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Studies on the Binding of Soluble Antigens to a Unique Ribonucleoprotein Fraction of *Macrophage Cells**

A. Arthur Gottlieb†

ABSTRACT: The response of macrophages to an immunogenic synthetic copolymer of L-glutamic acid, L-alanine, and L-tyrosine has been studied. This synthetic antigen is taken up by rat macrophages *in vitro* and is complexed exclusively to a distinct ribonucleoprotein of the macrophage. This ribonucleoprotein, which has previously been shown to exist only in macrophages, is separable from the rest of the cell's ribonucleic acid by virtue of a unique density in cesium sulfate.

Glu-Ala-Tyr can be partially released from the ribonucleoprotein of the macrophage by solutions of high ionic strength. The relation of these findings to those of other investigators is discussed.

A low molecular weight RNP[1] fraction of macrophages has been shown to be associated with particulate antigens to which the macrophage cells are experimentally exposed (Gottlieb, 1968a; Bishop, 1968). The RNP fraction derived from cells exposed to T2 bacteriophage specifically stimulates the production of neutralizing antibody against this bacterial virus. This ribonucleoprotein is characterized by an effective buoyant density of 1.588 g/cm^3 in cesium sulfate solution. Other physical properties are to be reported subsequently (Gottlieb and Straus, 1969).

Roelants and Goodman (1968) have studied the association of poly-γ-D-glutamic acid with RNA from macrophage cells. These workers reported that poly-γ-D-glutamic acid was associated with the 4–5S fraction of RNA in a strong and possibly covalent linkage and that they could not detect a specific association of the polypeptide with the light-density RNP fraction of macrophage RNA.

In an attempt to clarify the relation between antigen and RNP of the macrophage, we report here additional

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[1] RNP = ribonucleoprotein.

studies on the linkage of a soluble immunogenic copolymer with the RNP particle of macrophages.

Methods and Materials

Polypeptides. A synthetic linear random copolymer containing the α-amino acids, L-glutamic acid, L-alanine, and L-tyrosine (GAT) in the proportions of 60:30:10 was purchased from Pilot Biochemical Co., Watertown, Mass. The molecular weight of this copolymer, which will be referred to in this report as Glu-Ala-Tyr copolymer, was 50,000. This was the same material reported by Pinchuk *et al.* (1968) to be immunogenic and capable of reversing genetic unresponsiveness in nonresponder mice.

This polymer was radioiodinated by the method of Greenwood *et al.* (1963). After chromatography on Bio-Gel P-4, the polymer had a specific activity of 9.4×10^6 cpm/μg.

Exposure of Macrophages to [^{125}I]GAT Copolymer. Macrophages were prepared from rats as previously described (Gottlieb *et al.*, 1967). To a suspension of 5×10^8 macrophages in 5.0 ml of Eagle's minimum essential medium was added 2.5 μg of the [^{125}I]GAT copolymer. The mixture was incubated for 18 hr at 37° with shaking. For labeling of the RNA moiety, the cells were incubated with 1.5 mCi of carrier-free $Na_3{}^{32}PO_4$ for 18 hr, as previously described (Gottlieb *et al.*, 1967).

Preparation of RNA. RNA was harvested from the macrophage cells by shaking the cells which were suspended in 0.01 M sodium acetate buffer, 0.1 M NaCl, and 0.3% sodium dodecyl sulfate with redistilled phenol for 20 min at 4°. The RNA was precipitated with ethanol from the aqueous phase and recovered by centrifugation prior to treatment with electrophoretically purified DNase (Worthington) for 1 hr at 37° in 0.01 M Tris buffer (pH 7.4) containing 0.002 M $MgCl_2$. To remove oligodeoxyribonucleotides, the mixture was passed on a column of Bio-Gel P-4. The RNA, which was eluted as a single peak, was then mixed with a 20-fold volume of 0.02 M Tris buffer (pH 8.2) containing 0.01 M NaCl and placed on a 1.0×12 cm column of TEAE-cellulose in the same buffer. Elution of the RNP complex was carried out using an NaCl gradient of 0.1–0.5 M. The complex was characteristically eluted at 0.32 M NaCl. The column effluent was monitored for ^{125}I radioactivity by counting aliquots in the tritium window of a Packard EX-314 Tri-Carb scintillation counter.

