REPLICATION OF BACTERIOPHAGE RNA: PURIFICATION OF THE REPLICATIVE INTERMEDIATE BY AGAROSE COLUMN CHROMATOGRAPHY[1]

R. L. Erikson and J. A. Gordon

Department of Pathology

University of Colorado Medical School

Denver, Colorado 80220

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During the replication of bacteriophage RNA some viral-specific RNA synthesized in the infected cell displays characteristics expected of double-stranded RNA (Kelly and Sinsheimer, 1964; Weissmann and Borst, 1963; Weissmann, et al., 1964). In addition, the sedimentation behavior of some forms of viral-specific RNA suggests that a structure consisting of single-stranded RNA attached to a double-stranded RNA core (Fenwick et al., 1964) is also isolated from infected cells. Such a structure has been termed the replicative intermediate (RI) (Erikson et al., 1964), to distinguish it from the purely double-stranded replicative form (RF). The technique commonly used to purify the virus-specific double-stranded RNA, RNase treatment of nucleic acids extracted from infected cells in order to hydrolyze single-stranded

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RNA, appears to also result in degradation of RI, producing non-sedimentable fragments and RNase-resistant molecules which have a sedimentation coefficient of 13 S, the same as that of RF (Fenwick et al., 1964).

In this communication we describe the utilization of agarose in bead form to separate the replicative intermediate of R17 bacteriophage from cellular RNA. Beaded agarose fractionates nucleic acids by allowing molecules with larger molecular volumes to filter through more rapidly than those with smaller molecular volumes, and experiments on the fractionation of cellular from poliovirus RNA by the use of beaded agarose have been described (Öberg et al., 1965).

Escherichia coli were grown in 100 ml nutrient broth containing 1 μc $^{32}PO_4$ per ml until the cell concentration reached 5 x 10^8/ml. The culture was centrifuged, washed once and converted to spheroplasts by the technique described by Spiegelman et al. (1965), diluted into 23 ml sucrose nutrient broth (SNB) (Spiegelman et al., 1965) containing 7 μg/ml protamine sulfate and infected at a multiplicity of 30 plaque forming units of the RNA-containing phage R17 per cell. After 5 min at room temperature the culture was diluted into 40 ml SNB at 37°C containing actinomycin D (0.2 μg/ml). Thirty minutes later ^{3}H-uridine (Nuclear-Chicago, 20,000 mc/mM) was added (10 μc/ml) and in 10 seconds the culture was poured into a chilled flask containing 0.4 ml 1 M KCN. Incorporation of ^{3}H-uridine into uninfected cells is less than 5% of that into infected cells.

Nucleic acids were prepared from the pelleted culture as described previously (Erikson et al., 1965). Ribosomal RNA (^{32}P) and ^{3}H-labeled RNA were precipitated from the nucleic acid preparation (concentration about 4 mg/ml) by NaCl (1 M, 4°C, 12 hours) (unpublished procedure by R. M. Franklin and M. L. Fenwick). The precipitate was collected by centrifugation, dissolved in STE (0.15 M NaCl, 5 x 10^{-2} M tris, pH 7.2, and 10^{-3} M EDTA), re-precipitated by the addition of 2 volumes of cold ethanol and stored at 4°C in STE.

The distribution of a sample of this RNA after sedimentation through a sucrose gradient is shown in Fig. 1.

