (11/11)
	0.01	100 (11/11)
	0.001	74 (17/23)
	0.0001	55 (11/20)[b]
	0.0001	45 (9/20)[c]
Ribosomal prepn + buffer	0.1	100 (20/20)
	0.01	87 (27/31)
	0.001	61 (8/13)[b]
		0 (0/6)
Adjuvant control		
Uninoculated control		26 (5/19)

[a] Challenge dose: 10,000 LD_{50} of SR-11.
[b] $P < 0.05 > 0.01$.
[c] $P > 0.05$.

TABLE 4. *Challenge dependency on the degree of immunity obtained by ribosomal immunization in Swiss mice*[a]

Prepn	Amt injected (mg, dry wt)	Challenge dose (LD_{50})[b]		
		10^4	10^5	10^6
RIA live immunization[c]	4×10^{5}[d]	100 (20/20)	—[e]	—[e]
Ribosomal immunization	0.1	100 (20/20)	100 (5/5)	—[e]
	0.1	87 (27/31)	83 (9/12)	0 (0/6)
	0.001	61 (7/12)	50 (4/8)[f]	0 (0/6)
Uninoculated controls		0 (0/12)	0 (0/7)	0 (0/10)

[a] Challenged with virulent SR-11.
[b] Results expressed as per cent survival (no./total) at 30 days postchallenge.
[c] Immunized sc; challenged ip.
[d] Bacteria.
[e] Not determined.
[f] $P < 0.05 > 0.01$.

the tabular data. As can be seen, resistance to 10^5 LD_{50} was demonstrable at all three immunization levels ($P < 0.05 > 0.01$). Since live immunized standards were not challenged beyond 10^4 LD_{50}, no comparative resistance was determined.

Enzymatic treatment of the ribosomal immunogen. In an attempt to partially define the immunogenic moiety, the ribosomal preparations were treated with trypsin, Pronase, ribonuclease, and deoxyribonuclease for 12 hr at 4 C and for 30 min at 37 C to assure complete degradation of the respective macromolecular components. Swiss mice were immunized with 0.1 and 0.01 mg of the respective preparations. Sham-treated ribosomal preparations and enzyme-buffer controls were also included to determine the effect of the treatment on the immunogenic capacity of the preparation and to observe whether any immune response is attributable to the enzyme and not to the treated preparations. At 15 days postimmunization, all treated animals were challenged with 5×10^5 LD_{50} of virulent SR-11. The results of two such experiments were pooled and are presented in Table 5. Since there was no significant difference found between the enzyme-buffer and the uninoculated controls, the results were combined. As is shown, little, if any, significant decrease in the efficacy of the ribosomal vaccine was observed by treatment with any of the four enzymes when 0.1-mg immunizing doses were employed ($P < 0.001$). The lack of any significant immune response upon immunization with the 0.01-mg dose may be attributed to the massive challenge employed in this assay. Furthermore, the immunogen appeared to be stable to the treatment at 37 C for 30 min, as was shown by the survival of the sham-treated immunized mice ($P < 0.001$).

TABLE 5. *Enzymatic treatment of the ribosomal immunogen obtained from S. typhimurium*[a]

Treatment	Amt injected (mg, dry wt)	Per cent survival (no./total) 30 days postchallenge
Untreated	0.1	100 (11/11)
	0.01	8 (1/12)
Sham-treated[b]	0.1	77 (7/9)[c]
	0.01	8 (1/12)[d]
Ribonuclease	0.1	86 (6/7)[c]
	0.01	25 (3/12)[d]
Deoxyribonuclease	0.1	100 (8/8)[c]
	0.01	44 (5/12)[d]
Pronase	0.1	100 (11/11)[c]
	0.01	50 (6/12)[d]
Trypsin	0.1	66 (8/12)[c]
	0.01	9 (1/11)[d]
Normal controls[e]		18 (7/40)

[a] Challenge with 5×10^5 virulent SR-11.
[b] Treated overnight at 4 C and for 30 min at 37 C.
[c] $P = 0.001$; deviation significance from enzyme buffer controls.
[d] $P = 0.05$; deviation significance from enzyme-buffer controls.
[e] Represents combined values for enzyme-buffer and uninoculated controls.