Equilibrium Centrifugation in Cesium Sulfate. Aliquots of up to 100 μg of RNA, RNP, or GAT copolymer were dissolved in 0.01 M Tris buffer (pH 7.4) or, for certain studies described below, in 0.01 M acetate buffer (pH 5.0) and adjusted to an initial density of 1.57 g/cm^3 by addition of solid Cs_2SO_4 (Gallard-Schlesinger Co.). Volumes of 4.5–5.0 ml were centrifuged in a Spinco Model L2-65B ultracentrifuge for 44 hr at 40,000*g* at 25° using a type no. 65 fixed-angle rotor. After centrifugation, the tubes were punctured and 15-drop fractions were collected through a no. 23 gauge needle. Densities were calculated from the refractive index of the samples (Hearst and Vinograd, 1961), after which 0.3 ml of buffer was added and the optical density of each sample was measured at 260 mμ in a Gilford Model 2400 spectrophotometer. The samples were then assayed for radioactivity by dissolving them in Bray's (1960) solution.

Polyacrylamide Gel Disc Electrophoresis. The method of Bishop *et al.* (1967) was employed, except that the stacking gel was omitted. Electrophoresis was carried out on 60-mm gels at 5–10 mA/gel using a Canalco analytical gel electrophoresis apparatus.

In these studies, several buffer systems were employed in the gels and reservoir. Buffer E (0.04 M Tris, 0.02 M sodium acetate, and 0.001 M sodium EDTA, adjusted to pH 7.4 with glacial acetic acid) provided a medium of low ionic strength at a pH near neutrality. Buffer G (0.02 M Tris and 0.2 M glycine, pH 8.6, in 7.5 M urea) provided an alkaline medium which has been useful in the gel electrophoresis of proteins (Leboy *et al.*, 1964). Buffer ST (0.01 M Tris, 0.1 M NaCl, and 0.001 M EDTA, pH 7.4) and buffer 4 ST (0.01 M Tris, 0.4 M NaCl, and 0.004 M EDTA, pH 7.4) provided media of higher ionic strength near neutrality. Samples of RNA, RNP, or GAT copolymer were loaded onto the gels in the same buffer employed in the particular gel under study. The total volume of the sample did not exceed 35 μl. After completion of electrophoresis, the gels were frozen on Dry Ice and sliced into 1.2-mm sections using a device consisting of 11 razor blades bolted together on a machine screw with 1-mm washers as spacers. The gel sections were dried on filter paper and assayed for ^{125}I or ^{32}P radioactivity in 10 ml of toluene–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid. The ^{125}I radioactivity was measured in the tritium channel at an efficiency of 36%. The crossover of ^{32}P counts into the tritium channel was 12%, and no reciprocal crossover of ^{125}I label into the ^{32}P channel was observed.

Results

Two techniques were employed to locate the ^{125}I label in RNA derived from macrophage cells exposed to [^{125}I]GAT copolymer. These procedures provided a clear picture of the nature of the association of label from [^{125}I]GAT copolymer with macrophage RNA. In addition, a purified RNP derived from macrophages which were not exposed to the labeled antigen was isolated and iodinated directly *in vitro* in order to study the behavior of the preexisting protein component of the macrophage RNP complex. This material will be referred to as [^{125}I]RNP.

