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Purification of Immunogenically Active Ribonucleic Acid Preparations of *Salmonella typhimurium*: Molecular-Sieve and Anion-Exchange Chromatography[1]

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Immunogenic *Salmonella typhimurium* ribonucleic acid (RNA) preparations, prepared by differential centrifugation, phenol extraction at 65 C, and ethanol precipitation from 0.5% sodium dodecyl sulfate solution, maintained their immunogenicity through lyophilization. As measured by survival, differential pathogen counts 5 days postchallenge, or clearance of the infecting organism from the tissues, immunization with 50 μg (dry weight) of the lyophilized preparation proved as effective as immunization with 0.1 LD_{50} of attenuated *S. typhimurium* cells. Chromatography of the immunogenic fraction through Biogel P-6 (exclusion limit > 4,600) or through Biogel P-300 (exclusion limit > 300,000) resulted in only one immunogenically active protein of the eluate found in the void volume of the columns. Diethylaminoethyl (DEAE) cellulose anion-exchange chromatography of the RNA preparations showed that the immunogenic activity was eluted from the column at 0.8 to 1.0 M NaCl in a linear 0.1 to 2.0 M NaCl gradient. Nonimmunogenic, protein-containing minor peaks were eluted at 0.1 to 0.5 M NaCl. Serial fractionation of the crude RNA preparations over Biogel P-6 to DEAE cellulose to Biogel P-300 molecular-sieve or anion-exchange columns did not alter the immunogenicity of the RNA preparation. Incorporation of the column fractions into Freund's incomplete adjuvant did not increase their relative effectiveness in eliciting anti-salmonella resistance. Chemical analysis of the immunogenic preparations indicated that they were lacking in detectable protein, lipid, and deoxyribonucleic acid. These results suggest that the immunogenic moiety of the crude nucleic acid fraction is either RNA or an as yet undefined polysaccharide of greater than 300,000 molecular weight.

Within the past 5 years, an increasing body of evidence has accumulated to indicate that bacterial subcellular preparations are effective in inducing resistance to several bacterial parasites. Youmans and Youmans have elegantly demonstrated that ribosomal and crude ethanol-precipitated ribonucleic acid (RNA) preparations of *Mycobacterium tuberculosis* induce an effective immune response in mice to challenge infection with the homologous organism (42-45). In more recent work (46), they have further suggested that the immunogenic activity of the fractions may be associated with one particular species of nucleic acid.

[1] Preliminary results of this investigation were presented at the Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

Immunization with bacterial ribosomal preparations has also been found to be an effective means of prophylaxis for infections with *Staphylococcus aureus* (41), *Pseudomonas aeruginosa* (40), *Diplococcus pneumoniae* (30), and *Salmonella typhimurium* (35). The immunogenic fractions of these organisms consist of approximately 60% RNA, 30% protein, and 10% "contaminant" material on a dry weight basis. The active moiety in these preparations, however, has not been defined.

In recent work from this laboratory, evidence was presented to indicate that immunization with phenol-extracted RNA preparations obtained from *S. typhimurium* protects mice against subsequent challenge with virulent bacilli (36). In view of the heterogeneous chemical composi-

tion reported for the immunogenic preparations [97% RNA, < 0.5% protein, < 0.02% deoxyribonucleic acid (DNA), and approximately 2% miscellaneous carbohydrate], the identity of the active moiety in these fractions has remained obscure. This communication details results obtained through purifying the immunogenic RNA preparations by molecular-sieve and anion-exchange chromatography. The data strongly suggest that the active moiety is associated with a substance(s) of a molecular weight greater than 300,000.

MATERIALS AND METHODS

Organism. *S. typhimurium* strains SR-11 and RIA were employed throughout this investigation. The LD_{50} values of these strains for mice were less than 10 and approximately 5×10^4 bacteria per animal, respectively (36).

Animals. CF-1 female, albino Swiss mice obtained from Carworth Farms, Portage, Mich., and weighing between 18 and 25 g were used in all experiments. Mice were housed 10 per cage with food and water available ad lib.

Preparation of crude RNA fractions. The procedure employed for the preparation of the immunogenic RNA fractions was that described previously with modification (35, 36). An 8-liter broth culture of *S. typhimurium* SR-11 in mid- to late-log growth was harvested by continuous-flow centrifugation at 40,000 × *g* in a Sorvall refrigerated high-speed centrifuge. The harvested bacteria were then washed in 0.01 M sodium phosphate buffer, *p*H 7.19, at 25 C, and suspended in 3 volumes (v/v) of cold 0.01 M phosphate buffer containing 3×10^{-2} M $MgCl_2$ and 0.25% (w/v) sodium dodecyl sulfate (SDS). Polyvinyl sulfate (3×10^{-4}%, w/v) was included in the extraction buffer to minimize endonuclease activity. The bacterial mass was then homogenized for 1 min in a Waring Blendor and was subsequently passed through a French pressure cell at 10,000–14,000 psi. Although this is a more efficient fractionation method (90+% breakage), sonic treatment of the bacterial mass for 10 min at 70 w may be used as a substitute method.

The homogenate was subjected to two preliminary centrifugations at 27,000 and 45,000 × *g* in a Sorvall RC-2B refrigerated centrifuge. After the 45,000 × *g* centrifugation, the supernatant fluid was centrifuged at 105,000 × *g* for 3 hr in a Beckman model L refrigerated ultracentrifuge. The pellet from this centrifugation was suspended to a concentration of 50 mg (moist weight) per ml in 0.01 M phosphate buffer, *p*H 7.19, at 25 C, and was incubated with 5 μg of deoxyribonuclease per ml for 30 min at 15 C.

After incubation, one volume of hot (65 C), buffer-saturated phenol (suspended in and equilibrated three times with 0.01 M phosphate buffer, *p*H 7.19, at 25 C, containing 0.5% SDS) was added to this suspension, and the mixture was agitated vigorously for 10 min at 65 C. The aqueous phase was separated from the phenol phase by centrifugation for 5 min at 2,100 rev/min in an International HN-S centrifuge, and was decanted (the phenol phase was discarded). Extraction of the aqueous phase with 1 volume of hot buffer-saturated phenol was repeated at least four times or until the aqueous-phenol interphase was free from denatured protein. Subsequent to the final centrifugation, the upper two-thirds of the aqueous phase was carefully decanted and brought to 0.1 M NaCl by the addition of 1 M NaCl in 0.01 M phosphate buffer. Two volumes (v/v) of cold (−20 C) absolute ethanol were added to the preparation, and the mixture was permitted to stand overnight at −20 C.

The following day, the nucleic acid precipitate was collected by centrifugation at 10,000 × *g* for 5 min in a Sorvall refrigerated centrifuge (model-RC-2B), and was suspended in 0.01 M phosphate buffer, *p*H 7.1, at 25 C, containing 0.1 M NaCl. Two volumes (v/v) of absolute ethanol were again added, and the mixture was stored at −20 C for 4 hr. After collecting the precipitate and resuspending the pellet in 50 ml of 0.01 M phosphate buffer containing 0.1 M NaCl, an equal volume of 1.0% SDS (in 0.01 M phosphate buffer, *p*H 7.19, at 25 C, containing 0.1 M NaCl) was added, and the mixture was incubated for 30 min at 25 C under moderate agitation. The nucleic acid was then precipitated out of solution by the addition of 2 volumes of cold absolute ethanol. This mixture was stored at −20 C for 4 hr, collected by centrifugation at 10,000 × *g* as described above, and suspended in 50 ml of phosphate-NaCl buffer. The precipitation and collection of the nucleic acid were repeated an additional four times. The final pellet was then suspended in either sterile, pyrogen-free isotonic saline or 0.01 M phosphate-NaCl buffer, and was lyophilized immediately.

The yield of the nucleic acid fraction obtained by this procedure was approximately 0.2 to 0.4% of the moist weight of the cell mass. Qualitatively, the fraction exhibited an adsorption spectrum between 210 and 310 nm, which is comparable to that reported for nucleic acids (14, 25), and an A_{260}/mg equal to 20. The ratios of optical density at 260 nm (OD_{260}) to OD_{280} and OD_{260} to OD_{235} were approximately 2.138 ± 0.087 and 2.104 ± 0.093, respectively. Chemically, the dry weight of the crude nucleic acid fraction was composed of approximately 98.5% RNA, 1% miscellaneous carbohydrate, less than 1% protein, no detectable DNA, and no detectable lipid. The chemical analyses used in determining these concentrations were the same as those described previously (36).

