Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. IV. The Association with Peritoneal Exudate Cell Ribonucleic Acid of the Polypeptide in Immunogenic and Nonimmunogenic Forms*

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ABSTRACT: Poly-γ-D-glutamic acid has not been found to be immunogenic in pure form but elicits antipolypeptide antibodies when complexed with methylated albumin. In either form, tritiated polypeptide was taken up by rabbit peritoneal exudate cells, composed largely of macrophages, *in situ* or in the test tube, and was found at equal levels in ribonucleic acid (RNA) purified by four extractions with phenol and repeated precipitation with ethanol. The label was found exclusively in the 4–5S RNA fraction, which was the only type perceptibly synthesized after the introduction of polypeptide, determined by incorporation of uridine-^{14}C. There was no association between RNA and polypeptide mixed *in vitro*, but association comparable to that obtained with intact cells occurred with cell-free extracts. By using ^{35}S-labeled T2 bacteriophage instead of polypeptide, antigen was again found only in 4–5S RNA. After total hydrolysis of RNA–polypeptide complexes followed by high-voltage electrophoresis on DEAE-cellulose paper, the ^{3}H label was found only in glutamic acid. Using uridine-^{14}C-labeled RNA, it was shown that the associated antigen was still in a polymer form by treatment of the complex with RNase followed by molecular sieving.

The labeled polypeptide could not be dissociated from RNA by sucrose density gradient centrifugation, adsorption chromatography, gel electrophoresis, equilibrium sedimentation in cesium sulfate, or in competition by 10^{4}–10^{5}-fold excesses of unlabeled poly-γ-D-glutamic acid, poly-α-D-glutamic acid, poly-α-L-glutamic acid, or D-glutamic acid. These results indicate that the association of antigen with macrophage RNA is an active biologic process and that the association is strong and possibly covalent. Association with macrophage RNA does not appear to distinguish between antigens on the basis of immunogenicity.

The sequence of events initiated by an antigen which terminates with the appearance of antibody is still largely a mystery. Considerable interest has been engendered by the provocative report that RNA, extracted from peritoneal exudate cells which had been incubated with T2 bacteriophage, could induce the synthesis of neutralizing antibody by unprimed lymphoid cells (Fishman and Adler, 1963). This activity was sensitive to RNase but refractory to proteolytic enzymes. Subsequently, several investigators demonstrated the presence of antigen in similar RNA preparations, using the same (Friedman *et al.*, 1965) and other (Askonas and Rhodes, 1965) antigens, raising questions about the role of RNA in the experimental system. However, a more recent report, if corroborated, definitely assigns to the donor RNA an informational capacity (Adler *et al.*, 1966).

The capsular polypeptide of *Bacillus anthracis* (poly-γ-D-glutamic acid) has thus far not been found to elicit detectable serum antibody in rabbits when injected in pure form at doses ranging from 0.01 to 10 mg. On the other hand, animals immunized with the polypeptide complexed with an immunogenic carrier, methylated albumin, regularly respond with antibodies which precipitate with the polypeptide itself (Goodman and Nitecki, 1967). In order to assess the significance of the association of antigen with peritoneal cell RNA in the immune response, the uptake by these cells of poly-γ-D-glutamic acid in its immunogenic form and in the form in which it is at best a very weak immunogen was compared. The quantitative association of the polymer with cellular RNA, as well as the tenacity and specificity of this association, was investigated.

Materials and Methods

Polypeptides. (1) Poly-γ-D-glutamic acid: The purification and characterization of the capsular polypeptide of *B. anthracis* (strain M36) has been described (Goodman and Nitecki, 1966). It has an average molecular weight of 33,500 by end-group analysis. (2) Synthetic poly-α-D-glutamic acid (average mol wt 42,000) was from Yeda, Israel. (3) Synthetic poly-α-L-glutamic acid (average mol wt 6400) was kindly provided by Dr. Paul Maurer. (4) D-Glutamic acid was obtained from Mann Research Laboratories.

Poly-γ-D-glutamic was radiolabeled by exposure to

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tritium gas (New England Nuclear Corp.). After removal of the degradation products by exhaustive dialysis and chromatography on Sephadex G-50, the high molecular weight material had a specific activity of 51 μCi/mg.

The optical purity of the radiolabeled polypeptide was ascertained by gas–liquid partition chromatography (Westley, 1967). The hydrolyzed polypeptide was treated with *N*-trifluoracetyl-L-prolyl chloride to form *N*-trifluoracetyl-L-prolylglutamic acid dimethyl esters and analyzed on a Varian Aerograph 1200, using a column with dimensions of 60 $\times$ $^1/_8$ in. (Goodman *et al.*, 1968). During the analyses, the temperature was maintained at 200° and the nitrogen flow was 30 cc/min. Under these conditions, the retention times for the D and L isomers were 10.7 and 12.0 min, respectively. The *N*-trifluoracetyl-L-prolyl chloride reagent contained 3.5% of the D-prolyl form as determined by assay with sterically pure (−)-ephedrine. The corrected values for analysis of the polypeptide before and after tritiation were indistinguishable and gave a maximum of 3% for the L isomer. The precision of the method is about ±3%.

Rabbit Peritoneal Exudate Cells. New Zealand white rabbits weighing 2.5–3 kg were injected intraperitoneally with 50 ml of sterile liquid petrolatum. After 4 days the animals were sacrificed and their peritoneal exudates were harvested with 200 ml of a solution (I) containing phosphate buffer (0.015 M, pH 7.4), NaCl (0.14 M), heparin (1:20,000), and 3% normal rabbit serum. The oil was removed in a separatory funnel and the cells were centrifuged and washed with solution I. The yield of cells per rabbit varied from 2 $\times$ 10^8 to 10^9; 80–90% represented large mononuclear cells with the morphology of macrophages. The remainder consisted of small lymphocytes and polymorphonuclear leucocytes. The number of cells harvested without prior injection of petrolatum was about 1% of that obtained when petrolatum was used.