Density Gradient Ultracentrifugation in Cesium Sulfate of [^{125}I]RNP, [^{125}I]GAT Copolymer, and RNA from [^{125}I]GAT-exposed Cells. In the experiment illustrated in Figure 1a, a mixture of [^{32}P]RNP (labeled in the RNA moiety) and [^{125}I]RNP was banded in the same tube, and the profiles of the ^{32}P and the ^{125}I label are shown. It is clear that a significant fraction of the ^{125}I label banded at the density of the RNP as defined by the position of the ^{32}P label. The density computed from the refractive index at this point agrees precisely with the value previously reported

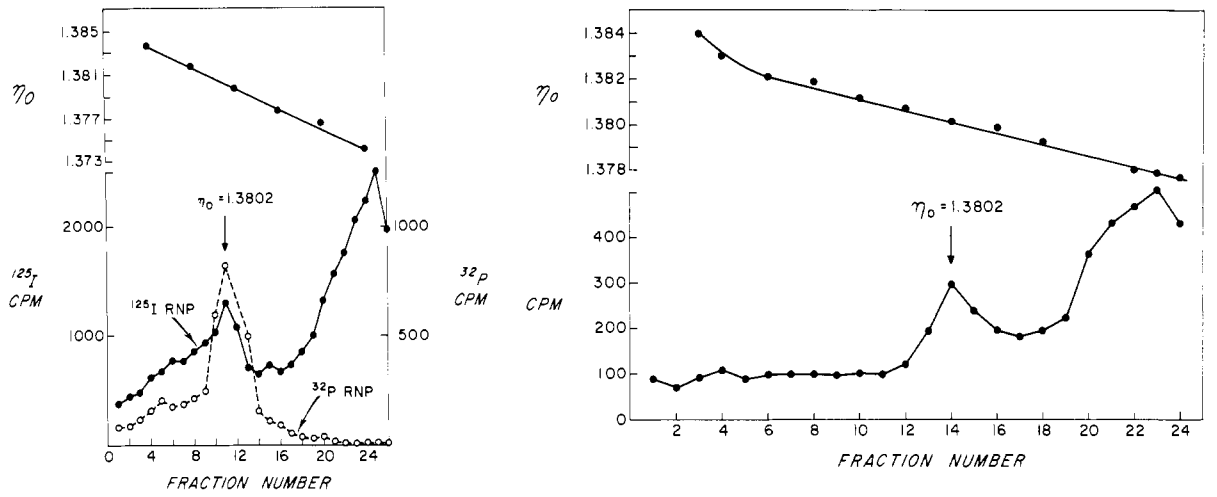

FIGURE 1: Equilibrium centrifugation in Cs_2SO_4 for 44 hr at 44,000 rpm, 25°. (a, left) Mixture of RNP labeled with ^{32}P in the RNA backbone and RNP labeled with ^{125}I in the protein moiety. (b, right) RNA from macrophages exposed to L-[^{125}I]Glu-Arg-Tyr copolymer. η_0 refers to the refractive index of the cesium sulfate solution at measured points in the gradient. A refractive index of 1.3802 corresponds to a density of 1.588 g/cm^3.

for RNP (1.588 g/cm^3). A large fraction of the ^{125}I label is noted at the top of the gradient and appears to be protein which is released by high concentrations of cesium sulfate. If [^{125}I]GAT copolymer is centrifuged in a similar gradient, this material also bands at the top of the gradient and is clearly distinguishable from the RNP complex.

The RNA derived from cells exposed to [^{125}I]GAT copolymer contained substantial amounts of ^{125}I label. Typically, 5×10^8 macrophage cells yielded about 1.0 mg of RNA, which had a specific activity of 770 cpm/μg. This level of polymer radioactivity in the RNA did not rise further if incubation of the cells with labeled L-GAT copolymer was continued beyond 16 hr. It was also noted that incubation of 5×10^8 cells with 3 mg of L-GAT copolymer having a specific activity of 50 μCi/mg, as in the studies of Roelants and Goodman (1968), yielded an RNA containing 34 cpm of ^{125}I/ug of RNA.

In the studies presented in this report, only the highly labeled RNA derived from macrophages exposed to L-[^{125}I]GAT copolymer in the absence of exogenous carrier copolymer was employed. If the RNA from these cells was subjected to cesium sulfate density ultracentrifugation, the results shown in Figure 1b were obtained. It is clear that most of the ^{125}I label is found in the low density region of the gradient and appears to be derived from GAT copolymer. A reproducible fraction of the label is found in the density region of the gradient corresponding to the RNP complex. In contrast to the findings of Roelants and Goodman (1968), we did not observe significant label at the density at which the bulk RNA of the macrophage bands ($\rho = 1.676$ g/cm^3). We observed no variation in these profiles upon changing the buffer employed in the gradient from Tris-HCl (pH 7.4) to NaAc–HAc (pH 5.0).