Molecular-sieve chromatography. Biogel P-6 (molecular weight exclusion limit > 4,600) and Biogel P-300 (molecular weight exclusion > 300,000) were obtained from BioRad Laboratories, Richmond, Calif. The appropriate columns were charged with 50 mg of the crude RNA preparation suspended in 5 ml of 0.01 M phosphate buffer, *p*H 7.19, at 25 C, containing 0.1 M NaCl. A Biogel P-6 column 50 by 1.5 cm was eluted at a flow rate of 60 ml per hr with the suspending phosphate-NaCl buffer. A Biogel P-300 column 30 by 2.5 cm was similarly eluted at a flow rate of 8 ml per hr. The eluates of the columns were monitored through an Isco dual-beam ultraviolet analyzer and recorder (model UA-2) at OD_{254}, and fractions were collected in 2- and 5-ml quantities from the P-6 and P-300 columns, respectively. Soluble yeast RNA (Nutritional

Biochemicals Corp., Cleveland, Ohio) and Blue Dextran 2,000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were employed as standards to compare with the elution profile of the *S. typhimurium* RNA preparations and to determine the void volume of the column, respectively.

Anion-exchange chromatography. Diethylaminoethyl (DEAE) cellulose was obtained from Sigma Chemical Co., St. Louis, Mo., and was prepared according to the protocol described elsewhere (26). A column (30 by 1.5 cm) was charged with 10 mg (dry weight) of the immunogenic RNA preparation contained in 5 ml of 0.01 M phosphate buffer containing 0.1 M NaCl. A 200-ml linear 0.1 to 2 M NaCl gradient was passed through the column at a flow rate of 0.25 ml per min, and the fractions were collected in 5-ml quantities. The eluate was monitored as described above.

Immunizations. Mice were immunized subcutaneously with 50 μg (dry weight) of the lyophilized RNA preparation, or of the pooled and lyophilized column eluates contained in 0.1 ml of sterile, pyrogen-free isotonic saline (Baxter Laboratories, Morton Grove, Ill.). When adjuvant was employed, the fractions were incorporated into 3 volumes (v/v) of Freund's incomplete adjuvant and injected subcutaneously in 0.4 ml. The fractions, at these dosage levels, were not toxic for mice. Animals receiving attenuated salmonella were injected subcutaneously with 0.1 LD_{50} of *S. typhimurium* strain RIA contained in 0.1 ml of sterile saline.

Challenge. At 15 or 30 days after immunization with preparations incorporated into either saline or adjuvant, treated and unimmunized control mice were injected intraperitoneally with 48 and 70 LD_{50} of virulent *S. typhimurium* SR-11, contained in sterile, pyrogen-free isotonic saline. Viable plate counts on Brain Heart Infusion agar defined the specific number of bacteria injected.

Immune assay. For the detection of demonstrable anti-salmonella resistance, two or all three of the following assays were employed.

(i) Survival: The end point for survival was designated as 30 days postinfection. The statistical significance of differences between the results obtained with control and treated mice was determined by chi-square analysis (27).

(ii) Differential pathogen counts at 5 days postchallenge (4): Challenged animals were individually killed by cervical dislocation; the animals were skinned, their feet and tails were removed, and the gastrointestinal tract was excised. The weight of the carcass was determined, and sterilized distilled water was added to yield a total volume of 100 ml. The suspension was homogenized for 3 min in a Waring Blendor, and 10-fold serial dilutions of the homogenate were prepared in sterile, isotonic saline and plated in 0.1-ml quantities on SS Agar (Difco). After incubation at 37 C for 24 hr, the number of viable bacteria in each dilution was determined, and the number of bacteria in the original homogenate was calculated. The number of bacteria per gram of tissue for each animal was determined as described elsewhere (33). Statistical significance of differences between values obtained for the treated and untreated groups were determined by rank order evaluation (39).

(iii) Pathogen tissue clearance: At 2, 4, 7, 10, 15, and 30 days postchallenge, the number of bacteria per gram of tissue for infected normal and treated mice was determined as defined above. The data are presented as the mean number of bacteria per gram of tissue versus the time postchallenge at which the animals were sacrificed.

Chemical analyses. Protein was determined by the Lowry technique (20) with bovine serum albumin (Nutritional Biochemicals Corp.) as the standard. These results were confirmed by polyacrylamide disc electrophoresis on 2 mg (dry weight) of the fractions in 5% gels by the procedure of Maurer (21). DNA and RNA were defined by the diphenylamine (11) and orcinol tests (25), respectively. Sperm DNA and purified yeast soluble RNA obtained from Nutritional Biochemicals Corp. were employed as standards. Lipid was qualitatively determined by thin-layer chromatography as described previously (36). Total carbohydrate concentrations were assayed by the phenol-sulfuric acid method of Dubois et al. (12), with D-ribose (Nutritional Biochemicals Corp.) as the standard.

RESULTS

Vaccine standardization. One limitation anticipated in attempts to purify the active moiety in the crude *S. typhimurium* RNA preparations was the previously reported temperature lability of the activity upon storage of the fraction at either 4 or -20 C (36). Because of the use of the modified extraction procedure employed in this study, and the immediate lyophilization of the crude fraction, the immunogenicity of the preparations in the lyophilized state required definition. Accordingly, mice were immunized with 50 μg (dry weight) of the RNA that had been stored in this manner for 1, 2, or 3 months. Animals immunized with 0.1 LD_{50} of attenuated *S. typhimurium* strain RIA or with 50 μg (dry weight) of the freshly prepared RNA served as positive controls. After 15 days, all animals were challenged with 65 LD_{50} of virulent *S. typhimurium* SR-11. As measured by survival (Table 1), the immunogenicity of the lyophilized RNA preparations was comparable to that observed in the animals immunized with either the freshly prepared RNA or the attenuated salmonellae. There was no definable correlation between a loss in immunogenicity and the time of storage for the immunogenic RNA preparations. Similarly, there was no significant decrease in the efficacy of the lyophilized fractions as measured by the more sensitive (15) differential pathogen count immune assay (Fig. 1).

One criticism of the use of the preceding immune assays in determining the efficacy of a vaccine preparation is the inability of either survival or differential pathogen counts to detect

a sterilizing immunity in the immunized mice (3, 7, 8). To define the comparative efficiency of the subcellular vaccine preparations in eliciting this type of response, 25 mice were immunized with 0.1 LD_{50} of attenuated *S. typhimurium* RIA, with 50 μg (dry weight) of the freshly prepared RNA, or with the RNA fraction lyophilized 2 months previously. Unimmunized animals served as normal controls. At 15 days postimmunization, the animals were challenged with 65 LD_{50} of virulent *S. typhimurium*. At 2, 4, 7, 10, 15, and 30 days postimmunization, five mice from each group were sacrificed, and the number of bacteria per gram of tissue was determined. The results of these experiments are presented in Fig. 2. Animals immunized with the RIA, freshly prepared RNA, or the lyophilized RNA preparation cleared infecting bacteria from the tissue below the limit of detectability within 30 days postchallenge. In contrast, the normal animals showed a steady increase in bacterial counts, and by 10 to 15 days postchallenge all of the animals had succumbed to infection. These results clearly indicate that the resistance elicited with the immunogenic subcellular fraction is comparable to the sterilizing response induced with the attenuated salmonellae.

TABLE 1. *Comparative immunogenicity of lyophilized RNA preparations as measured by survival*[a]

Immunizing prepn	Storage time (months)	Survival at 30 days (%)[b]
S. typhimurium strain RIA[c]	—	85.0 (17/20)
S. typhimurium RNA		
Freshly prepared[d]	—	80.0 (16/20)[e]
Lyophilized[d]	1	95.0 (19/20)
	2	80.0 (16/20)
	3	86.7 (13/15)
Unimmunized controls	—	8.0 (4/50)

[a] Challenged 15 days postimmunization with 65 LD_{50} of *S. typhimurium* strain SR-11.
[b] The number of survivors/total is given in parentheses.
[c] Immunized with 0.1 LD_{50}.
[d] Immunized with 50 μg (dry weight) of RNA.
[e] $P < 0.001$.