Exposure of Peritoneal Exudate Cells to M36-^{3}H Polypeptide. Rabbit peritoneal exudate cells were exposed to M36-^{3}H polypeptide either *in situ* or in the test tube. The polypeptide was introduced in either of two insoluble forms: as a precipitate with alum (M36-^{3}H-alum) (Porter, 1958) or complexed with methylated bovine serum albumin M36-^{3}H-MBSA) (Goodman and Nitecki, 1967). For *in situ* experiments, 20–110 μCi of polypeptide was injected intraperitoneally 2.5 hr before peritoneal exudates were harvested. For experiments in the test tube, 2 $\times$ 10^8–10^9 peritoneal cells from individual rabbits were suspended in 2 ml of solution I and incubated for 1 or 5 hr at 37° with 110–166 μCi of polypeptide, after which they were pelleted and washed with the same solution.

For cell-free experiments, 5 $\times$ 10^8 cells were suspended in 0.015 M sodium phosphate buffer (pH 7.4)–0.1 M NaCl and homogenized by repeated stroking in a Dounce tissue grinder. The grinder disrupts more than 99% of the cells with a single stroke. The homogenate was incubated with 11.2 μCi of M36-^{3}H-alum for 1 or 2.5 hr at 37° prior to purification of RNA.

Preparation of RNA. The preparation of RNA from peritoneal exudate cells was based on the method described by Glišin and Glišin (1964), as modified by Gottlieb *et al.* (1967), and involved four extractions with phenol. Washed cells were suspended for 15 min in 5 ml of solution II (0.01 M sodium acetate buffer (pH 5.0), 0.1 M NaCl, and 0.5% sodium dodecyl sulfate) and homogenized in a Dounce tissue grinder. The material was extracted with an equal volume of liquified phenol saturated with solution II for 15 min in the cold. The interphase was resuspended in 5 ml of solution II with 0.001 M $MgCl_2$ and again extracted with phenol. The water phases of both extractions were pooled and reextracted with phenol and the interphase was again suspended in 5 ml of solution II and phenol was extracted at 60° for 6 min.

The pooled water phases were treated with electrophoretically purified DNase (Worthington Biochemical Corp.) for 20 min at room temperature, followed by two additional extractions with equal volumes of phenol in the cold. Two volumes of ethanol were added to the last water phase and the solution was stored at −20° overnight.

The RNA was recovered by centrifugation, resuspended in a minimal volume of solution II, and dialyzed overnight against 10^3 volumes of solution II. This yielded a product with a 260 mμ/280 mμ extinction ratio of 2.0 in quantities of 150–267 μg/rabbit.

Each time a sample of RNA from a given preparation was used, the RNA was precipitated by addition of two volumes of ethanol. After centrifugation and decantation of the supernatant, the RNA was dissolved in the desired buffer. Some preparations were reprecipitated as many as five times. In some experiments, RNA was labeled with ^{14}C by adding 5 μCi of uridine-2-^{14}C (New England Nuclear Corp.) to a suspension of peritoneal exudate cells at the time of addition of M36-^{3}H polypeptide.

Sucrose Density Gradient Ultracentrifugation. RNA in solution II was layered on top of a 12-ml, 5–30% sucrose linear gradient (0.01 M acetate buffer (pH 5.0), 0.1 M NaCl, and 0.001 M EDTA) and centrifuged in a Spinco L2 preparative ultracentrifuge (SW41 rotor for 6 hr at 190,000*g* or SW50 rotor for 24 hr at 78,000*g*). The absorbency at 260 mμ in the gradient was measured with a Gilford recording spectrophotometer and fractions of 12 drops were collected. Unless otherwise stated, 10 μl from each tube was counted in a Beckman scintillation system after addition of 10 ml of a scintillation fluid consisting of 100 g of napthalene and 5 g of 2,5-diphenyloxazole brought to 1 l. with dioxane. Individual fractions were conserved at −20° after addition of two volumes of ethanol.

Cellulose Chromatography. RNA-^{14}C–M36-^{3}H complexes were chromatographed on cellulose columns using an ethanol–buffer system which resolves the major molecular species of RNA (Barber, 1966; Franklin, 1966). Whatman cellulose CF11 was repeatedly suspended in STE buffer (0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.001 M EDTA) containing 35%ethanol and the fine particles were decanted. The cellulose was stirred overnight in STE buffer containing 1% β-mercaptoethanol and resuspended twice in 35% ethanol–STE. A 10 $\times$ 0.3 cm column was poured and washed with

TABLE I: The Association of M36-^{3}H with Peritoneal Cells and Peritoneal Cell RNA.

Method of Exposure to M36-^{3}H			Total Dose of M36-^{3}H (μCi)	% Total ^{3}H in cells	% Intracellular ^{3}H on RNA	M36[a]/4S RNA[b] (μg/μg)	M36[a]/4S RNA[b] per Intracellular ^{3}H (μg/μg μCi)
In situ intraperitoneal injection	With oil	Alum precipitated	64	2.5	1.8	0.007	4.4×10^{-3}
		MBSA precipitated	20	1.25	2.8	0.002	8.0×10^{-3}
	Without oil	Alum precipitated	86	0.05	7.2	0.004	9.0×10^{-2}
		MBSA precipitated	30	0.26	5.5	0.004	5.1×10^{-2}
In the test-tube: cell suspensions		Alum precipitated	86	21	17.7	0.14	7.6×10^{-3}
			86	18	14.5	0.14	9.0×10^{-3}
			115[c]	13	5.8	0.08	5.4×10^{-3}
		MBSA precipitated	115	7.6	9.6	0.04	4.6×10^{-3}
Cell-free preparations		Alum precipitated	11.2		5.7	0.11	1.0×10^{-3}
			11.2		4.8	0.10	0.9×10^{-3}
Control							
4S RNA + alum precipitated			86[e]		0.003	0.0007	8.0×10^{-6}
4S RNA + M36-^{3}H			104[e]		0.06	0.001	9.0×10^{-6}
28S RNA + M36-^{3}H			104[e]		0.06	0.001[d]	9.0×10^{-6}[d]