Electrophoretic Behavior of [^{125}I]RNP, [^{125}I]GAT Copolymer, and RNA from [^{125}I]GAT Exposed Cells on Polyacrylamide Gels. Samples of [^{125}I]RNP, [^{125}I]GAT copolymer, and RNA from macrophages exposed to [^{125}I]GAT copolymer were run in parallel in a variety of gel systems. As shown in Figure 2, the [^{125}I]RNP and the [^{125}I]GAT copolymer had distinct and different electrophoretic mobilities on polyacrylamide gels run in buffer E (pH 7.4). In this buffer, the R_F of the [^{125}I]RNP complex was 0.36 and that of the GAT copolymer was 0.29. The ^{125}I label in RNA derived from macrophages exposed to [^{125}I]GAT copolymer migrated as a single peak at an R_F of 0.36 in this system. The ^{125}I label observed in this case could not have been derived by spurious association of ^{125}I label with other RNAs of the macrophage, since this RNP preparation *did not* exhibit ^{125}I label in the region of the cesium sulfate gradient correspond-

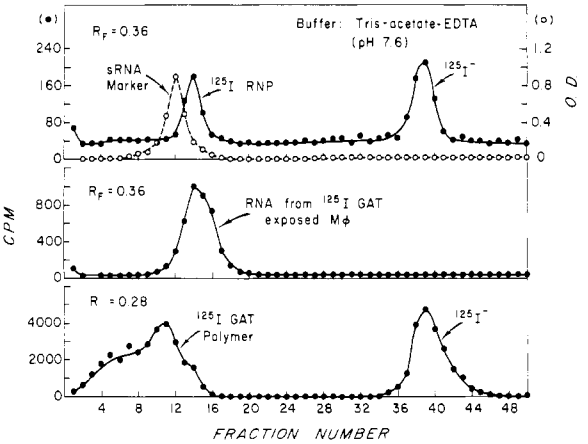

FIGURE 2: Electrophoresis in polyacrylamide gels at pH 7.6. Top: [^{125}I]RNP; middle: RNA from [^{125}I]Glu-Arg-Tyr-exposed macrophages (MØ); bottom: [^{125}I]Glu-Arg-Tyr copolymer.

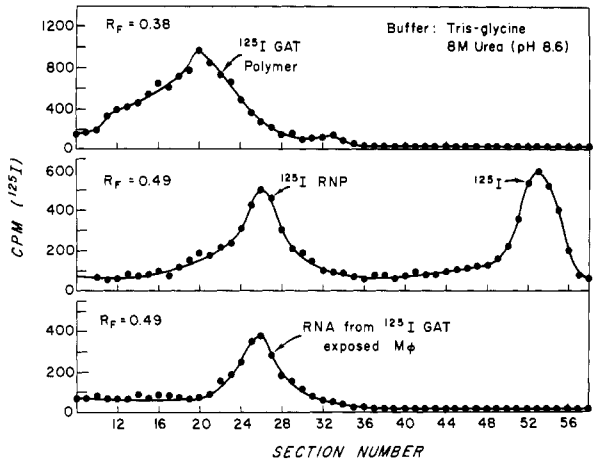

FIGURE 3: Electrophoresis in polyacrylamide gels in urea at pH 8.6. Top: [^{125}I]Glu-Arg-Tyr copolymer; middle: [^{125}I]RNP; bottom: RNA from [^{125}I]Glu-Arg-Tyr-exposed macrophages.