Molecular-sieve chromatography, Biogel P-6. Small-molecular-weight contaminant carbohydrate, phenol, and residual SDS (10) were excluded from the lyophilized RNA preparations by molecular-sieve chromatography over Biogel P-6 (molecular weight exclusion limit > 4,600). Characteristic OD_{254} profiles of two immunogenic RNA fractions, yeast RNA, and Blue Dextran 2000 are presented in Fig. 3. The majority of the material absorbing at 254 nm in the immunogenic RNA fraction was eluted in the void volume of

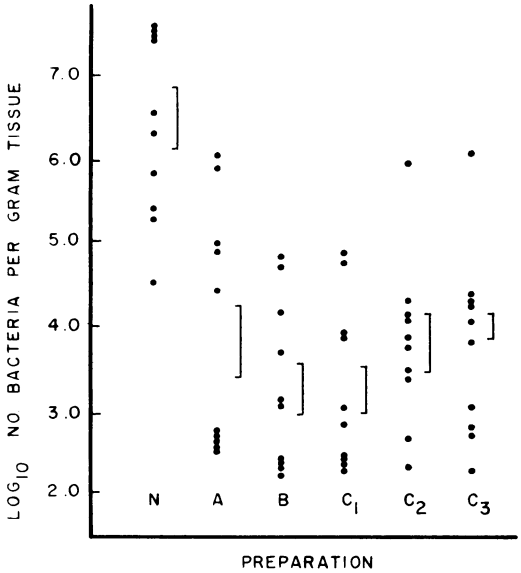

FIG. 1. *Comparative immunogenicity of lyophilized S. typhimurium RNA preparations as measured by differential pathogen counts 5 days postchallenge. N = normal, unimmunized controls; A = animals immunized with 0.1 LD_{50} of S. typhimurium strain RIA; B = animals immunized with 50 μg of freshly prepared RNA; and C_1, C_2, C_3 = animals immunized with 50 μg (dry weight) of lyophilized RNA stored for 1, 2, and 3 months, respectively. Each dot represents the value obtained from one mouse; the bracketed lines define the geometric mean ± the standard error.*

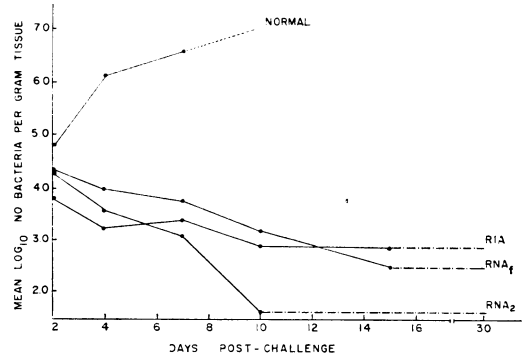

FIG. 2. *Comparative immunogenicity of lyophilized S. typhimurium RNA preparations as measured by clearance of the challenge inocula from the tissues. Each dot represents the geometric mean of 5 mice. Normal-unimmunized controls; RIA = mice immunized with 0.1 LD_{50} of S. typhimurium strain RIA; RNA_f = 50 μg of freshly prepared RNA; RNA_2 = 50 μg (dry weight) of an RNA preparation lyophilized for 2 months. The dot-dash lines indicate numbers below the limits of detection; the dashed line for the controls indicates that all animals had died.*

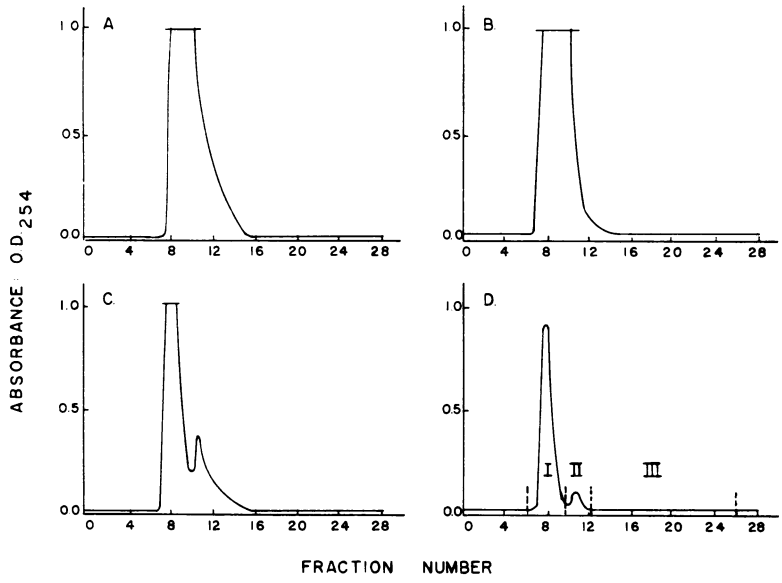

FIG. 3. *Biogel P-6 elution profiles for Blue Dextran 2000 (A), yeast soluble RNA (B), and immunogenic S. typhimurium RNA preparations (C, D). A 50 by 1.6 cm column was eluted at a flow rate of 60.0 ml per hr with 0.01 M phosphate buffer containing 0.1 M NaCl, pH 7.19, at 25 C. The dotted lines in D define the column fractions pooled for lyophilization and immunization.*

the P-6 column as determined by comparison with the Blue Dextran 2000 elution profile. This compared favorably with the profile obtained for the purified yeast RNA standard. A second absorbing peak, following the one in the void volume, was consistently found in all of the salmonella preparations.

To examine the immunogenicity of the column eluate, fractions were pooled as shown in Fig. 3 and lyophilized; then 50 μg (dry weight) was injected into 60 mice. In each group, 30 mice received the fractions incorporated into Freund's incomplete adjuvant, 30 received the preparation in saline. At 15 days postimmunization, the animals injected with the preparations suspended in saline were challenged with 62 LD_{50} of *S. typhimurium* SR-11. At 30 days postimmunization, the mice that received the fractions incorporated into adjuvant were similarly infected. Unimmunized animals served as normal controls. The results from these experiments are presented in Table 2 and Fig. 4. As measured by either survival or the ability of the immunized animal to inhibit or retard bacterial multiplication, the only portion of the eluate found to be immunogenic was that portion containing the RNA located in the void volume of the column. Adjuvant did little to increase the efficacy of any of the fractions in inducing resistance to the challenge infection.

Molecular-sieve chromatography, Biogel P-300. Subsequent elution of the immunogenic RNA preparations through Biogel-300 (molecular weight exclusion limit > 300,000) with 0.01 M phosphate buffer, *p*H 7.19, at 25 C, containing 0.1 M NaCl, gave a characteristic OD_{254} elution profile as shown in Fig. 5. Fractions from the column were pooled and lyophilized, and 30 mice were injected with 50 μg of one of the respective preparations incorporated into adjuvant. An additional 30 mice received the pooled suspension in saline. At 30 or 15 days after immunization, the animals were challenged with 48 LD_{50} of virulent SR-11. The survival of the infected animals and

TABLE 2. *Immunogenicity of pooled Biogel P-6 column fractions as measured by survival*[a]

Immunizing prepn	Suspending diluent	Survival at 30 days (%)[b]
S. typhimurium RNA	Saline	90 (18/20)[c]
	Adjuvant	70 (14/20)[c]
Fraction I	Saline	80 (16/20)[c]
	Adjuvant	65 (13/20)[d]
Fraction II	Saline	0 (0/20)
	Adjuvant	15 (3/20)[e]
Fraction III	Saline	10 (2/20)
	Adjuvant	5 (1/20)
Unimmunized controls	—	5 (1/20)

[a] Challenged at 15 (saline) or 30 (adjuvant) days postimmunization with 62 LD_{50} of *S. typhimurium* SR-11.

[b] The number of survivors/total is given in parentheses.

[c] $P < 0.001$.

[d] $0.01 > P > 0.001$.

[e] $P > 0.1$.