[a] The quantity of M26 is based on specific radioactivity. [b] The quantity of RNA is based on optical density at 260 mμ. [c] Incubated with cell for 5 hr. [d] M36-^{3}H/28S RNA. [e] Total dose is considered intracellular in calculations.

35% ethanol–STE. M36-^{3}H or RNA-^{14}C–M36-^{3}H complexes obtained from sucrose gradients was dissolved in 0.3 ml of the same buffer and applied to the column. Fractions containing 20 drops were collected in scintillation vials. After 25 fractions were collected, the ethanol concentration of the buffer was lowered to 15%, and after an additional 25 fractions the column was eluted with the buffer itself.

Equilibrium Centrifugation in Cesium Sulfate. The technique described by Bishop *et al.* (1965) was used. Solutions of Cs_2SO_4 (Stanley H. Cohen Co.) in 0.005 M Tris buffer (pH 7.4) or 0.01 M acetate buffer (pH 5.0)–0.001 M EDTA, ρ 1.60 at 5°, containing either 33 μg of RNA-^{14}C–M36-^{3}H complexes or a mixture of 33 μg of RNA-^{14}C and 2.6 μg of M36-^{3}H, in volumes of 5.0 ml were centrifuged in a Spinco Model L ultracentrifuge for 80 hr at 35,000 rpm in an SW50 rotor at 5°. After the run, the bottoms of the tubes were punctured and fractions of 5 drops for density analysis and 15 drops for radioactivity determination were collected. Densities were calculated from the refractive index of the samples (Hearst and Vinograd, 1961).

Polyacrylamide Gel Disc Electrophoresis. RNA-^{14}C–M36-^{3}H complexes were electrophoresed at 5 mA for 45 min in 5% polyacrylamide gel columns (pH 7.8) (Loening, 1967). The gels were frozen and sectioned into 1-mm slices with a McIlwain cytostat. The slices were put in glass counting vials and dissolved in 30% H_2O_2 at 37°. Radioactivity was determined after addition of NCS solubilizer (Nuclear Chicago Corp.) to the scintillation fluid.

High-Voltage Electrophoresis. M36-^{3}H polypeptide and RNA polypeptide complexes were hydrolyzed with 6 N HCl and electrophoresed on DEAE-cellulose paper at pH 3.5, 2500 V, for 2.5 hr (Goodman *et al.*, 1968). The papers were dried and cut into 1 × 2 cm sections for radioactivity measurements in the liquid scintillation system.

Molecular Sieving. Sephadex G-200 in microcolumns with a total volume of 2.5 ml was used to evaluate the association of ^{3}H and ^{14}C in RNA-^{14}C–M36-^{3}H complexes before and after treatment with beef pancreatic ribonuclease (Boehringer & Mannheim Corp.), used at a concentration of 300 μg/ml. Fractions containing 5 drops were collected from the columns by elution with 0.1 M NaCl.

Competition Experiments. Quantities of RNA–M36-^{3}H complex containing 2000–5000 cpm were mixed with 10^4–10^5 times as much unlabeled polypeptide or glutamic acid, on a weight basis, to determine the specificity and tenacity of the association. The mixtures were incubated in volumes of 0.4 ml of 0.015 M sodium phosphate buffer (pH 7.4)–0.14 M NaCl, at room temperature for 24 hr. RNA was precipitated with two volumes of

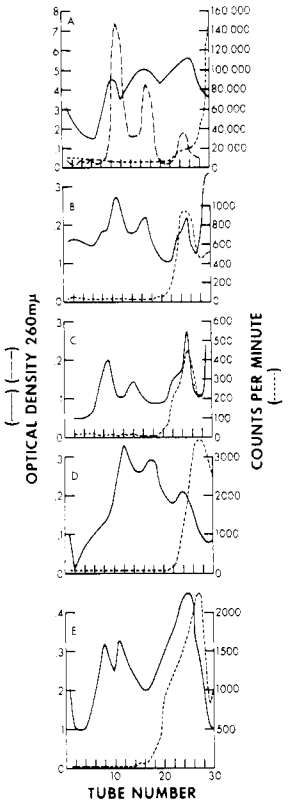

FIGURE 1: Sucrose density gradient ultracentrifugation of cellular RNA and M36-^{3}H. Centrifuged for 6 hr at 190,000*g*. The top of the gradient is at the right. (A) – – –, Hela cell RNA showing typical 28S, 16S, and 4S peaks; ----, free M36-^{3}H; ——, peritoneal exudate cell RNA from cells unexposed to M36-^{3}H. (B) RNA from peritoneal exudate cells incubated with M36-^{3}H–alum for 2.5 hr *in situ*. (C) RNA from peritoneal exudate cells incubated with M36-^{3}H–MBSA for 2.5 hr *in situ*. (D) RNA from suspensions of peritoneal exudate cells incubated with M36-^{3}H–alum for 1 hr in the test tube. (E) RNA from suspensions of peritoneal exudate cells incubated with M36-^{3}H–MBSA for 1 hr in the test tube.

ethanol, and radioactivity in the precipitate and in the supernatant was determined. Controls in which RNA from cells which had not been exposed to polypeptide was mixed *in vitro* with labeled polypeptide were included.