Comparison of the immunogenicity of ribosome vaccines with endotoxin. In view of reports concerning the immunogenicity of detergent-treated endotoxin in infections of *Escherichia coli* (16) and *S. typhimurium* (30), and the proposed "O" antigen association of the protective antigen reported by Jenkin and Rowley (18), it was imperative that the immunogenic effects of endotoxin be assayed and compared with the ribosomal immunogen. Swiss mice were immunized with purified LPS, untreated endotoxin preparations, and

SDS-treated crude endotoxin preparations in amounts comparable to those previously found immunogenic with the ribosomal preparations. All mice were challenged 15 days postimmunization with 1,000 LD_{50} of the virulent SR-11. The results from two such experiments were pooled and are presented in Table 6. No significant protection was afforded by endotoxin immunizations ($P > 0.05$) in contrast to the highly significant protection obtained by immunization with the ribosomal preparation ($P < 0.001$).

Dialysis. Ribosomal preparations were dialyzed at 42 C against either 600 volumes of 10^{-4} M $MgCl_2$-phosphate buffer for 24 hr or against doubly distilled demineralized water for 48 hr. Swiss mice were subsequently immunized with various amounts of the dialysates and were challenged with 100 LD_{50} of SR-11. The results of two such experiments were pooled and are presented in Table 7. Although no significant effect on the immunogenicity of the preparation was observed with 0.1- or 0.01-mg doses dialyzed in chloride buffer, a significant reduction in immunity was apparent by immunization with 0.1 mg of the preparation dialyzed in doubly distilled demineralized water. It appears, therefore, that the immunogen is dialyzable. However, further studies have indicated that the partial reduction in immunogenicity is due to degradation of the preparation and not to dialyzing out the specific immunogenic moiety (*unpublished data*).

Resolution on sucrose density gradients. Ribosomes were resolved into their subcomponent parts on 5 to 20 per cent linear sucrose gradients to determine whether the immunogen is unique

TABLE 6. *Ribosomal immunogen compared to endotoxin immunizations*[a]

Immunization prepn	Amt injected (mg, dry wt)	Per cent survival (no./total) 30 days postchallenge
Ribosomal prepn	0.01	80 (8/10)
	0.001	100 (13/13)
Commercial LPS	0.05	33 (2/6)[b]
	0.005	17 (1/6)
Crude endotoxin (Sr-11)	0.05	10 (1/10)
	0.005	20 (2/10)[b]
	0.0005	10 (1/10)
SDS-treated crude endotoxin	0.05	10 (1/10)
	0.005	20 (2/10)[b]
	0.0005	20 (2/10)
Unimmunized controls		13 (2/15)

[a] Challenge dose: 1,000 LD_{50} of SR-11.
[b] $P > 0.05$.

TABLE 7. *Effect of dialysis on the immunogenicity of the ribosomal preparations obtained from S. typhimurium*[a]

Treatment	Amt injected (mg)	Per cent survival (no./total) 30 days postchallenge
Untreated ribosomes	0.1	100 (10/10)
	0.01	100 (18/18)
Dialyzed in $MgCl_2$ (10^{-4} M) for 24 hr	0.1	100 (9/9)
	0.01	75 (6/8)[b]
Dialyzed in doubly distilled demineralized water for 48 hr	0.1	50 (4/8)[c]
Untreated controls		10 (1/10)

[a] Challenge dose: 100 LD_{50} of SR-11.
[b] $P > 0.05$; deviation significance from untreated ribosomes. $P < 0.01 > 0.001$; deviation significance from untreated controls.
[c] $P < 0.05 > 0.01$; deviation significance from both ribosomal immunized and untreated controls.

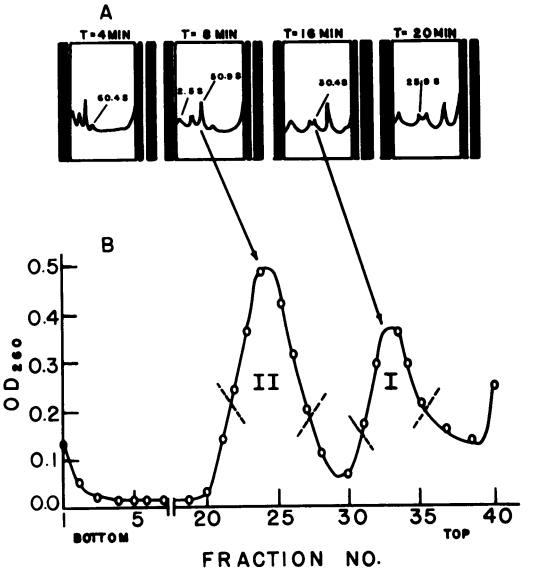

FIG. 1. (*A*) *Diagrammatic reproduction of sedimentation velocity analysis of the particulate preparation employed in the separation of ribosomal subunits through rate-zonal centrifugation. S values are reported for the respective boundaries at 20 C in 10^{-4} M $MgCl_2$-0.01 M sodium-phosphate buffer, pH 7.1 at 4 C.* (*B*) *Resolution of the ribosomal immunogen on 5 to 20% linear sucrose density gradients. Arrows indicate corresponding peaks as determined by sedimentation velocity analysis. Dotted lines outline the boundary of the fractions pooled for crude RNA preparations.*