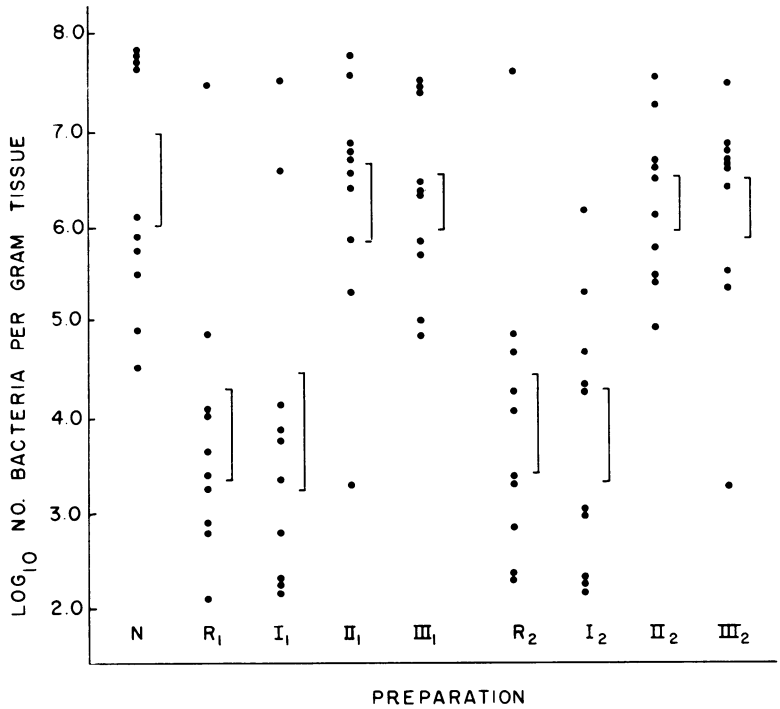

FIG. 4. *Immunogenicity of pooled Biogel P-6 column fractions as measured by differential pathogen counts 5 days postchallenge. N = unimmunized normal controls. R_1, I_1, II_1, III_1 = animals immunized with 50 μg of S. typhimurium RNA, fraction I, fraction II, and fraction III injected in saline, respectively. R_2, I_2, II_2, III_2 = animals immunized with the respective preparations incorporated into Freund's incomplete adjuvant. Each dot represents the value obtained from one animal; the bracketed lines define the mean ± the standard error.*

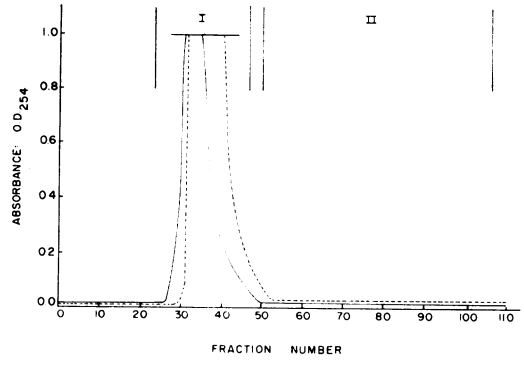

FIG. 5. *Biogel P-300 elution profile of immunogenic S. typhimurium RNA (solid line) and Blue Dextran 2000 (dashed line). The 30 by 2.5 cm column was eluted with 0.01 M phosphate buffer, pH 7.19, at 24 C, containing 0.1 M NaCl at a flow rate of 8 ml/hr. Fractions were collected in 5-ml quantities. Vertical lines designate those fractions pooled for lyophilization and immunization.*

the differential pathogen counts at 5 days postchallenge are presented in Table 3 and Fig. 6, respectively. The only mice found to be both resistant to the challenge and capable of inhibiting the multiplication of the organism were those

TABLE 3. *Immunogenicity of Biogel P-300 fractions as measured by survival*[a]

Immunizing prepn	Suspending diluent	Survival at 30 days (%)[b]
S. typhimurium RNA	Saline	80 (16/20)
	Adjuvant	70 (14/20)
Fraction I	Saline	90 (18/20)
	Adjuvant	75 (15/20)[c]
Fraction II	Saline	25 (5/20)
	Adjuvant	10 (2/20)
Unimmunized controls	—	15 (3/20)

[a] Challenged either 15 (saline) or 30 (adjuvant) days postimmunization with 48 LD_{50} of *S. typhimurium* SR-11.

[b] The number of survivors/total is given in parentheses.

[c] $P < 0.001$.

immunized with the nucleic acid-containing eluate of the column. Adjuvant appeared to make little difference in the degree of immunity obtained with the respective fractions.

DEAE cellulose anion-exchange chromatography. On the premise that a polar contaminant was nonspecifically absorbed to the RNA in the crude fractions and was consequently responsible

for the immunogenicity of the preparations, the RNA fractions were purified through anion-exchange chromatography. Yeast RNA and 50 mg of purified *S. typhimurium* endotoxin (37) were employed as standards for the comparison of elution profiles. As shown in Fig. 7, the yeast RNA eluted off the column at 0.8 to 1.0 M NaCl as a homogeneous peak. In contrast, elution of the endotoxin preparation with this buffer system resulted in four absorbing peaks at 254 nm. The three minor peaks were eluted at 0.2 M, 0.4 M, and greater than 1.8 M NaCl. The major peak was eluted at 0.85 to 1.0 M NaCl. Chemical analyses of these fractions indicated that the major peak was high in carbohydrate content, but lacking in detectable protein. Similarly, this fraction exhibited a 210 to 310 nm absorption spectrum characteristic of nucleic acids ($OD_{260}:OD_{280}$ = 1.947; $OD_{260}:OD_{235}$ = 1.876). These data seem to indicate that the major peak of the endotoxin eluate was composed of nucleic acid contaminant carried over throughout the Westphal phenol extraction procedure and would be in support of the work of other investigators (2, 16, 38).

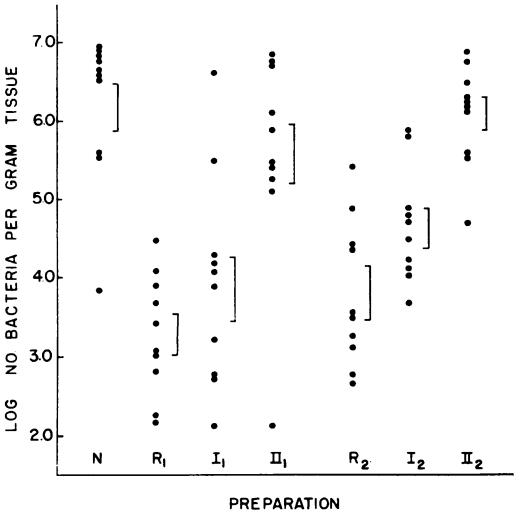

FIG. 6. *Immunogenicity of Biogel P-300 column fractions as measured by differential pathogen counts 5 days postchallenge. N = unimmunized controls. R_1, I_1, II_1 = animals immunized with 50 μg (dry weight) of S. typhimurium RNA, fraction I, and fraction II in saline, respectively. R_2, I_2, II_2 = animals immunized with the respective preparations incorporated into Freund's incomplete adjuvant. Each dot represents the value obtained from one animal; the bracketed lines define the mean ± the standard error.*

In contrast, the immunogenic *S. typhimurium* RNA preparation gave a homogeneous peak eluted at 0.8 to 1.0 M NaCl. This major peak was eluted at these NaCl concentrations consistently in all preparations examined and matches that elution profile observed with the yeast RNA. In several tests of other immunogenic RNA preparations, however, barely detectable minor peaks were observed to be eluted at 0.1 to 0.5 M NaCl (Fig. 8: A_1–A_4). Chemical analyses of these fractions indicated that they were primarily protein, that they were lacking in detectable carbohydrate, and that they absorbed ultraviolet light maximally at 280 nm. In contrast, the major peak of