^{35}S-Labeled T2 Bacteriophage. T2 phage were grown in *E. coli* strain B in M9 medium (Adams, 1959) supplemented with glycerol (0.4%) and gelatin (10 μg/l.), in which $MgSO_4$ was replaced by $MgCl_2$. ^{35}S (1 mCi) as Na_2SO_4 was added per 100 ml of medium (New England Nuclear Corp., specific activity 1.75 mCi/mg). Bacteria were infected at a multiplicity of 0.1 and treated with chloroform after 4.5 hr.

The lysate was purified by successive low- and high-speed centrifugation and zonal sedimentation in sucrose. The product was dialyzed against sodium phosphate buffer (0.015 M, pH 7.4)–0.14 M NaCl. The final phage preparation, suspended in 4 ml, contained 3.2×10^{10} PFU[1]/ml with a specific radioactivity of 0.16 μCi/10^{10} particles. The phage suspension (2 ml) was incubated with 5×10^8 peritoneal exudate cells at 37° for 30 min, after which the cells were washed and RNA was extracted as described.

[1] PFU = plaque-forming units.

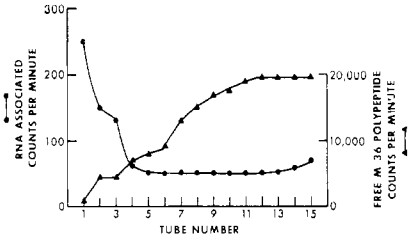

FIGURE 2: Sucrose density gradient ultracentrifugation for 24 hr at 78,000*g* of free M36-^{3}H and RNA–M36-^{3}H complexes. Sedimentation is to the left.

Results

Association of M36-^{3}H with Peritoneal Exudate Cells and Peritoneal Exudate Cell RNA. Table I gives the quantities of M36-^{3}H in microcuries which were injected intraperitoneally into rabbits, exposed to suspensions of cells in the test tube, or mixed with cell-free extracts. The cell-associated radioactivity was determined by summation of the activities in the pooled phenol phases, the interphases, and the final water phase of the extraction procedure. Attempts to directly determine the radioactivity of intact cells led to clumping and severe quenching in the scintillation medium. The radioactivity associated with RNA was measured after dialysis of the preparations against solution II, which contained sodium dodecyl sulfate.

In general, no correlations could be made between total dose of M36-^{3}H and the quantities of cell-associated and RNA-associated radioactivity (Table I).

In sucrose density gradients, tritium appeared exclusively in the region of 4–5S RNA, whether alum- or MBSA-precipitated polypeptide was used or whether the association occurred *in situ* or in the test tube (Figure 1). As little as 0.05% of the total bound radioactivity could have been detected in other regions of the gradient. In the test-tube experiments, the label was consistently displaced with respect to the 4–5S RNA peak (Figure 1D,E), whether incubation of M36-^{3}H with cells lasted for 1 or 5 hr, and appeared to be associated with a fraction of smaller molecular size. However, since the free polypeptide remained at the top of the gradient (Figure 1A), even after centrifugation for 24 hr, which pellets the RNA-associated radioactivity (Figure 2), the displacement was probably due to a shift in sedimentation caused by the associated polypeptide. The shift was inconspicuous in *in situ* experiments perhaps because only about 10% as much polypeptide was RNA associated.

Free M36-^{3}H and RNA-^{14}C–M36-^{3}H complexes obtained from sucrose gradients were both eluted from cellulose columns in the 35% ethanol–STE buffer fraction. However, the ^{3}H and ^{14}C labels in the complexes were eluted simultaneously, whereas M36-^{3}H itself was removed distinctly earlier (Figure 3), providing

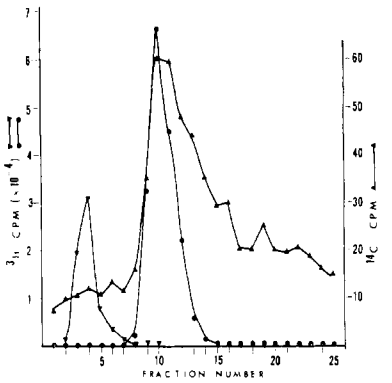

FIGURE 3: Chromatography on cellulose columns of M36-^{3}H polypeptide (▼——▼) and M36-^{3}H–RNA-^{14}C complexes (▲——▲ and ●——●). The free polypeptide is eluted prior to the RNA-associated polypeptide, which appears with the RNA. Elution was with 35% ethanol–STE buffer.

additional evidence of firm association between antigen and RNA.

The results of equilibrium sedimentation in cesium sulfate gradients are shown in Figures 4 and 5. Each of the components in a control mixture of RNA-^{14}C and M-36^{3}H banded at separate positions in the gradient at pH 5.0 (Figure 4A) and at pH 7.4 (Figure 5A). The polypeptide remained at the top of the gradients while the principal RNA band appeared at ρ 1.68. Two additional minor RNA bands were found in the pH 7.4 gradient at ρ 1.61 and 1.52. These could be RNA–protein complexes, but the polypeptide was clearly not associated with either of them. Resolution was less satisfactory in the pH 5.0 gradient as larger samples were taken for analysis. This gradient was charged with less material in order to minimize the possibility of "trapping" polypeptide in the RNA bands. At pH 5.0 the two labels were essentially coincident throughout the gradient (Figure 4B). Most of the ^{3}H was in the major RNA band at ρ 1.68, but minor bands at ρ 1.58 and 1.48 were found. At pH 7.4 there was evidence of dissociation of the radiolabels after 80-hr centrifugation (Figure 5B). This bears a gross resemblance to the alkaline lability of aminoacyl-tRNA bonds (Gilbert, 1963).