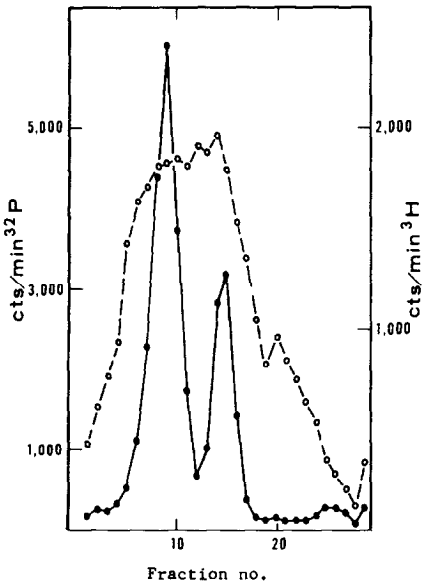

Fig. 1. Sedimentation analysis of RNA from infected spheroplasts. A 0.2 ml sample of the RNA described in the text was layered on a 4.4 ml sucrose gradient (20 - 7% w/v), placed in a SW50 rotor and spun for 2.5 hours at 50,000 rev/min at 10°C in a Spinco model L2 ultracentrifuge. The fractions were collected directly into vials for simultaneous counting of both ^{32}P and ^{3}H in a Packard Tri-Carb liquid scintillation spectrometer. (^{32}P ——●——), (^{3}H ---o---).

The 23 S and 16 S ribosomal RNA are shown by the position of the ^{32}P and the pulse-labeled RNA is shown by the ^{3}H. In contrast, sedimentation analysis of the 1 molar NaCl supernatant revealed no labeled RNA which sedimented faster than 13 S.

Four percent agarose beads (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey)[2] suspended in STE were allowed to sediment in a glass column 0.9 cm by 50 cm fitted with a sintered glass disk. After the column was washed with 300 ml STE the packed height was 43 cm, and the flow rate was about 8 ml per hour. The top of the column was protected by a filter paper disk. 150 µg of the RNA described in Fig. 1 were applied in 0.2 ml STE to the column and eluted with STE. All of these operations were carried out at room temperature. The elution profile of the RNA is shown in Fig. 2. A large peak of ^{3}H-labeled RNA elutes with the void volume, which was determined by the position of the leading peak upon the chromatography of blue dextran (molecular weight about 2 million); however, most of the ribosomal RNA (^{32}P) and some ^{3}H-labeled RNA pass through the column more slowly. It would be anticipated that the RI because of its partially double-stranded structure and its high molecular weight (Erikson et al., 1965) would elute more rapidly than would the smaller single-stranded E. coli ribosomal RNA. Therefore the following experiments were carried out to establish that RNA eluting in the first peak has the properties of the RI.

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[2] Agarose beads are, at present, available in limited experimental quantities from Pharmacia.

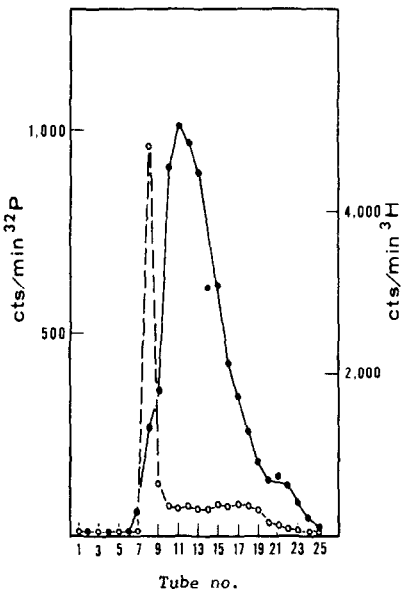

Fig. 2. Chromatography of the RNA described in Fig. 1 through 4% agarose beads. One-ml fractions were collected, and 0.1 ml of each fraction was used for radioactivity determination. (^{32}P —●—), (^{3}H ---o---).

The RNA was precipitated from tube 8 and from tubes 10-15 (Fig. 2), dissolved in 0.2 ml STE and rechromatographed. Again the RI (tube 8; Fig. 2) passed through the column in nearly the void volume but the small amount of ribosomal RNA initially in this fraction was retarded. The ribosomal RNA and the small amount of ^{3}H-labeled RNA which moved together (tubes 10-15; Fig. 2) displayed exactly the same behavior during the second cycle. The RNA was again collected and analyzed by sucrose gradient sedimentation.