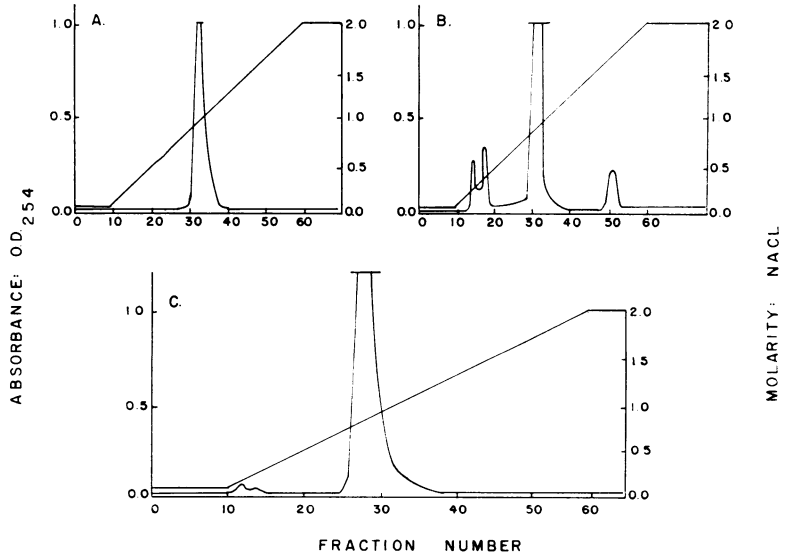

FIG. 7. *DEAE cellulose anion-exchange chromatography profiles of yeast soluble RNA (A), S. typhimurium endotoxin (B), and immunogenic RNA preparations (C). A 30 by 1.0 cm column was eluted with a linear 0.1 to 2.0 M NaCl gradient in 0.01 M phosphate buffer, pH 7.19, at 24 C. Fractions were collected in 5.0-ml samples.*

the RNA preparations did not contain detectable protein, exhibited a significant carbohydrate content, and gave an absorption spectrum characteristic of nucleic acids (OD_{260}:OD_{280} = 2.231; OD_{260}:OD_{235} = 2.178) with a corresponding A_{260}/mg of 20 (Fig. 9). Comparable elution profiles and chemical results were also obtained when excessively large amounts of the initial RNA preparation (>100 mg) were employed as the column charge, indicating that the immunogenic preparations were composed primarily of RNA.

The DEAE cellulose eluate was pooled according to the protocol shown in Fig. 8 and lyophilized. Animals were immunized subcutaneously

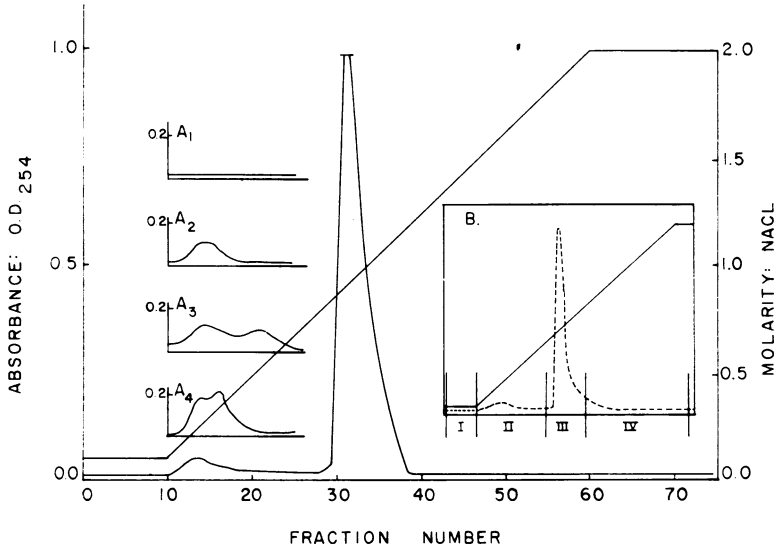

FIG. 8. *Immunogenic S. typhimurium RNA elution profile on a 30 by 1 cm DEAE cellulose anion-exchange column. The column was eluted with a linear 0.1 to 2.0 M NaCl gradient in 0.01 M phosphate buffer, pH 7.19, at 24 C. Inserts A_1, A_2, A_3, and A_4 show varying 0.1 to 0.5 M NaCl elution profiles obtained with different RNA preparations; insert B shows the fractions pooled for lyophilization and immunization.*

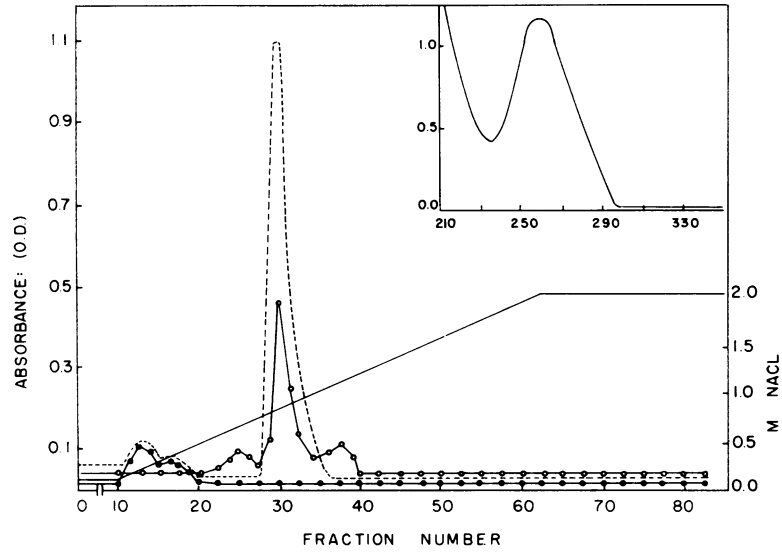

FIG. 9. *Chemical analyses of DEAE cellulose anion-exchange elution fraction of immunogenic S. typhimurium RNA. Dashed line, OD_{254} absorbance; ●, protein; ○, total carbohydrate. Insert shows 210 to 310 nm absorption profile of fraction no. 30.*

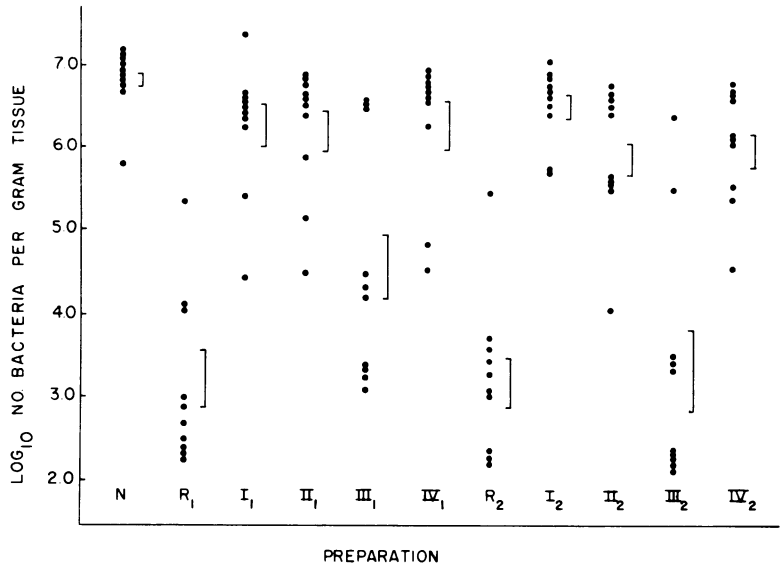

FIG. 10. *Immunogenicity of DEAE cellulose anion-exchange fractions as measured by differential pathogen counts 5 days postchallenge. N = unimmunized normal controls. R_1, I_1, II_1, III_1, IV_1 = animals immunized with 50 μg (dry weight) of S. typhimurium RNA, fraction I, fraction II, fraction III, and fraction IV in saline, respectively. R_2, I_2, II_2, III_2, IV_2 = animals immunized with the respective preparations incorporated into adjuvant. Each dot represents the value obtained from one animal; the bracketed lines define the mean ± the standard error.*

with 50 μg (dry weight) of one of the pooled fractions. Twenty-five animals of each group received the preparation in saline, and 25 animals received the fractions incorporated into Freund's incomplete adjuvant. At 15 or 30 days postimmunization, the animals were challenged intraperitoneally with 70 LD_{50} of virulent *S. typhimurium*. Unimmunized animals again served as normal controls, and the immune assays employed were both differential pathogen counts at 5 days postchallenge (Fig. 10) and survival (Table 4). Regardless of the incorporation of the fractions into adjuvant, the only pooled eluate found to be immunogenic was that containing the RNA of the bacteria.