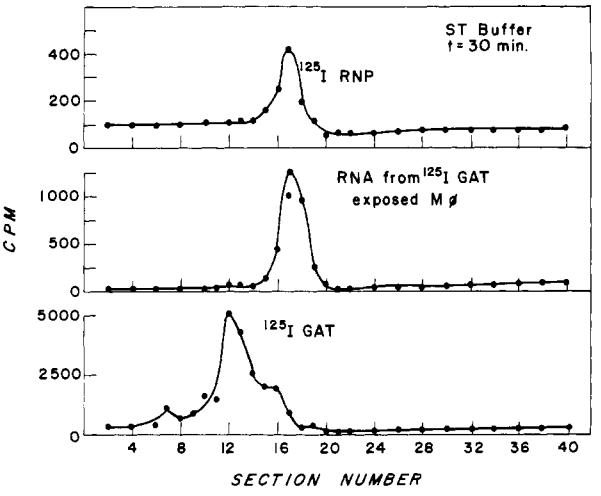

FIGURE 4: Electrophoresis in polyacrylamide gels in saline–Tris buffer (ST). Top: [^{125}I]RNP; middle: RNA from [^{125}I]Glu-Arg-Tyr-exposed macrophages; bottom: [^{125}I]-Glu-Arg-Tyr copolymer.

ing to the bulk RNA of the cell (Figure 1b). As shown in Figure 3, when a Tris-glycine buffer (pH 8.6) was employed, the [^{125}I]RNP migrated as a single peak with an R_F of 0.49, while the R_F of the [^{125}I]GAT copolymer was 0.38. Again, the ^{125}I label in RNA derived from macrophages exposed to ^{125}I GAT copolymer migrated identically with that of the [^{125}I]RNP complex itself. The position of these three molecules relative to each other was not influenced by prolonging the run such that the [^{125}I]iodide marker ran off the gel.

From these studies, it is clear that the ^{125}I label in this RNA preparation is distinguishable from that of the [^{125}I]GAT polymer in both the E and G buffer systems, which are both of low ionic strength. In view of the density gradient studies which suggested that a large fraction of the ^{125}I label present in the [^{125}I]RNP and in RNA derived from macrophages exposed to [^{125}I]GAT was released by solutions of high ionic strength, it was of interest to examine the behavior of these materials on gels of increasing ionic strength. Accordingly, [^{125}I]RNP, [^{125}I]GAT, and RNA from [^{125}I]GAT-exposed cells were first subjected to electrophoresis in buffer ST (0.01 M Tris, 0.1 M NaCl, and 0.001 M EDTA). As shown in Figure 4, the ^{125}I label in the RNA obtained from [^{125}I]GAT-exposed macrophages migrated identically with purified RNP and could be easily distinguished from that of the copolymer. If the electrophoresis was carried out in a buffer of higher ionic strength 4 ST (0.01 M Tris, 0.4 M NaCl, and 0.004 M EDTA), a portion of the [^{125}I]RNP label was found to dissociate from the complex. This is shown in Figure 5, where it is also clear that the ^{125}I label in the RNA from [^{125}I]GAT-exposed cells does not undergo such a dissociation. The dissociated material appeared to be free of RNA, as judged by the absence of ^{32}P label in the region of the dissociated peak. Electrophoresis in gels of ionic strength higher than that of the 4 ST gel was impractical because of the high conductance of these gel systems which resulted in prolonged migration times. These results would appear to reflect a firmer type of binding of exogenous antigens as compared with the endogenous protein associated with the RNP of macrophages.

Discussion

The association of antigens with RNA of the macrophage is of interest to students of antibody formation because of the demonstrated immunogenicity, in several experimental systems, of antigen–RNA complexes from these cells. In previous studies, all of the immunogenicity attributed to the RNA of macrophages infected with T2 bacteriophage was found in the RNP

