Separation by Polyacrylamide Gel Electrophoresis of RNA from the Spleens of Immunized Mice[1] (35401)

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(Introduced by R. W. Wissler)

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After immunization, a complex series of events occurs in the lymphoid organs of immunocompetent animals which often leads to the biosynthesis and release of antibody protein. Among such events are the proliferation of precursors of antibody-forming cells (1) and the activation of portions of nuclear DNA for the transcription of "new" species of RNA (2). These newly formed RNAs include messengers for the synthesis of antibody H chains and L chains.

RNA molecules of varying molecular weights may be separated according to size and shape by electrophoresis in polyacrylamide gels. The gel acts as a molecular sieve and the rate of migration of large molecules through the gel is a function of their molecular weight. The rate of migration of molecules of known size may be compared with that of unknown and reasonable estimates of their molecular weights may be made. A detailed structural analysis of antibody protein has been completed (3). Most classes of immunoglobulins consist of two light chains whose individual molecular weight is 22,000 and two H chains whose individual molecular weight is approximately 60,000. The molecular weights of their corresponding RNA messengers is approximately 220,000 and 600,000.

In this study, the pattern of RNA synthesized in the spleens of mice after immunization as detected by polyacrylamide gel electrophoresis was determined. Two RNA peaks of special interest were noted.

Material and Methods. Animals and immunization; labeling of cellular RNA. Eight- to 12-week-old CD-1 mice (Charles River, Wilmington, Mass.) were used in all experiments. They were maintained in air-conditioned animal quarters and fed Purina mouse chow and water *ad libitum*. Approximately 10^8 washed sheep RBC were injected intraperitoneally at varying times before the mice were killed.

A suspension of spleen cells was prepared by gently forcing the spleens through a no. 40 stainless steel screen in cold Eagle's medium. The packed cell volume was determined and the cells were resuspended in fresh medium containing 100 μCi/ml of tritium-labeled uridine (New England Nuclear, Boston, Mass.) The ratio of cells:medium was constant for both experimental and control groups. One ml of packed cells was suspended in 5 ml of medium containing labeled uridine. The suspension was incubated for 60 min at 37° in a water bath equipped with a shaking device.

Separation of RNA: sucrose gradient. After incubation, RNA was extracted from the spleen cells by the hot phenol method (4) as described previously (5). This method yields total cellular RNA.

The RNA was separated in a linear 20–5% density gradient of sucrose in saline–Mg^{2+} (10^{-4} *M*) by centrifugation in a 25.1 rotor (Beckman-Spinco) at 23,000 rpm for 16 hr at 12°. After centrifugation, fractions from the gradient were collected from a hole punctured in the bottom of the tube. The optical density of each fraction was determined (Fig. 1).

Separation of RNA: polyacrylamide gel electrophoresis. RNA from that portion of the gradient which corresponded to 4S to 18S

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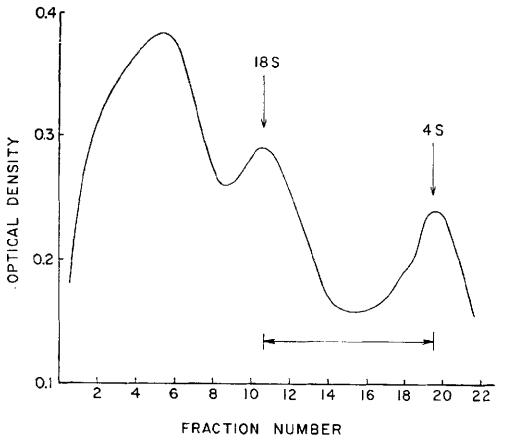


FIG. 1. Density gradient centrifugation of RNA: Mice were injected with sheep RBC. RNA from their spleens was centrifuged in a density gradient of sucrose. That RNA which sedimented in the region indicated by the vertical lines was collected for further separation by electrophoresis. Optical density at 260 mμ.

was pooled and precipitated with 2 vol of absolute ethanol. It was separated further by polyacrylamide gel electrophoresis (6).

Gels were prepared with recrystallized acrylamide as described by Weinberg *et al.* (7). In brief, 2.25 ml of monomeric acrylamide (20% aqueous solution) and 30 mg of N,N^1-methylenebisacrylamide were mixed in 4 ml of Tris–acetate buffer (Tris, 0.12 *M*; sodium acetate, 0.06 *M*; EDTA, 0.006 *M*) at pH 7.4; 1.2 ml of glycerol; and 4.55 ml of water. Polymerization began after the addition of 100 μl of ammonium persulfate (10% aqueous solution) and 10 μl of tetramethylethylenediamine. Immediately after mixing and before polymerization occurred, the mixture was poured into 0.4 × 7-cm tubes. The final concentration of the acrylamide was 3.75%.