Serial column purification. Although purification of the immunogenic RNA preparations through elution of the fraction over Biogel P-6, Biogel P-300, or DEAE cellulose had indicated that the immunogenicity of the subcellular fraction was associated with the nucleic acid in the preparation, the ability of a serially purified nucleic acid fraction to induce substantive antisalmonella resistance had not been examined. Consequently, when the crude sample was employed as the column charge, it was possible that a contaminant of the initial preparation was continually carried throughout the fractionation into the immunogenic eluate. To exclude this possibility, the nucleic acid peak obtained off the Biogel P-6 (cf. Fig. 3, peak I) was applied to a DEAE cellulose column and eluted with a linear 0.1 to 2.0 M NaCl gradient in 0.01 M phosphate buffer, *p*H 7.19, at 25 C. The fractions representing the major portion of the nucleic acid peak obtained from anion-exchange chromatography

TABLE 4. *Immunogenicity of DEAE cellulose fractions as measured by survival*[a]

Immunizing prepn	Suspending diluent	Survival at 30 days (%)[b]
S. typhimurium RNA	Saline	93.3 (14/15)
	Adjuvant	80.4 (12/15)[c]
Fraction I	Saline	20.0 (3/15)[d]
	Adjuvant	13.2 (2/15)
Fraction II	Saline	13.3 (2/15)
	Adjuvant	19.6 (3/15)
Fraction III	Saline	86.7 (13/15)
	Adjuvant	72.6 (11/15)[c]
Fraction IV	Saline	0.0 (0/15)
	Adjuvant	13.2 (2/15)
Unimmunized controls	—	6.7 (1/15)

[a] Challenged either 15 (saline) or 30 (adjuvant) days postimmunization with 70 LD_{50} of *S. typhimurium* SR-11.

[b] The number of survivors/total is given in parentheses.

[c] $0.01 > P > 0.001$.

[d] $P > 0.05$.

(cf. Fig. 9, peak III) were pooled and eluted through Biogel P-300. The fractions of the P-300 eluent containing the nucleic acid were pooled (cf. Fig. 5, peak I), lyophilized, and injected subcutaneously in saline into normal mice (50 μg, dry weight, per animal). Unimmunized animals and mice immunized with 50 μg (dry weight) of the crude RNA, 0.1 LD_{50} of attenuated *S. typhimurium* strain RIA, or 50 μg of the pooled and lyophilized non-nucleic acid-containing fractions of the P-300 column (cf. Fig. 5, peak II) served as controls. At 15 days postimmunization, the animals were challenged with 52 LD_{50} of virulent *S. typhimurium* SR-11. As measured by the ability of the immunized animals to inhibit or retard bacterial multiplication 5 days postchallenge (Fig. 11), fraction I was the only portion of the P-300 eluent that induced a resistance comparable to that induced with the crude RNA and attenuated salmonella vaccine preparations. The non-nucleic acid-containing protein of the gradient (fraction II) did not elicit substantive resistance. Similarly, immunization with fraction I resulted in elimination of the pathogen from the tissue (Fig. 12), whereas the normal controls and animals immunized with 50 μg of fraction II supported the growth of the challenge inocula and had succumbed to infection within 15 days postchallenge.

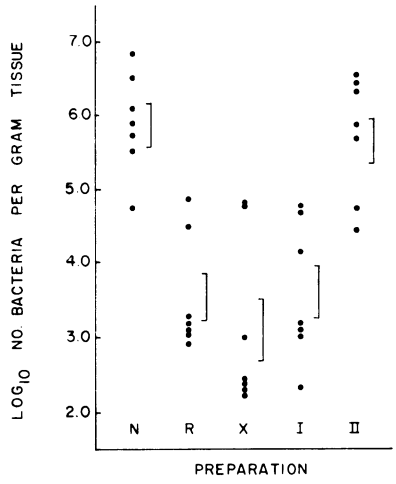

FIG. 11. *Immunogenicity of serially purified RNA as measured by differential pathogen counts 5 days postchallenge. N = unimmunized controls. X, R, I, II = animals immunized with 0.1 LD_{50} of attenuated S. typhimurium strain RIA, 50 μg (dry weight) of immunogenic S. typhimurium RNA, 50 μg of fraction I, or 50 μg dry weight of the non-OD_{254}-absorbing portion of the Biogel P-300 column, respectively. Each dot represents the value obtained from one animal; the bracketed lines define the mean ± the standard error.*

Chemical assay. Chemical tests were conducted on 50-mg (dry weight) samples of the various immunogenic column eluates. Six samples of Biogel P-6 fraction I, five samples of DEAE cellulose fraction III, three samples of Biogel P-300 fraction I, and four samples of the serial purification void volume were tested. Protein was present (<0.008%) only in fraction I of the Biogel P-6 eluates by the Lowry method. How-

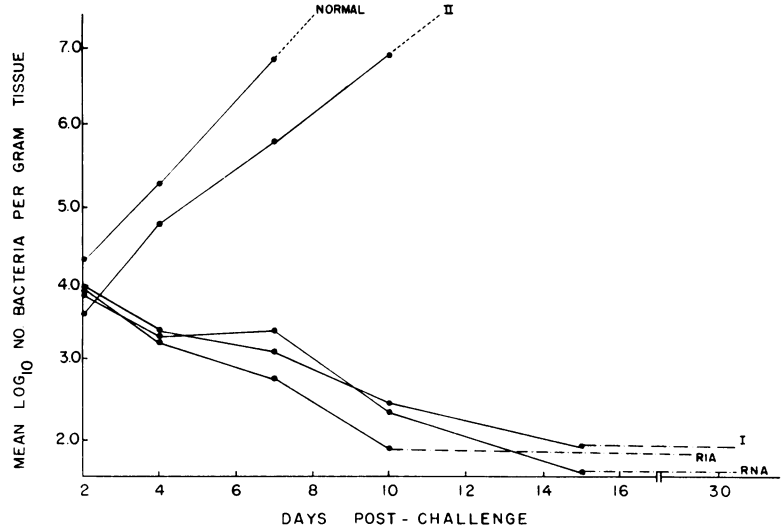

FIG. 12. *Immunogenicity of serially purified RNA as measured by clearance of the challenge inocula from the tissues. Normal = unimmunized controls. Animals immunized with 0.1 LD_{50} of attenuated S. typhimurium strain RIA (RIA), 50 μg (dry weight) of immunogenic S. typhimurium RNA (RNA), and 50 μg of fraction I (I), or non-OD_{254}-absorbing fraction II (II), respectively. Dot-dash lines represent none detected; dashed lines, all animals had died. Each point represents the mean obtained from five mice.*

ever, protein was not detectable (<0.001% of the preparation) in this fraction by analytical polyacrylamide disc electrophoresis (2 mg, dry weight, of preparation examined), nor was it detectable in any of the other samples by either method. DNA (determined by the diphenylamine test) and lipid (determined by thin-layer chromatography) were not detectable in any of the samples. In view of the defined sensitivity of the colorimetric tests for the qualitative presence of the respective components (11, 20, 25, 27) and the empirical confirmation of these sensitivities in our own laboratories, if protein, DNA, or lipid were present they would be expected to be found in amounts of less than 0.5 μg of material per 50 mg of RNA. Consequently, these substances would represent less than 0.001% of the dry weight of the fraction.

Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (12) to be 24.28 ± 0.43% (dry weight) of the serial purification void volume, and pentose was determined by the orcinol method to be 25.13 ± 0.59%. It should be stated, however, that validation of these relative percentages resides with the completion of more extensive analytical assays. Work along these lines is currently in progress.

DISCUSSION

In previous reports from this laboratory, immunization with hot phenol-SDS extracted RNA preparations of *S. typhimurium* has been shown to be an effective means of inducing immunity to experimental salmonellosis of the mouse (32–34, 36). The data presented in this investigation fully support the contention that these subcellular extracts elicit a true protective immune response as defined by Collins (7–9). Mice immunized with 50 μg (dry weight) of the phenol-extracted RNA were able to resist subsequent infection by the homologous pathogen. The resistance was exemplified by the ability of the challenge animals both to survive the infection and to suppress the growth of the bacteria at 5 days postchallenge. The possibility that these results are only a reflection of the ability of the infected animal to retard infection and are not characteristic of a true immune state was eliminated by the data demonstrating that, upon immunization with the crude RNA, the pathogen was eliminated from the tissues of the infected animal. This sterilizing type of immune response was comparable to that elicited upon immunization with the attenuated salmonellae.