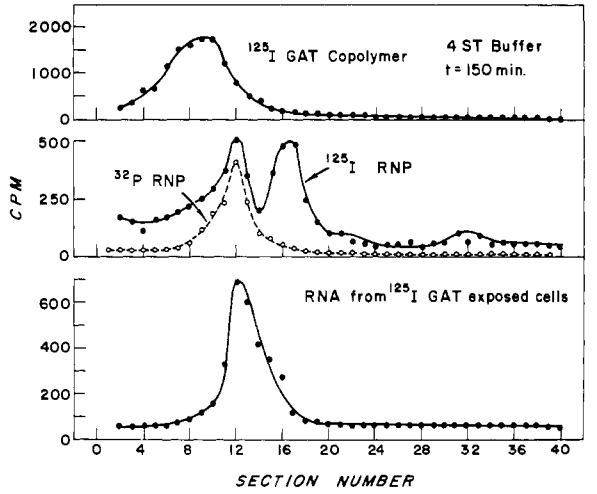

FIGURE 5: Electrophoresis in polyacrylamide gels in 4 ST buffer. Top: [^{125}I]Glu-Arg-Tyr copolymer; middle: [^{125}I]-RPN plus ^{32}P-labeled RNP; bottom: RNA from [^{125}I]Glu-Arg-Tyr-exposed macrophages.

complex. Moreover, significant amounts of labeled antigen were associated with the RNP complex as judged by cesium sulfate density gradient studies, but not with other RNAs of the macrophage. In the case of labeled T2 bacteriophage and sheep red blood cells, no distinct protein peak was seen in the cesium sulfate density gradients, but protein fragments containing radioactive label were distributed as small molecules along the gradient (Gottlieb, 1968a,b).

In contrast to these studies with particulate antigens, the association of radiolabeled soluble antigens with the RNP complex appears to be somewhat different. In cesium sulfate density gradients, a large fraction of the soluble GAT label which had been associated with the total RNA derived from the macrophages banded as copolymer at the light end of the gradient. This study indicates that a large fraction of the soluble antigen associated with the RNP could be released by solutions of high ionic strength.

An important piece of evidence regarding the nature of the soluble antigenic label associated with the macrophage RNA is provided by the behavior of this label on disc electrophoresis. In gels having an ionic strength equivalent to 0.1 M NaCl or less, the label associated with the total RNA fraction derived from macrophages exposed to [^{125}I]GAT copolymer migrated identically to purified RNP complex and could be readily distinguished from GAT copolymer. Thus, under the conditions of isolation, which utilize buffers containing 0.1 M NaCl, all of the soluble antigenic label is associated with the RNP. If the salt concentration is raised by placing the RNP in 2.3 M cesium sulphate, a large fraction of the copolymer label associated with the RNP is liberated from the complex.

In light of these results, the findings of Roelants and Goodman (1968) may be reinterpreted. These workers isolated RNA from macrophages and subjected it to column chromatography on cellulose in a buffer containing 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.001 M Versene. In their system, RNA containing polypeptide antigens was shown to be separable from the free polypeptide. The RNA obtained following passage through such a column exhibited minor bands at densities of 1.48–1.61, but no distinctive localization of the antigenic label to these bands was noted. However, the density of individual bands and the degree of association of label with each minor band was not confirmed by rebanding these fractions in a second density gradient. Moreover, in view of our findings regarding the behavior of label derived from GAT copolymer in the RNP under conditions of high salt concentration, it might be expected that most of the label would be found at the light end of the gradient.

In the studies carried out by Roelants and Goodman, 3 mg of copolymer having a specific activity of 50 μCi/mg was added to 10^8 cells, whereas we employed 2.5 μg of GAT copolymer having a specific activity of 18.5 μCi/ug. In view of the comparatively low specific activity of the polymer employed in their study, a small amount of residual label in the RNP band might have been overlooked. Alternatively, the macrophage may not be able to link a polypeptide consisting of D-amino acids as efficiently to the RNP complex, even though the D-polypeptide may enter the cell.

It is of interest to note that Roelants and Goodman concluded that their copolymer was undergraded after association with RNA. This is in contrast to our results (Gottlieb and Straus, 1969) which indicate that the RNP has a molecular weight of about 12,000. Since the protein moiety represents 28% of the complex, the associated polypeptides must also be of small molecular weight. Moreover, these authors state that the D-amino acid polymer label in their RNA preparations migrated behind 4S RNA on gel electrophoresis. In our study, it is clear that labeled RNP migrates ahead of tRNA, consistent with our observations on the molecular weight of the RNP complex. Furthermore, no label from the RNP is noted to migrate behind tRNA. In these results presented in this report, it is clear that none of the antigenic label derived from the L-GAT copolymer employed in this study is complexed to any species of RNA other than the RNP complex.