to one particular component. Analytical ultracentrifugations were done on the particulate preparation used to charge the gradients (32), the *S* values were determined, and the OD_{260} peaks were observed in the gradient fractions designated accordingly (Fig. 1A and B). NA concentrations in each of the respective gradient fractions were determined by spectrophotometric assay at 260 nm (11; 1 OD_{260} unit = 50μg of NA), and Swiss mice were immunized with various NA equivalents from fractions collected at the top of each of the respective gradient peaks (Fig. 1B). At 15 days postimmunization, all animals were challenged with 10 LD_{50} of virulent SR-11. The results of two such experiments were pooled and are presented in Table 8. Both the 50*S* (approximately) and 30*S* (approximately) subunit fractions, individually and in combination, maintained some immunogenic activity comparable to that obtained with 0.001 mg of NA equivalence of the ribosomal preparation. Furthermore, the immunogen appeared to remain localized with the ribosomal subunits, as indicated by the inability of the gradient controls to induce significant immunity to challenge infection with the virulent salmonellae ($P > 0.05$).

Crude NA preparations (36) were obtained from each of the pooled subfraction peaks (Fig. 1B) and from the entire ribosomal preparation by precipitation of the NA in 66% ethyl alcohol and 0.01 M NaCl (32) for 4 hr at −20 C. After collection of the precipitates by centrifugation at 15,000 × *g* for 10 min at 4 C and resuspension of the sediment in 0.01 M phosphate buffer (*p*H 7.1 at 4 C), Swiss mice were immunized with various NA equivalents from each of the pooled fractions and in combination. The results from two such experiments are pooled and presented in Table 9. Significant immunity was induced by immunization with 6.5 to 1.2 μg of NA equivalents of the crude preparations ($P < 0.01 > 0.001$), indicating that the specific immunogen is associated with the NA of the ribosomal preparations. Immunogenicity was lost, however, when 0.6 μg of NA equivalents of the respective preparations was administered, further indicating that some of the immunogen was lost during pooling and ethyl alcohol precipitation of the fractions (compare Tables 8 and 9).

TABLE 8. *Immunogenicity of linear sucrose gradient-resolved OD_{260} peaks*[a]

Prepn	Amt injected[b]	Per cent survival (no./total) 30 days postchallenge
Ribosomal prepn	100	100 (15/15)
	10	100 (20/20)
	1	55 (11/20)[f]
Peak I (30*S*)	3.5	84 (14/15)
	0.2	100 (10/10)
Peak II (50*S*)	3.5	100 (15/15)
	0.2	90 (9/10)[d]
Peak I + peak II	3.5	94 (14/15)
	0.2	100 (10/10)
Gradient control[e]		25 (2/8)[f]
Uninoculated controls		20 (4/20)

[a] Challenge dose: 10 LD_{50} of SR-11.
[b] Micrograms of equivalence NA.
[c] $P < 0.05 > 0.01$.
[d] $P < 0.001$.
[e] Gradient control: each mouse received 0.2 ml of a portion of the gradient not absorbing at OD_{260}.
[f] $P > 0.05$.

Chemical assay. The chemical data on the preparations found to be immunogenic are presented in Table 10. The RNA concentration of the crude ethyl alcohol-precipitated NA and the ribosomal preparations varied from 70 to 80% and protein concentrations varied from 15 to 25%. DNA (4% ± 4%) was found in some of the ribosomal fractions but was not detectable in the ethyl alcohol precipitates. In all preparations, there was from 2 to 10% undefined "contaminant" material on a dry weight basis. No correlation was found, however, between the degree of contamination and the immunity obtained at marginal immunizing doses.

