BIOCHEMISTRY OF FRIEND VIRUS-INFECTED CELLS

I. VIRION-SPECIFIC RNA

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A single stranded RNA has been isolated from at least two mouse leukemia viruses (Duesberg and Robinson, 1966; Baluda, 1967). Sucrose sedimentation analyses resolve a major component with a $S_{20,w}$ of 70S in 0.1 M salt and a molecular weight of 12×10^6, computable from the empirical relation of Spirin (1963). The prediction that this macromolecule may be a "prototype" genome or a portion thereof, is supported by the observations presented in this communication for Friend's agent (Friend, 1957).

MATERIALS AND METHODS

Media and chemicals: SSS is Eagle's Spinner salt solution (GIBCO, Grand Island, N. Y.) PFS is identical to SSS except for NaH_2PO_4 which is reduced to 0.001 M. C^{14}-Leucine (240 uc/umole) Deoxyribonuclease I (RNA-ase free) and Ribonuclease B were obtained from Bioschwarz, Orangeburg, N. Y. H^3-Choline chloride (150 uc/umole) and carrier-free $P^{32}O_4$ (1 uc/ug) were purchased from Amersham-Searle, Des Plaines, Ill. Ribonuclease-free sucrose was obtained from Mann Research Laboratories, New York, New York. Sodium dodecylsulfate (SDS), recrystallized from propanol, was a gift from Dr. Norman Simmons. NTE buffer contains 10^{-2} M Tris (hydroxymethyl)-aminomethane - HCl (pH 7.4), 10^{-3} M Ethylenediaminotetraacetic acid and 0.15 M NaCl.

Virus: The strain of Friend virus (FV) used in this study has been maintained in inbred DBA/2/Crgl mice by serial inoculation. An ID_{50} of $10^{4.0}$

can be obtained 10 days after inoculation of cell-free virus prepared from 10% (w/v) spleen sonicates.

Propagation of radioactively labeled virions: Spleens from mice infected 180 hours previously with $10^{3.0}$ ID_{50} of FV were used as a source of virus-infected cells. Single-cell suspensions were obtained by gentle homogenization of 1-2 mm^3 fragments in a Ten Broeck grinder containing 10 volumes of PFS, followed by further dispersal through a tuberculin syringe. The cells were washed 3 times with PFS and then resuspended at a density of 3 x 10^7 nucleated cells per ml. Dialyzed calf serum, H^3-choline, C^{14}-leucine and P^{32} were added to 25 ml volumes of the cell suspension at concentrations of 2%, 25 uc/ml, 2 uc/ml, and 300 uc/ml respectively. After 240 min, at 37° C, the cells were washed with warm PFS and resuspended in SSS containing unlabeled choline (1.6 umole/ml) and leucine (0.08 umole/ml) plus 1% calf serum, penicillin (200 units/ ml) and streptomycin (0.02 mg/ml). After 18-20 hours exposure to the "chase" medium the suspensions where chilled for 30 min. at 0° C and then made 10 mM and 1 mM with respect to Tris-HCl and EDTA by the addition of 10X NTE buffer. A cell-free suspension was then obtained by centrifugation (8000 g; 10 min; 4° C). The pellet was retained for subsequent extraction of ribosomal RNA. Virus in the supernate was precipitated by the addition of an equal volume of saturated ammonium sulfate (pH 7.0). The precipitate was collected by centrifugation (30000 g; 5 min) and solubilized in NTE (10% of the supernate volume). After further clarification (8000 g; 10 min) the concentrated solutions were diluted with NTE and 15 ml layered onto 12 ml of 0.6 M sucrose on top of 3 ml of 2.0 M sucrose in NTE in a Spinco SW25.1 rotor tube. The virus was concentrated to the density interface between these solutions by centrifugation at 25 Krpm for 120 min. The interface bands were withdrawn, diluted 1;4 with NTE, and again centrifuged for 15 min. at 8000 g.

Purification of virus: Partially purified virus in 4 ml volumes of this supernate was then centrifuged to density equilibrium in 26 ml of a pre-

formed sucrose gradient (0.6 - 2.0 M) in NTE. After 6 hours at 25 Krpm, the gradients were fractionated by dripping from the bottom of the tube and 25 and 150 uliter were removed from each of 30 fractions for determination of acid-insoluble radioactivity and infectivity, respectively. Infectivity was checked after a 1:4 dilution of each 150 uliter sample with NTE. Two-tenths ml were inoculated into each of three 20-25 day-old DBA/2 mice. Average spleen weight per group 10 days after inoculation was used to quantitate infectivity.

Extraction of virus nucleic acid: SDS was added to the purified virus (Fractions 10-14; Figure 1) and 1 mg of sRNA to a final concentration of 1%. After 10 min. at room temperature the mixture was deproteinized by 3 succesive extractions with equal volumes of phenol in 0.1 M sodium phosphate buffer. Nucleic acid was precipitated from the final aqueous phase by the addition of several drops of 50% acetic acid and 2 volumes of cold ethanol. The precipitate was pelleted by centrifugation (30000 g; 30 min; 0^{o} C), and then solubilized in 2 ml of NTE.