In examining the chemical composition of the immunogenic RNA preparations, three alternatives exist with respect to the chemical nature of the active moiety in the immunogenic fraction. First, it is entirely possible that the activity is inherent in a contaminant molecular species of the fraction. On the basis of the chemical test employed to determine the constituents in the crude immunogenic preparations (36), this would suggest that an immunologically unique small-molecular-weight carbohydrate, polysaccharide, or protein is the immunogen. Consequently, it is not improbable that in the crude fractions a large concentration of immunogenic carbohydrate or polysaccharide does exist and is responsible for the activity of the preparations. In additional support of this thesis, earlier work from this laboratory demonstrated that the major contaminant of the fraction was nonribose carbohydrate (36). It is important to note, however, that small-molecular-weight (<4,600) nonpolar material was removed from the crude preparations via molecular-sieve chromatography over Biogel P-6. Although this would appear to diminish the probability that carbohydrate was involved, it does not exclude the possibility that a charged derivative was absorbed to the highly polar nucleic acid eluted in the void volume of the column. In addition, regardless of ionic charge, if the molecular weight of the carbohydrate was greater than 4,600 it would not be separable from the RNA through this means. It should be stated, however, that via the Biogel P-300 molecular sieve and serial purification scheme, polar material and residues of less than 300,000 molecular weight should have been differentially eluted from the nucleic acid. Similarly, chemical analysis of the serially purified RNA suggests that the total carbohydrate of the preparation can be accounted for by the pentose concentration empirically determined for the RNA of the fraction (approximately 25% on a dry weight basis). These data strongly indicate that a carbohydrate contaminant is not responsible for the activity of the fraction.

Along similar lines, the possibility that a protein or RNA-absorbed peptide fragment is responsible for the immunogenicity of the RNA must be considered, since the crude fractions are known to contain from 1.0 to 0.1% of the cellular protein. Protein, however, was separated from the nucleic acid through anion-exchange chromatography. In addition, the proteins eluted from the preparation were not immunogenic, regardless of whether they were or were not incorporated into adjuvant. Similarly, through the quantitation of protein in the sample via polyacrylamide disc electrophoresis and colorimetric assay, it was demonstrated that, if present, proteinaceous material accounted for less than 0.001% of the immunogenic preparation. Assuming that the percentage stands up under more rigorous analytical tests, these data in effect would require that a unique peptide must be active in less than 0.0005 μg per 50-μg (dry weight) immunizing dose. Although this is feasible, the degree of resistance ob-

served subsequent to immunization, coupled with the necessity of converting an entire animal to an immune state, renders this possibility unlikely.

The possibility that a contaminant molecular species is responsible for the immunogenicity of the fraction would be further limited if one were to consider the comparative efficacy of immunizing with equivalent quantities of the crude and purified RNA preparations. Immunization with 50 μg (dry weight) of the purified fraction elicited an immune state comparable to that obtained with 50 μg of the crude RNA. In conjunction with this equal immunizing potency, it is interesting to note that the concentration of contaminant material in the fractions was reduced from approximately 3% to less than 0.003% of the dry weight of the preparation. Whereas there existed an approximate 1,000-fold decrease in non-nucleic acid material, a commensurate decrease in the immunizing potency of the fraction was not observed. This fact alone would strongly indicate that a contaminant molecule acting independently of the nucleic acid is not responsible for the activity of the fraction.

The second possible general explanation for the immunogenicity of both the crude and purified subcellular preparations is that a molecule in close association with the RNA of the preparation is responsible for the observed anti-salmonella resistance. This in effect would be attributing to the RNA the function of acting in an adjuvant-like manner, and as such would be in support of the work of several other laboratories (15, 19, 22). If the RNA was acting in this capacity, however, one might expect that the efficacy of the preparation would be further increased by the addition of a well-defined adjuvant to the preparation. In contrast, it was observed that the addition of Freund's incomplete adjuvant did not enhance the immunogenicity of the fraction. In a like manner, serial purification and recycling of the immunogenic preparations over DEAE cellulose would be expected to result in the elution of some of this absorbed material into other fractions of the eluate. This was not found to be the case. The possibility cannot be excluded on the evidence accumulated to date, however, that a contaminant may exist so strongly bound to the RNA (perhaps covalently) as to preclude its being eluted at a salt concentration less than that which is required for the elution of the RNA.

The third possibility as to the chemical nature of the immunogen, and the one that appears to be most strongly suggested by the evidence presented in this and previous reports from this laboratory, is that the immunogenicity of the fraction is in some manner intimately associated with the RNA of the pathogen. This concept has recently been further supported by the work of Houchens and Wright, who demonstrated that ribosomes and RNA of *S. typhimurium* were the only subcellular preparations of salmonellae capable of increasing the mean survival time and total survival of animals challenged with the homologous parasite (Bacteriol. Proc., p. 111, 1971).

If the data continue to withstand the test of time as they have over the past several years, then one must necessarily ask the question as to how the RNA elicits an immune state. Ushiba et al. (31) and Margolis and Digley (Bacteriol. Proc., p. 112, 1971) have suggested that immune cells may be characterized by the presence of a surface-associated bactericidal globulin. As a consequence, the function of an immunogen in inducing immunity would be to selectively induce the production of this cell-bound antibody. In recent studies, however, Houchens and Wright (Bacteriol. Proc., p. 111, 1971) have been unable to demonstrate the presence of cell-associated globulins in mice immunized with subcellular ribosomes or RNA. In light of these data, other mechanisms should possibly be explored to explain the efficacy of the immunogenic subcellular preparations.

It is possible that a bactericidal effect mediated by antiviral interferon could be responsible for the observed response. It is well documented that gram-negative bacterial preparations and subcellular fractions can and do induce the production of interferon in immunized animals (1, 28, 29). Foreign nucleic acids are also documented to act in a comparable fashion (13, 23). Similarly, the antibacterial-antifungal effect of interferon or interferon-like substances has received recent support from several laboratories (23, 24, 28). These data suggest that one response to be anticipated in animals immunized with salmonella RNA would be the production of interferon. It is important to note, however, that the degree of resistance induced with the salmonella fractions cannot be explained on the basis of a low-level resistance characteristic of the interferon-mediated bactericidal response (28). Similarly, endotoxin and synthetic polynucleotides are known to be effective inducers of interferon production (23, 24, 28). These substances, however, do not elicit a response comparable to that induced with the immunogenic RNA preparations (35, 36, 47; *unpublished data*). By this reasoning, therefore, it would seem improbable that nucleic acid-induced interferon is responsible for the RNA-elicited anti-salmonella response.

As a final alternative mechanism, it should be considered that the bacterial RNA may possibly be exerting some control over the genetic material of normal cells, thereby resulting in a transformed population of immune monocytes. Although this

mechanism may appear to be highly improbable on the evidence available to date, Brown and Coffey have recently demonstrated the ability of synthetic polyribonucleotides to derepress gene functions in a eukaryotic cell (5). Along similar lines, Huebner and Todaro have suggested that C-type RNA virus, responsible for the spontaneous appearance of cancer in BALB/C mice, may result in the transformed cell state via an influence on the genetic composition of the cell (18). The possibility that situations comparable to these exist in the cellular immune response elicited with the immunogenic RNA fractions of salmonellae is indeed an interesting thought, and may provide a fruitful area of investigation in the near future.