Disc electrophoresis in polyacrylamide gels showed that M36-^{3}H remained near the gel origin whereas 4S RNA banded near the bottom. With RNA-^{14}C–M36-^{3}H complexes, the two isotopes were distributed throughout the gel between the positions of the components in pure form. However, discrete bands of RNA–polypeptide complex were not seen, perhaps due to heterogeneity of the polypeptide or to the relatively alkaline gel.

The most valid basis of comparing the quantitative association of polypeptide with RNA in the various experiments seemed to be in terms of the amount of M36-^{3}H per unit quantity of RNA per microcurie of intracellular ^{3}H, expressed as micrograms per microgram per microcurie. In the *in situ* experiments, this ratio varied from 4.4×10^{-3} to 9.0×10^{-2} (Table I). Petrolatum, which increased the numbers of cells by about 100-fold, decreased the ratio by a factor of about 10.

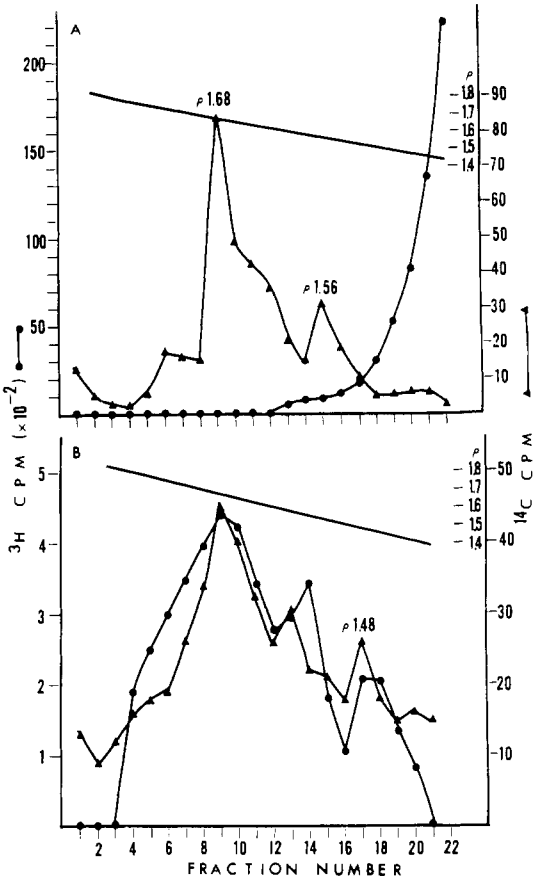

FIGURE 4: Equilibrium centrifugation in Cs_2SO_4 for 80 hr at 35,000 rpm, 5°, pH 5.0. (A) Mixture of RNA-^{14}C and M36-^{3}H polypeptide. (B) RNA-^{14}C–M36-^{3}H complexes.

Of particular interest, MBSA exerted no apparent quantitative effect on the association. In each of the two sets of *in situ* experiments, with oil and without oil, the value for the MBSA-complexed polypeptide was comparable to that for the polypeptide–alum complex.

Experiments performed with suspensions of cells gave about a tenfold greater uptake, probably because they were carried out with quantities of cells and M36-^{3}H comparable to *in situ* experiments but in a volume of 2.0 ml rather than the peritoneal cavity, thus enhancing the efficiency of contact by the cells. The proportion of intracellular ^{3}H associated with 4S RNA was 10- to 20-fold greater than in the *in situ* experiments in which oil was used to harvest the cells (Table I). Again, the presence of MBSA did not implement association. The degree of association was comparable for incubation times of 1 and 5 hr. While less than 4% of the polypeptide exposed to suspensions containing at least 10^{8} cells/ml was found in purified RNA, the ratio of polypeptide to 4–5S RNA, on either a weight or molar basis (since the molecular weights of the two are similar), was as high as 0.14. As the distribution of ^{3}H was asymmetric with respect to 4–5S RNA, the polypeptide was associated with only a part of this RNA, putting the ratio even higher.

As many as five reprecipitations with ethanol of the RNA complexed with M36-^{3}H did not result in significant diminution of the associated radioactivity.

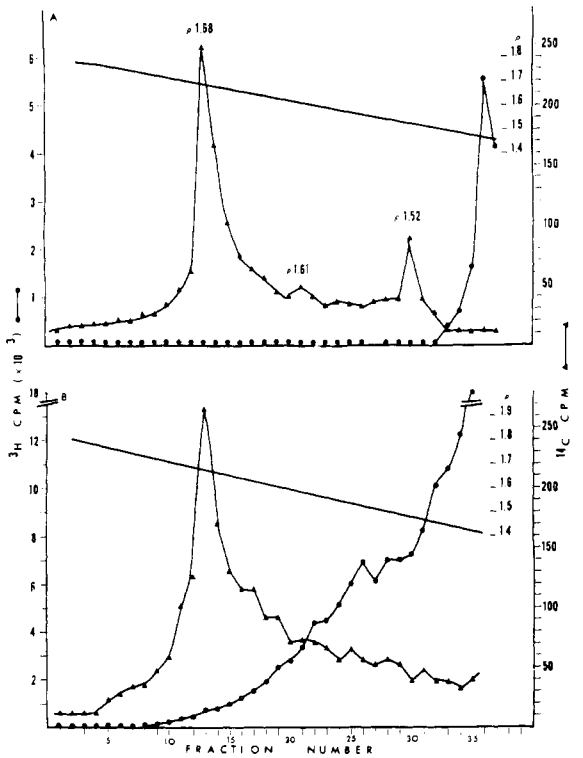

FIGURE 5: Equilibrium centrifugation in Cs_2SO_4 for 80 hr at 35,000 rpm, 5°, pH 7.4. (A) Mixture of RNA-^{14}C and M36-^{3}H. (B) RNA-^{14}C–M36-^{3}H complexes.