Extraction of ribosomal RNA: The cell pellet obtained in the initial clarification was resuspended in 10 ml of STE and dispersed by sonication for 10 secs. (Branson Model S75). Following lysis with SDS at 1% and 3 extractions with phenol as described for viral nucleic acid, DNA was removed with a stirring rod from the aqueous phase during the drop-wise addition of 0.5 - 1 volume of ethanol. The flocculus obtained during the addition of 1-2 volumes of ethanol was pelleted by centrifugation, solubilized in 3 ml of 10^{-2} M Tris-HCl and 0.01 M $MgCl_2$ and then exposed to 150 ug of DNA-ase for 30 min. at 37^{o} C. DNA-ase was then removed by 2 further phenol-SDS extractions and the RNA in the aqueous phase recovered by ethanol precipitation at - 20^{o} C. This preparation was pelleted by centrifugation and redissolved in 5 ml of NTE.

Preparative sedimentation of viral and cell RNA: Linear sucrose gradients were used to resolve the major components of each P^{32}-labeled preparation.

Unlabeled mouse ribosomal RNA (250 ug) was mixed with virion-derived RNA in 1 ml of NTE and 0.3 ml amounts layered onto 4.7 ml of a 0.15 - 0.60 M gradient in NTE in a SW39 rotor tube. After 150 min. at 36 Krpm, a 10 uliter sample was removed from each of 20 fractions for the determination of acid-insoluble radioactivity. Samples of 150 uliter were diluted with water to 1 ml and assayed for optical density at 260 mu. The distribution of P^{32} in cell RNA was determined after 0.3 ml amounts from the "stock" preparation were layered onto an identical gradient and centrifuged for 330 min. at 36 Krpm. This gradient was not calibrated with unlabelled marker RNA. Acid-insoluble radioactivity and OD_{260} determinations were performed as described for virion-specific RNA.

Base composition determination: The remainder of fractions 3-5 (Figure 2A) containing the rapidly sedimenting peak (70S) were pooled, as were fractions 18-20 and 0.6 ml of each were mixed with 50 ug of sRNA and then incubated with 0.2 ml of 0.3 M KOH for 16 hours at 37° C. Similar samples from a pool of fractions 4-6 and 8-10 (Figure 2B) from the gradient containing cell RNA were treated identically. The latter were assumed to be representative of the 28S and 18S ribosomal species. The hydrolysates were neutralized with 6 N perchloric acid and after 2 hours at 0° C, $KClO_4$ was removed by centrifugation. The method of Weiss (1960) was used to separate each mixture of ribonucleotides. A 50 uliter sample was spotted on Whatman 3 MM paper and subjected to flat plate electrophoresis in 0.025 M citrate buffer (pH 3.8) at 400 volts for 20 hours. Individual mono-nucleotides were located by ultraviolet absorbance and the distribution of P^{32} in the electrophenogram was determined in 0.5 x 1 cm sections.

Determination of radioactivity: Samples from gradient fractions were co-precipited with 200 ug of bovine serum albumin by the addition of 0.5 ml of cold 0.5 M Trichloroacetic acid (TCA). The precipitates were collected under vacuum on filter paper washed with several volumes of 0.2 TCA and then placed in vials containing 0.5 ml of NCS (Nuclear-Chicago, Des Plaines, Ill.)

and 10 ml of toluene-based scintillation fluid. Paper strips from the electropherogram with 1 ml of NCS were dispersed in 15 ml of scintillation fluid. Liquid spectrometry was performed with a Nuclear-Chicago Mark I 3-channel model.