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LITERATURE CITED

1. Absher, M., and W. P. Stinebring. 1969. Endotoxin properties of poly I: poly C., an interferon stimulator. Nature (London) 223:715–717.
2. Albizo, J. M., and M. J. Surgalla. 1970. Isolation and biological characterization of *Pasteurella pestis* endotoxin. Infect. Immunity 2:229–236.
3. Badakhsh, F. F., and M. Herzberg. 1969. Deoxycholate-treated, nontoxic, whole-cell vaccine protective against experimental salmonellosis of mice. J. Bacteriol. 100:738–744.
4. Berry, L. J. 1955. Effect of malonate on growth rate of *Salmonella typhimurium* in mice. Ann. N.Y. Acad. Sci. 62: 327–348.
5. Brown, D. G., and D. S. Coffey. 1971. Release of nuclear template restrictions by specific polyribonucleotides. Science 171:176–178.
6. Chaparas, S. D., D. E. Thor, H. P. Godfrey, H. Baer, and S. R. Hedrick. 1970. Tuberculin-active carbohydrate that induces inhibition of macrophage migration but not lymphocyte transformation. Science 170:637–639.
7. Collins, F. M. 1968. Cross-protection against *Salmonella enteritidis* infection in mice. J. Bacteriol. 95:1343–1349.
8. Collins, F. M. 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in nonvaccinated mice challenged by various routes. J. Bacteriol. 97:667–675.
9. Collins, F. M., and M. Milne. 1966. Heat-labile antigens of *Salmonella enteritidis*. II. Mouse-protection studies. J. Bacteriol. 92:549–557.
10. Curling, J. M. 1970. The use of Sephadex in the separation, purification, and characterization of biological materials, p. 417–484. *In* G. A. Kerkut (ed.), Experiments in physiology and biochemistry, vol. 3. Academic Press Inc., New York.
11. Dische, Z. 1955. Color reactions of nucleic acid components, p. 285–306. *In* E. Chargaff and J. N. Davidson (ed.), The nucleic acids. Academic Press Inc., New York.
12. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. *In* A. Nowotny (ed.), Basic exercises in immunochemistry. 1969. Springer-Verlag, New York.
13. Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Nat. Acad. Sci. U.S.A. 58:1004–1010.
14. Fraenkel-Conrat, H. L. 1966. Preparation and testing of tobacco mosaic virus-RNA, p. 480–487. *In* L. Cantone and D. R. Davis (ed.), Procedures in nucleic acid research, vol. 5. Harper and Row, Evanston, Ill.
15. Friedman, H. P., A. B. Stavitsky, and I. M. Soloman. 1965. Induction *in vitro* of antibodies to T_2 phage antigens in the ribonucleic extract employed. Science 149:1106–1107.
16. Fukushi, K., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner, and E. Ribi. 1964. Extraction and purification of endotoxin from enterobacteriaceae: a comparison of selected methods and sources. J. Bacteriol. 87:391–400.
17. Godfrey, H. P., H. Baer, and S. D. Chaparas. 1969. Inhibition of macrophage migration by a skin-reactive polysaccharide from BC6 culture filtrates. J. Immunol. 102:1466–1473.
18. Huebner, R. J., and G. T. Todaro. 1969. Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Nat. Acad. Sci. U.S.A. 64:1087–1094.
19. Johnson, A. G., and G. Hoekstra. 1969. Acceleration of the primary antibody response, p. 187–190. *In* R. T. Smith, R. A. Good, and P. A. Miescher (ed.), Ontogeny of immunity. Univ. of Florida Press, Gainesville.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
21. Maurer, H. R. 1968. Disc-electrophorese, p. 77. Walter de Gruter and Co., Berlin.
22. Merritt, K., and A. G. Johnson. 1965. Studies on the adjuvant action of bacterial endotoxins on antibody formation. VI. Enhancement of antibody formation by nucleic acids. J. Immunol. 94:416–422.
23. Remington, J. S., and T. C. Merigan. 1968. Interferon: protection of cells infected with an intracellular protozoan (*Toxoplasma gondii*). Science 161:804–806.
24. Remington, J. S., and T. C. Merigan. 1970. Synthetic polyanions protect mice against intracellular bacterial infection. Nature (London) 226:361–363.
25. Schneider, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. J. Biol. Chem. 161: 293–303.
26. Sober, H. A., F. J. Gutter, M. M. Wyckoff, and E. A. Peterson. 1969. *In* A. Nowotny (ed.), Basic exercises in immunochemistry, p. 10–12. Springer-Verlag, New York.
27. Sokal, R. R., and F. J. Rohlf. 1969. Biometry, p. 150–172. W. H. Freeman and Co., San Francisco.
28. Somer, P. De, E. De Clercq, C. Cocito, and A. Billiau. 1970. The interferon inducer from brucella. Ann. N.Y. Acad. Sci. 173:274–281.
29. Stinebring, W. R., and J. S. Youngner. 1964. Patterns of interferon appearance in mice injected with bacteria or bacterial endotoxin. Nature (London) 204:712–714.
30. Thompson, H. C. W., and I. S. Snyder. 1971. Protection against pneumococcal infection by a ribosomal preparation. Infect. Immunity 3:16–23.
31. Ushiba, D., T. Nakae, T. Akiyama, and Y. Kishimoto. 1966. Characterization of "clearance" factor and "cell-bound" antibody in experimental typhoid. J. Bacteriol. 91:1705–1712.
32. Venneman, M. R., and L. J. Berry. 1971. Experimental salmonellosis: differential passive transfer of immunity with serum and cells obtained from ribosomal and ribonucleic acid immunized mice. J. Reticuloendothel. Soc. 9:491–502.
33. Venneman, M. R., and L. J. Berry. 1971. Serum-mediated resistance induced with immunogenic preparations of *Salmonella typhimurium*. Infect. Immunity 4:374–380.
34. Venneman, M. R., and L. J. Berry. 1971. Cellular mediated resistance induced with immunogenic preparations of *Salmonella typhimurium*. Infect. Immunity 4:381–387.
35. Venneman, M. R., and N. J. Bigley. 1969. Isolation and partial characterization of an immunogenic moiety obtained from *Salmonella typhimurium*. J. Bacteriol. 100:140–148.
36. Venneman, M. R., N. J. Bigley, and L. J. Berry. 1970. Immunogenicity of ribonucleic acid preparations obtained from *Salmonella typhimurium*. Infect. Immunity 1:574–582.
37. Westphal, O., and K. Jamm. 1965. Bacterial lipopolysaccha-

rides, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry. Academic Press Inc., New York.
38. Wheat, R. W., M. Berst, E. Ruschmann, O. Lüderitz, and O. Westphal. 1967. Lipopolysaccharides of *Salmonella* T. mutants. J. Bacteriol. 94:1366–1380.
39. Wilcoxon, F., and R. A. Wilcox. 1964. Some rapid approximate statistical procedures. American Cyanamid Co., New York.
40. Winston, S. H., and L. J. Berry. 1970. Antibacterial immunity induced by ribosomal vaccines. J. Reticuloendothel. Soc. 8:13–24.
41. Winston, S. H., and L. J. Berry. 1970. Immunity induced by ribosomal extracts from *Staphylococcus aureus*. J. Reticuloendothel. Soc. 8:66–73.
42. Youmans, A. S., and G. P. Youmans. 1966. Preparation of highly immunogenic ribosomal fractions of *Mycobacterium tuberculosis* by use of sodium dodecyl sulfate. J. Bacteriol. 91:2139–2145.
43. Youmans, A. S., and G. P. Youmans. 1966. Effect of trypsin and ribonuclease on the immunogenic activity of ribosomes and ribonucleic acid isolated from *Mycobacterium tuberculosis*. J. Bacteriol. 91:2146–2154.
44. Youmans, A. S., and G. P. Youmans. 1967. Preparation and effect of different adjuvants on the immunogenic activity of mycobacterial ribosomal fractions. J. Bacteriol. 94:836–843.
45. Youmans, A. S., and G. P. Youmans. 1969. Factors affecting immunogenic activity of mycobacterial ribosomal and ribonucleic acid preparations. J. Bacteriol. 99:42–50.
46. Youmans, A. S., and G. P. Youmans. 1970. Immunogenic mycobacterial ribosomal and ribonucleic acid preparations: chemical and physical characteristics. Infect. Immunity 2:659–668.
47. Youmans, A. S., and G. P. Youmans. 1971. Failure of synthetic polynucleotides to affect the immunogenicity of mycobacterial ribonucleic acid and ribosomal protein preparations. Infect. Immunity 3:149–153.