Approximately 50 μg of RNA from the 4S to 18S fraction in the Tris–acetate buffer containing 15% glycerol and 0.5% sodium dodecylsulfate and 1 drop of methylene blue was added to the top of the gel. Electrophoresis was performed for 90 min at 5 mA/gel in an analytical gel electrophoresis apparatus (Canalco, Bethesda, Md.). After the leading edge of methylene blue reached the bottom of the tube, the gel was removed from the tube, frozen at −70° and sliced into 4-mm portions. The sp act of the RNA from each slice was determined by measuring the optical density (260 mμ) and determining the cpm for each fraction. Radioactivity was measured in a liquid scintillation counter.

The molecular weights of each fraction were estimated by reference to a standard curve (8) using 18S and 4S RNA present in the extracts as internal markers.

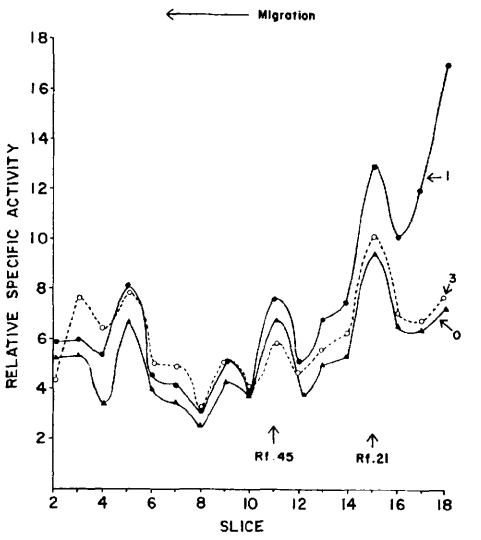
Results. Separation by polyacrylamide gel electrophoresis of RNA from the spleens of immunized mice. Mice were injected with sheep RBC; and 1 and 3 days afterwards a suspension of cells from their spleens was incubated for 60 min *in vitro* in a medium containing tritium-labeled uridine before extraction of total cellular RNA. After centrifugation in a density gradient of sucrose, RNA in that portion of the gradient which corresponded to the 4S to 18S pool was separated by electrophoresis in 3.75% polyacrylamide gels. The sp act of each separated fraction was determined (Figs. 2 and 3). Among the several peaks were two of especial interest—one whose $R_{f_{4S}}$ was a .45–.47 and another whose $R_{f_{4S}}$ was .21. The rate of synthesis of RNA in those peaks increased as much as three times on day 1 after immunization.

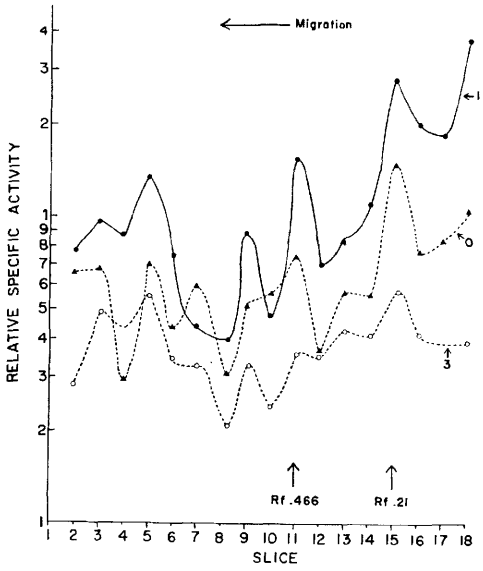
In another series of experiments, mice received 450 R before they were injected with sheep RBC. Control animals were immunized, but not irradiated. One day later, both groups of mice were sacrificed and suspensions of cells from their spleens were incubated in medium containing tritium-labeled uridine as described previously. RNA was separated first by density gradient centrifugation and then by gel electrophoresis. RNA synthesis in the spleens of irradiated mice was inhibited; the .21 and .45–.47 peaks were absent (Fig. 4).

Discussion. Mice were immunized with sheep RBC. After injection with this antigen the rate of RNA synthesis and total content of RNA in the spleen increased (13). The largest proportion of cells forming antibodies to sheep RBC was found 3 to 4 days after immunization.