At physiological ionic strengths, soluble L-GAT copolymer is attached exclusively to the RNP complex. At high ionic strengths, L-GAT copolymer is released from the complex in a form which is large enough to band at a density consistent with polypeptide. As noted previously, when RNA extracted from macrophages which have been exposed to labeled T2 bacteriophage is placed in a cesium sulfate density gradient, a significant fraction of the label bands at the density of the RNP complex. However, a considerable amount of label is distributed through the gradient and behaves as small polyelectrolyte molecules. Clearly, these labeled molecules which are derived from the T2 bacteriophage are not large enough to band, and their small size reflects a difference in the handling by the macrophage of particulate and soluble antigens. Presumably, these small polypeptide fragments are released from the RNP in the cesium sulfate gradient.

There seems to be little doubt that the immunogenicity of RNAs from antigen-treated macrophages is due to the presence of antigen in the RNA. Our previous work (Gottlieb *et al.*, 1967) has established that the RNP does not contain the base sequence information for the coding of antibody γ-globulin. The small size of the RNP also excludes an informational role for this molecule. The recent report (Adler *et al.*, 1966), in which macrophage RNA from rabbits of one allotype induced in lymphoid cells from rabbits of a second allotype the formation of antibodies with genetic determinants of the donor, is intriguing but this effect may reflect the presence of mRNAs from lymphocytes which are present in the peritoneal exudate cell population. Since this RNA species contains no antigen, it would appear to be distinct from the RNP complex described in this study.

The location of the RNP in sucrose gradients has been a point of controversy. In the report of Roelants and Goodman, nearly all of the antigenic label was found at the top of the sucrose gradient, and in some experiments the label was displaced to the light side of the 4S peak. In studies to be reported subsequently, it will

be shown that the RNP complex is specifically associated with the macrophage ribosome (D. C. Bishop and A. A. Gottlieb, manuscript in preparation). Thus, if the RNP is found in the 4S fraction, this can only be a result of release of the RNP from its *in vivo* form. These findings confirm earlier observations which indicated that the immunogenicity for T2 bacteriophage was located only in the 28S fraction of undegraded RNA in sucrose and the RNP complex in cesium sulfate gradients. It should be noted that although the RNP exists in macrophages which have not been exposed to T2 bacteriophage, this RNP is *not* demonstrably immunogenic for this antigen.

The data presented in this report are compatible with the observations of Saha *et al.* (1964). Their studies indicated that following the intravenous injection of [^{35}S]bovine serum albumin into rabbits, there was a progressive association of labeled antigen with the higher molecular weight RNA fractions of liver. Moreover, in the studies which these workers reported regarding the chromatography of liver nucleoprotein ($N - p^*$) fractions on DEAE-cellulose, maximal antigenic label was eluted between 0.1 and 0.5 M in a salt elution gradient. This result is strikingly similar to our studies in which the RNP is eluted from TEAE-cellulose at 0.32–0.34 M NaCl. It is also of interest that their nucleoprotein ($N - p^*$) fractions contained the largest amounts of labeled antigen. In the studies of Saha *et al.*, the source of these nucleoprotein ($N - p^*$) fractions cannot be determined, although it would seem likely that the cell of origin is the Kuppfer cell. Saha's studies are complicated by the presence of large amounts of hepatic RNA and concomitant metabolism of antigen by the hepatic cells.

In this report, we have presented direct evidence for the unique association of exogenous antigenic fragments with a particular ribonucleoprotein fraction of the macrophage. Since Kuppfer cells may be regarded as fixed macrophages, it seems reasonable to predict that a similar mechanism exists in the liver. The role of this antigen–RNP in the production of antibody *in vivo* remains to be elucidated, and intensive efforts in this direction are currently under way.

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