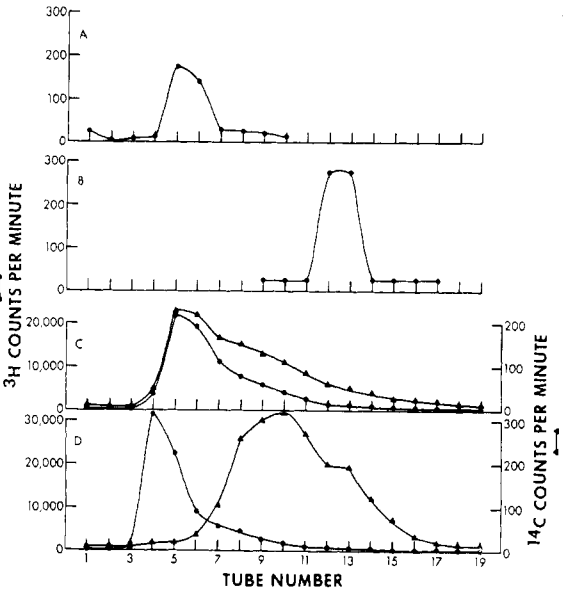

FIGURE 6: Chromatograms from a 2.5-ml column of Sephadex G-200, eluted with 0.1 M NaCl. (A) M36-^{3}H. (B) Totally hydrolyzed M36-^{3}H. (C) RNA-uridine-^{14}C–M36-^{3}H complexes. (D) RNA-uridine-^{14}C–M36-^{3}H complexes treated with RNase.

When M36-^{3}H–alum was mixed with a crude extract of homogenized PE cells for either 1 or 2.5 hr prior to purification of RNA, association was of the same order of magnitude as that obtained with suspensions of intact cells (Table I). The total dose of ^{3}H was considered intracellular in the calculations, which may give rise to a misleadingly low value, but the significant point is that association at a level at least 10^2 times that of controls (see below) was obtained in a cell-free system.

Control experiments were performed in which the RNA extracted from 2×10^8–10^9 cells was incubated with M36-^{3}H, either alum precipitated or soluble, in phosphate buffer (0.015 M, pH 7.4, 0.15 or 2.0 M NaCl) for 1 hr at 37° and then submitted to the same treatment used for extracting cellular RNA. However, cell suspensions were washed to remove the extracellular polypeptide, while in the controls phenol extraction necessarily took place in the presence of all the added polypeptide. Nevertheless, the micrograms of M36 per microgram of RNA per microcurie of ^{3}H for the controls were of the order of only 0.1–1% of the values for the cell suspensions or cell-free extract (Table I), whether 4S or 28S RNA was used.

The Nature of the Radioactive Label Associated with RNA. Hydrolyzed M36-^{3}H and 4–5S RNA–M36-^{3}H, either digested with RNase or hydrolyzed with 6 N HCl, were subjected to high-voltage electrophoresis on DEAE-cellulose paper. Under the conditions used, D-glutamic acid migrates rapidly toward the cathode. The position of D-glutamic acid was established by running an authentic sample on the same sheet of paper and staining with ninhydrin. The positions of the radioactive samples were assessed by cutting the remainder of the sheet into 1 × 2 cm sections which were counted by liquid scintillation. The recovery of radioactivity on paper was estimated by comparing identical quantities of each sample directly in scintillation fluid and on paper in scintillation fluid. In each case, the sample applied to paper gave only about 25% of the counts per minute given by the same sample put directly into fluid. After correction for quenching, 111% of the counts in hydrolyzed M36-^{3}H was found at the position of D-glutamic acid; 100% of the radioactivity in RNase-treated RNA–M36-^{3}H remained at the origin, and 76% of the counts in the hydrolyzed sample was found at the position of D-glutamic acid. Radioactivity was not detected at other locations on the papers. Thus, it appeared that the label in the 4–5S RNA was in glutamic acid.

To determine if the glutamic acid associated with 4–5S RNA was highly degraded or still in a polymer form, 4–5S RNA–M36-^{3}H, radiolabeled with uridine-^{14}C, was passed through a Sephadex G-200 column in native form and after treatment with RNase. The column was calibrated with hydrolyzed and unhydrolyzed M36-^{3}H to define the positions of the native polypeptide and D-glutamic acid (Figure 6). The RNA-^{14}C–M36-^{3}H complex was eluted at about the same position as the polypeptide itself. However, after RNase treatment, almost all of the ^{14}C was eluted later than the ^{3}H. Thus, the tritiated glutamic acid associated with 4–5S RNA was still in a polymer form, although this method does not permit precise comparison with the native polypeptide.

The 4–5S was the only type of RNA labeled when uridine-^{14}C was added to cell suspensions with the poly-

TABLE II: Competition Experiments to Determine Dissociability of M36-^{3}H from RNA.

	Source of RNA			
	M36-^{3}H–Alum		M36-^{3}H–MBSA	
Competitor[a]	Supernatant[b]	Precipitate[b]	Supernatant[b]	Precipitate[b]
Saline	10	90	10	90
Poly-α-L-Glu	10	90	5	95
Poly-α-D-Glu	5	95	3	97
M36-polypeptide	6	94	3	97
D-Glu	5	95	4	96

Control	Supernatant[b]	Precipitate[b]
RNA mixed *in vitro* with free M36-^{3}H	96	4

[a] Competitors used in quantities of 10^4–10^5 that of M36-^{3}H associated with RNA, calculated on the basis of specific radioactivity. [b] Precipitation by two volumes of ethanol. Values in per cent total counts per minute.

peptide. Therefore, antigen becomes associated only with the fraction which contains newly synthesized RNA. It would be of obvious interest to know if antigen becomes associated exclusively with newly synthesized RNA.