A suspension of spleen cells from either immunized or control animals was incubated

in vitro for 60 min in a medium containing tritium-labeled uridine. This brief period of exposure to labeled uridine was chosen so as to label those species of RNA undergoing the most rapid rate of synthesis. RNA from the cells was separated by density gradient centrifugation in sucrose. Much of the heavy ribosomal peak (greater than 18S) and lightly sedimenting fractions (less than 4S) were discarded. The remainder, consisting of the rapidly labeled fractions, were separated further by polyacrylamide gel electrophoresis.





FIGS. 2 and 3. Gel electrophoresis of RNA after immunization: RNA from the density gradient (Fig. 1) was separated further by electrophoresis in 3.75% polyacrylamide gels. After electrophoresis, the gel was frozen and sliced. RNA from each slice was eluted and its specific activity (cpm/OD 260 mμ) was determined. The numbers refer to days after injection with sheep RBC. The R_f values were determined relative to internal 4S and 18S markers. Slice no. 3 was considered to be 4S RNA and was assigned the value R_f 1.0. Slice no. 18 was considered to be 18S RNA and was assigned the value R_f0.

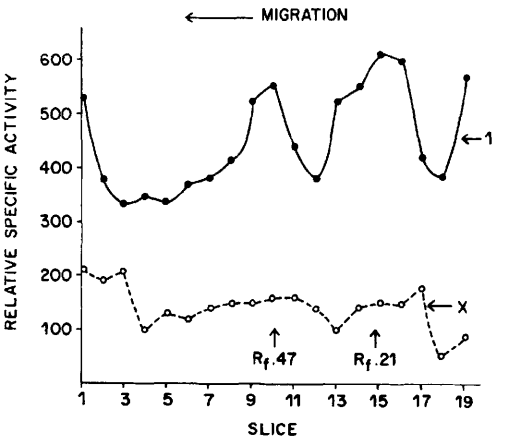


FIG. 4. Gel electrophoresis of RNA, after X-ray: Mice were injected with sheep RBC after they received 450 R of X-irradiation or were not X-irradiated before they were injected. RNA was extracted from their spleens 1 day later and separated first by density gradient centrifugation and then by polyacrylamide gel electrophoresis. X = mice X-irradiated before injection.

The rate of migration of RNA through a polyacrylamide gel by electrophoresis is related to the molecular size of the RNA and the concentration of the gel (9). Migration rate is a linear function of the log of the molecular weight of the RNA (10). Using this method, Bishop *et al.* (10) determined the molecular weights of viral nucleic acids. Mills *et al.* (11) determined the molecular weight of newly synthesized RNA in a cell free system, and Loening (12) applied the method to an analysis of RNA obtained from green plants. Labrie (7) isolated RNA with the properties of a hemoglobin messenger from rabbit reticulocytes from polyacrylamide gels.

In the present experiments, two species of RNA were obtained by gel electrophoresis which were of especial interest. Their R_f

values in 3.75% acrylamide gels, relative to 18S and 4S RNA "markers" were .21 and .45–.47. Such RNA corresponded to RNA with molecular weights of approximately 600,000 and 220,000, respectively.

The molecular weights of the antibody H chains is approximately 60,000 and of the L chain is 22,000. Their respective messenger RNAs would be, in theory, 600,000 and 200,000. RNA from the .21 and .45–.47 peaks may contain messenger RNAs for antibody, although they are undoubtedly contaminated by nonantibody specifying RNAs of similar size.

The suspicion that the .21 and .45–.47 peaks might contain RNA which was involved in the immune response was strengthened by the observation that the rate of synthesis of RNA which migrated in these regions of the gel increased 1 day after immunization. Further mice X-irradiated before immunization failed to form the two relevant peaks.

Summary. RNA from the spleens of immunized mice was separated by electrophoresis in polyacrylamide gels. Estimates of the molecular weight of various species of RNA was made by this method. Two peaks of especial interest were noted. They corresponded to RNAs with molecular weights of size approximating the theoretical requirements for messengers for antibody H chains and L chains. Their rate of synthesis as measured by the incorporation of ^{3}H-uridine increased after immunization and X-irradiation of the mice before they were injected with antigen inhibited the appearance of the relevant peaks. They were considered to contain messenger RNAs for antibody although undoubtedly contained nonantibody-specifying RNAs of similar molecular weight.

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