The distribution of the purified RI is depicted in Fig. 3a and that of the ribosomal RNA preparation in Fig. 3b. Since these patterns are very close to those found prior

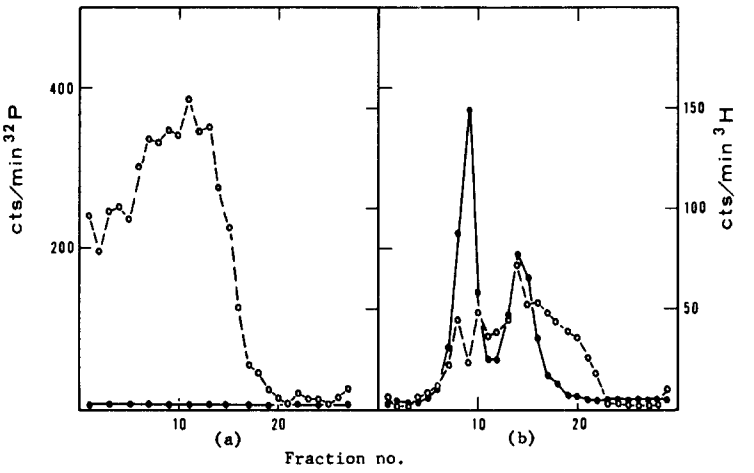

Fig. 3. Sedimentation analysis of RNA fractionated by an agarose column. (a) Replicative intermediate. (b) ^{32}P-labeled ribosomal and ^{3}H-labeled single-stranded RNA. Sucrose gradient sedimentation and analysis were carried out as described in the legend for Fig. 1. (^{32}P ——●——), (^{3}H ---o---).

to separation (Fig. 1), neither type of RNA has been degraded or appreciably altered by agarose column chromatography. Of the ^{3}H-uridine incorporated into RI in this short pulse (Fig. 3a) 73% has a sedimentation coefficient of 13 S after treatment with RNase (pancreatic ribonuclease, Worthington, 0.1 µg/ml, 10 min at 37°C), the rest being converted to nonsedimentable fragments. Similar sedimentation behavior has been described for RI which had not been freed of single-stranded RNA (Fenwick et al., 1964), and, therefore, the percentage of RNase-resistant material was lower. Results obtained with RI purified by Cs_2SO_4 equilibrium density-gradient centrifugation (Erikson, 1966) substantiate those demonstrated here. In contrast to the RI, the preparation containing the ribosomal and ^{3}H-labeled RNA retarded by the column (Fig. 3b) yields

no detectable resistant RNA, indicating that it is single-stranded.

In conclusion, the replicative intermediate synthesized in RNA phage-infected cells has been separated from single-stranded RNA by chromatography through beaded agarose and provides independent evidence for its previously described properties (Fenwick et al., 1964). This procedure makes possible the preparation of enough RI to elucidate the structure and biological properties of this viral-specific RNA component.

References

Erikson, R. L. in preparation (1966).
Erikson, R. L., Fenwick, M. L. and Franklin, R. M., J. Mol. Biol., 10, 519 (1964).
Erikson, R. L., Fenwick, M. L. and Franklin, R. M., J. Mol. Biol., 13, 399 (1965).
Fenwick, M. L., Erikson, R. L. and Franklin, R. M., Science, 146, 527 (1964).
Kelly, R. B. and Sinsheimer, R. L., J. Mol. Biol., 8, 602 (1964).
Öberg, B., Bengtsson, S. and Philipson, L., Biochem. Biophys. Res. Commun., 20, 36 (1965).
Spiegelman, S., Haruna, I., Holland, F. B., Beaudreau, G. and Mills, D., Proc. Nat. Acad. Sci. U.S., 54, 919 (1965).
Weissmann, C. and Borst, P., Science, 142, 1188 (1963).
Weissmann, C., Borst, P., Burdon, R. H., Billeter, M. A. and Ochoa, S., Proc. Nat. Acad. Sci. U.S., 51, 682 (1964).