Competition Experiments. Attempts were made to dissociate the labeled polypeptide from RNA–M36-^{3}H complexes with large excesses of unlabeled M36 polypeptide, other glutamyl polypeptides, and D-glutamic acid. There was no significant association of M36-^{3}H with RNA mixed *in vitro* (Table II). On the other hand, none of the unlabeled competitors could displace significant radioactivity from RNA–M36-^{3}H complexes derived from cells exposed to the polypeptide in either of the two forms used, when compared to controls which employed saline instead of competitor. Consequently, in conjunction with the other experimental data, it can be inferred that the association between RNA and polypeptide is very firm and possibly covalent.

Association of T2-^{35}S Bacteriophage with Peritoneal Exudate Cell RNA. Since Gottlieb *et al.* (1967) found "immune activity" in a 28S RNA from rat peritoneal exudate cells, the association of this antigen with rabbit peritoneal exudate cell RNA was examined. Only 0.65% of the 1.0 μCi of T2 incubated with peritoneal exudate cells was taken up by the cells. Of the total intracellular antigen, 9% was associated with purified RNA, a value consistent with those for M36-^{3}H. Sucrose density gradient profiles of the RNA showed detectable label only in the 4–5S fraction. However, because of the very low specific radioactivity of the bacteriophage, 1 cpm/2×10^4 particles, and the small percentage of total T2 taken up by the cells, less than about 30% of the total radioactivity found in the gradients could not have been detected in another fraction. Thus, it is not possible to exclude the existence of antigen in RNA other than the 4–5 S, but one can conclude that most of the antigen associated with RNA, as in the case of M36 polypeptide, is found in that fraction. It is interesting to note that the amount of ^{35}S found in the total RNA from 5×10^8 cells corresponds to 4×10 phage particles.

Discussion

The findings presented here confirm that antigen becomes associated with RNA from peritoneal exudate cells, that the associated complex appears exclusively in the 4–5S region which alone contains newly synthesized RNA, is very firm, and that association does not discriminate between molecules on the basis of immunogenicity. The cell preparations consist largely of macrophages, which are known to ingest foreign substances, and it seems reasonable that antigen becomes associated with RNA from cells of this type. Antigen has not yet been demonstrated in cells of the lymphoid series (Dutton, 1967). Greater association was obtained without the use of oil to increase the yield of cells, so it could not be attributed to an artifact introduced by this material.

The radiolabel was found exclusively in glutamic acid, on the basis of electrophoretic mobility, after purification of RNA and was still in a polymer form (Figure 3). The extent of degradation of other antigens prior to association with RNA is an open question, since the cells may lack enzymes capable of attacking poly-γ-D-glutamic acid.

That the polypeptide is bound to and not merely contaminating the preparations of RNA was shown by the following. (1) The very thorough purification, involving four extractions with phenol and repeated precipitation with ethanol, did not dissociate polypeptide from RNA; (2) RNA and polypeptide mixed *in vitro*, either under isoionic conditions or in the presence of 2 M sodium chloride to reduce electrostatic repulsion, showed no significant association; (3) RNA and complexed polypeptide were eluted together from cellulose columns, whereas free polypeptide was eluted at a different position; (4) density gradient centrifugation in sucrose or cesium sulfate discriminated between free polypeptide

and the complexes; and (5) the RNA–polypeptide complexes could not be dissociated by large excesses of the homologous and heterologous polypeptides or by D-glutamic acid. Those observations suggest that binding is an active biologic process. Moreover, cell-free extracts incubated with M36-^{3}H produced association comparable in degree to that obtained with intact cells. This aspect of the study is under intensive investigation.

The nature of the bonding between antigen and RNA is of obvious interest. A distinctive RNA–antigen band was not found in cesium sulfate gradients; instead, the polypeptide appeared in all the RNA bands (Figure 4) and, unexpectedly, did not significantly alter the position of the major band at ρ 1.68. However, a pronounced alkaline lability of the complex was indicated by the gradient run at pH 7.4 (Figure 5). Aminoacyl-tRNA bonds and growing polypeptide chain tRNA bonds are also alkaline labile, the latter being more resistant (Gilbert, 1963). A comparative study of the alkaline lability of antigen–RNA and aminoacyl-tRNA bonds is in progress.

Another point of fundamental interest lies in the association of polypeptide with newly synthesized RNA. In experiments employing ^{14}C-labeled RNA, association of M36-^{3}H only with newly synthesized RNA was being followed (Figures 3, 4, and 6). Does antigen become associated *only* with RNA synthesized after its introduction? Experiments to resolve this question are in progress.

Significant distinctions could not be drawn between the polypeptide precipitated with alum or with methylated albumin as far as association with RNA is concerned. In the former form, the polypeptide is at best very weakly immunogenic; we have not found serum antibodies in rabbits immunized with repeatedly administered doses of pure polypeptide ranging from 10 μg to 10 mg per injection. On the other hand, albumin complexes regularly elicit substantial quantities of antipolypeptide antibody (Goodman and Nitecki, 1967). Thus, at this stage of the "immune pathway," if antigen–macrophage RNA complexes are indeed involved, no distinction is made on the basis of immunogenicity.

It has been assumed that antibody is needed for the uptake of foreign substances by macrophages (Lennox and Cohn, 1967). Minute quantities of antibody to a large variety of antigens have been detected in normal individuals, and macrophages readily adsorb antibody to their surfaces. While it was not possible to exclude the presence of traces of antibody which react with poly-γ-D-glutamic acid in normal rabbits, antibodies to this immunogenically inert polymer have not been demonstrated in the absence of an immunogenic carrier; yet the polypeptide was taken up by peritoneal exudate cell suspensions equally in the presence or absence of MBSA. Association with RNA occurred in the absence of intact cells.