DISCUSSION

In contrast to the thesis that only living vaccines can induce a "protective" immunity in experi-

TABLE 9. *Immunization with crude ribosomal-associated RNA obtained from the ribosomal immunogen and from rate-zonal definition of the ribosomal subfractions*[a]

RNA prepn	Amt injected[b]	Per cent survival (no./total) 30 days postchallenge
Crude ribosomal	12	68 (13/19)[c]
	4	84 (16/19)
Peak I	6.5	94 (14/15)[d]
	0.6	40 (4/10)[e]
Peak II	6.5	100 (15/15)[d]
	0.6	20 (2/10)[e]
Peak I + peak II	12.6	94 (14/15)
	1.2	90 (9/10)[d]
Unimmunized controls		20 (2/10)

[a] Challenge dose: 100 LD_{50} of SR-11.
[b] Micrograms equivalence NA.
[c] $P < 0.05 > 0.01$.
[d] $P < 0.01 > 0.001$.
[e] $P > 0.05$.

TABLE 10. *Chemical assay of the immunogenic fractions obtained from S. typhimurium*

Prepn	Protein[a]	RNA[a]	DNA[a]	OD ratio	
				260:280	260:235
Ribosomal fractions (19)[b]	235.5 ± 20.4	692.0 ± 45.0	4.09 ± 4.32	1.890 ± 0.223	1.812 ± 0.302
Ribosomal NA, ethyl-alcohol-precipitated (2)	193.3 ± 14.3	795.2 ± 68.3	ND[d]	1.940 ± 0.321	1.972 ± 0.142
Peak I: 30*S* (4)	0.528 ± 0.015[e]	—[f]	—[f]	1.692 ± 0.012	1.540 ± 0.083
Peak II: 50*S* (4)	1.024 ± 0.231[e]	—[f]	—[f]	1.462 ± 0.120	1.363 ± 0.212

[a] Expressed as micrograms of material per milligram (dry weight) of preparation.
[b] Reported as mean average of number of preparations in parentheses.
[c] Standard deviation.
[d] Not detectable.
[e] Expressed as micrograms per microgram of NA.
[f] No determined.

mental salmonellosis (9) and in conjunction with the work of Youmans and Youmans (33–36), who showed that ribosomal and RNA preparations from pathogenic *Mycobacteria* can act as protective immunogens in experimental tuberculosis, it has been demonstrated that immunization with ribosomal and crude NA preparations obtained from *S. typhimurium* affords protection in mice to challenge infection with as much as 10^5 LD_{50} of the virulent bacilli. This finding is also supported by the recent work of Eisenstein, Winston, and Berry (Bacteriol. Proc., p. 87, 1968). The immunity obtained by immunization with the ribosomal preparation is dose-dependent with respect to the quantity of immunogen given and independent of the need for adjuvant. Similarly, the degree of immunity obtained at 15 days post-immunization is comparable to that obtained by immunization with living vaccines and greater than that obtained with heat-killed organisms.

As compared to the ribosomal vaccine, purified LPS, crude endotoxin, and detergent-treated endotoxin preparations were not effective in inducing a protective immune response; this finding supports the work of other investigators (5, 6, 25).

Enzymatic treatment of the ribosomal preparations for 12 hr at 4 C, followed by incubation at 37 C for 30 min (36) with trypsin, Pronase, deoxyribonuclease, and ribonuclease, showed that the specific immunogen in the preparation is not susceptible to enzymatic degradation by any of the enzymes in 0.1-mg (dry weight) immunizing doses. Similarly, rate-zonal resolution of the ribosomal preparation into its component fractions further demonstrated that the immunogen is not unique to a specific ribosomal particle but is peculiar to the ribosomal particle per se, as indicated by the lack of detectable immunogenicity found in the gradient controls.

Crude ethyl alcohol-precipitated RNA preparations obtained from both the ribosomal fraction and the subfraction components were shown to be effective immunogens. These results suggest that RNA may be the specific immunogenic moiety in the preparation and seem to contradict the fact that immunogenicity was not destroyed by treatment with pancreatic ribonuclease. However, in view of the limited degradation and cleavage obtained by treatment with pancreatic ribonuclease (36), as well as the quaternary structure of the NA bound in the ribosomal particles making it insensitive to ribonuclease degradation (32), the possibility that RNA is the immunogenic moiety is tenable. However, it is equally plausible that lipid, adsorbed carbohydrate, or both were carried over throughout the extraction procedures and act as the specific immunogen. This concept may be supported by the observation that the ribosomal preparations are immunogenic without adjuvant, suggesting an "adjuvant-like" role for the NA inherent in the preparation (4). At this time, therefore, it is not possible to define the specific moiety in the preparation responsible for the anti-salmonellae immunity, since the fractions did contain extraneous protein and other nondefined "contaminant" material.

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