The relationships between antigen, peritoneal exudate cell RNA, and the antibody response are confusing at present. Following the report that RNA from cells which had been incubated with T2 phage could induce antibody synthesis by unprimed lymphoid cells (Fishman and Adler, 1963), other investigators demonstrated antigen in similar preparations (Friedman *et al.*, 1965; Askonas and Rhodes, 1965). Fishman characterized the active fraction as a low molecular weight RNA; Askonas and Rhodes found antigen at the position of 4–5S RNA in sucrose density gradient profiles. Although antibody-inducing activity was sensitive to RNase, it was suspected that RNA served a nonspecific function in the experimental system.

Gottlieb *et al.* (1967) used unlabeled T2 phage and extended the study by stringently purifying rat peritoneal exudate cell RNA and assaying activity of density gradient fractions separately. Activity was found only in the 28S fraction, but was unimpaired upon degradation to a 4–6S form and was sensitive to both Pronase and RNase, although a much higher RNase:substrate ratio was needed than in the other studies cited. These results indicate that activity resides in preexisting RNA rather than in RNA synthesized following exposure to antigen. However, the presence of antigen in the active fraction was not established, as unlabeled T2 was used and the distinctive band in cesium sulfate gradients which bore activity and Pronase sensitivity was also found in control preparations from unexposed cells. In the present investigation, T2, like poly-γ-D-glutamic acid, was detectably associated only with 4–5S RNA from rabbit peritoneal exudate cells. The RNA preparations were examined immediately after purification and the ratios of the molecular species in the gradient profiles were analogous to those of undegraded RNA in the study of Gottlieb *et al.* Therefore, we do not feel that the antigen-associated 4S RNA in this study was a degradation product of a 28S RNA.

An informational role for macrophage RNA in the immune response remains an intriguing possibility, strengthened by a recent report in which peritoneal exudate cell RNA from rabbits of one allotype induced in lymphoid cells from an allelic type antibodies with genetic determinants of the donor (Adler *et al.*, 1966). RNA (4–5 S) is composed of about 70–80 nucleotides which might code for perhaps 25 amino acids. The variable regions of antibody polypeptide chains which determine specificity are limited to a sequence of no more than about 100 residues in the light chain. It is not yet clear whether the variable segment of the heavy chain is similar in extent. Amino acid sequence data indicate that about 40% of the residues in this region vary and that there is no gradient of variability along the sequence (reviewed by Lennox and Cohn, 1967). However, not all of the 40 variable positions may be involved in antibody specificity. If the small RNA acts directly in an informational capacity, as suggested by Adler *et al.* (1966), it might be postulated that it becomes inserted into a larger messenger in the small lymphocyte and codes for both specificity and allotypic amino acids. Insertion rather than separate translation is necessitated by evidence indicating that the polypeptide chains are single synthetic units (Lennox and Cohn, 1967). There is no known precedent for such an event in RNA, but the insertion of temperate bacteriophage DNA into the bacterial genome is an established analogy.

At this stage it is difficult to reconcile the data of the various investigators contributing to this subject. If

Fishman's and Gottlieb's active RNAs are the same, then the Pronase sensitivity and preexisting character of the material argue against, but do not preclude, an informational role. If the RNA which associates with polypeptide in the present study can be equated with the active material, its nondiscriminatory behavior toward weak and strong immunogens also makes a direct informational role suspect. On the other hand, there are indications that at least two kinds of active RNA are involved, one which is associated with antigen and another which is not (Fishman *et al.*, 1964). It has been postulated that the antigen-free RNA has informational significance. The interrelationships of these various observations, complicated by the use of cell preparations which do not consist exclusively of macrophages, remain to be elucidated.

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References

Adams, M. H. (1959), Bacteriophages, New York, N. Y., Interscience, p 446.

Adler, F. L., Fishman, M., and Dray, S. (1966), *J. Immunol. 97*, 554.

Askonas, B. A., and Rhodes, J. M. (1965), *Nature 205*, 470.

Barber, R. (1966), *Biochim. Biophys. Acta 114*, 422.

Bishop, J. M., Summers, D. F., and Levintow, L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1273.

Dutton, R. W. (1967), *Advan. Immunol. 6*, 254.

Fishman, M., and Adler, F. L. (1963), *J. Exptl. Med. 117*, 595.

Fishman, M., Van Rood, J. J., and Adler, F. L. (1964), Molecular and Cellular Basis of Antibody Formation, Sterzl, J., Ed., Prague, Publishing House of the Czechoslovak Academy of Sciences, p 491.

Franklin, R. M. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1504.

Friedman, H. P., Stavitsky, A. B., and Solomon, J. M. (1965), *Science 149*, 1106.

Gilbert, W. (1963), *J. Mol. Biol. 6*, 389.

Glišin, V. R., and Glišin, M. V. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1548.

Goodman, J. W., and Nitecki, D. E. (1966), *Biochemistry 5*, 657.

Goodman, J. W., and Nitecki, D. E. (1967), *Immunology 13*, 577.

Goodman, J. W., Nitecki, D. E., and Stoltenberg, I. M. (1968), *Biochemistry 7*, 706.

Gottlieb, A. A., Glišin, V. R., and Doty, P. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1849.

Hearst, J. E., and Vinogard, J. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1005.

Lennox, E. S., and Cohn, M. (1967), *Ann. Rev. Biochem. 36*, 365.

Loening, V. E. (1967), *Biochem. J. 102*, 251.

Porter, R. R. (1958), *Biochem. J. 59*, 405.

Westley, J. W. (1967), *Advan. Astronaut. Sci. 22*, 213.