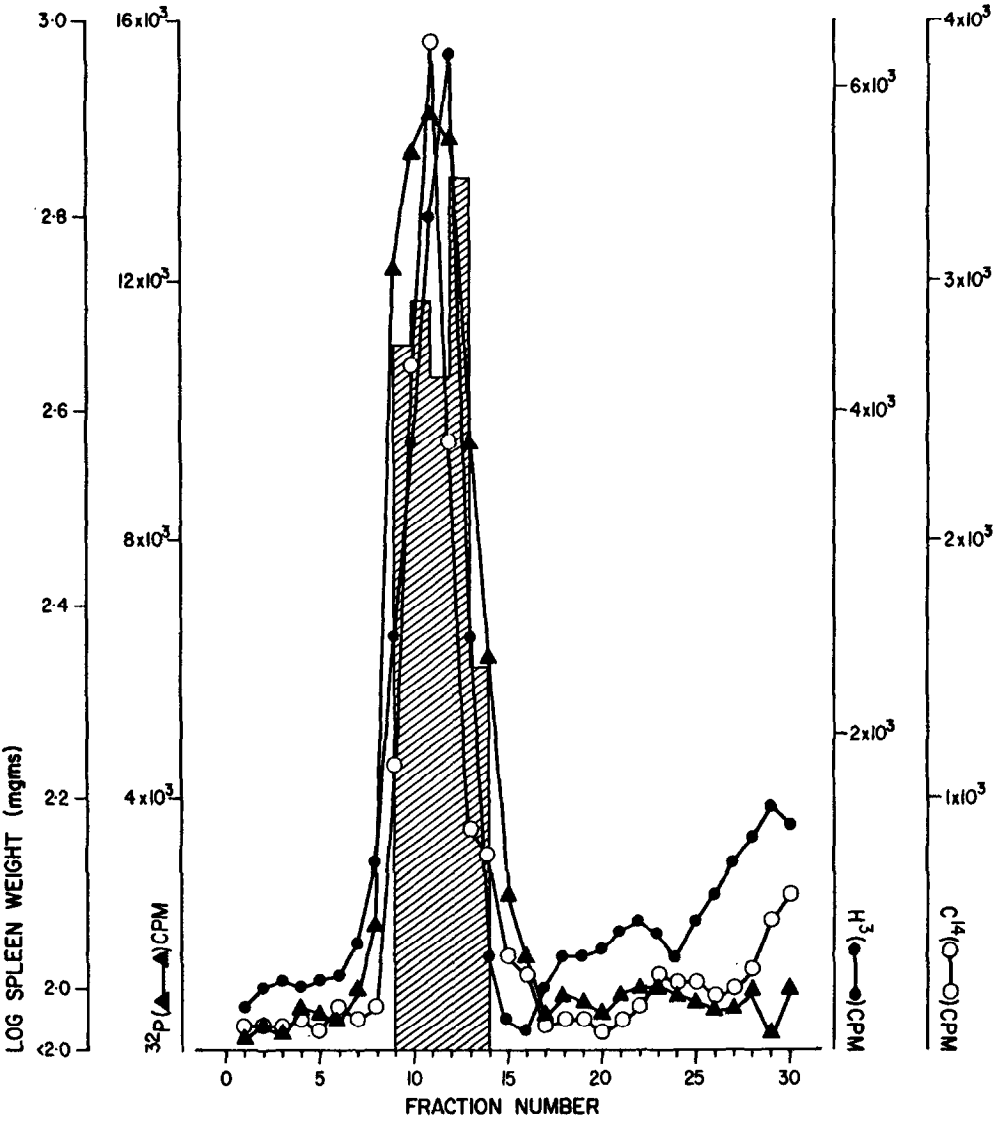

Figure 1. Distribution of P^{32}, H^3-choline and C^{14}-leucine in Friend virus at density equilibrium. Shaded area represents infectivity. Condition of centrifugation: SW25.1 rotor; 0.6 - 2.0 M sucrose in NTE buffer; 25 Krpm; 360 min; 4° C.

RESULTS AND DISCUSSION

The distribution of infectivity and labeled precursors of viral nucleic acid, protein and lipid at density equilibrium are shown in Figure 1. It would be tenuous to assign biologic significance to the assymetrical distribution of H^3-choline because the method of assay for infectivity is relatively imprecise. Modification of the standard Reed-Muench technique as described by Friend (1959), or appropriation of other methods of quantitation (Pluznik and Sachs, 1964), have not enhanced precision for these purposes. Furthermore the rationale for incorporating C^{14}-leucine and H^3-choline into virions in these experiments was to provide criteria for the purity of nucleic acid isolates obtained subsequently.

Sedimentation of RNA derived from purified virus is shown in Figure 2A. In this ionic environment (0.15 M salt), a discrete fast-moving component with an estimated $S_{20,w}$ of 68S can be resolved, if 28S RNA is used as a reference. A second component ($<$10S) is suggested by the presence of a considerable amount of radioactivity which does not sediment beyond 18S RNA. Figure 2B shows the sedimentation profile of RNA from virus-producing cells. As synthesis of cell-specific species was not experimentally inhibited,

TABLE I

Base composition of virion-specific and ribosomal RNA's from FV-infected cells

	Cytosine[a]	Adenine	Guanine	Uracil
Virus 68S	24.4 ± 0.4[b]	25.1 ± 0.2	29.1 ± 0.2	21.4 ± 0.2
Virus 10S	28.1 ± 0.2	19.2 ± 0.2	34.8 ± 0.3	17.9 ± 0.2
Ribosome 28S	29.2 ± 0.2	18.3 ± 0.3	33.9 ± 0.2	18.6 ± 0.2
Ribosome 18S	27.0 ± 0.2	20.5 ± 0.2	30.7 ± 0.3	21.8 ± 0.2

a) Based on P^{32} radioactivity in Cytidylic acid, Adenylic acid, etc.
b) Mean plus average variation of 4 determinations.

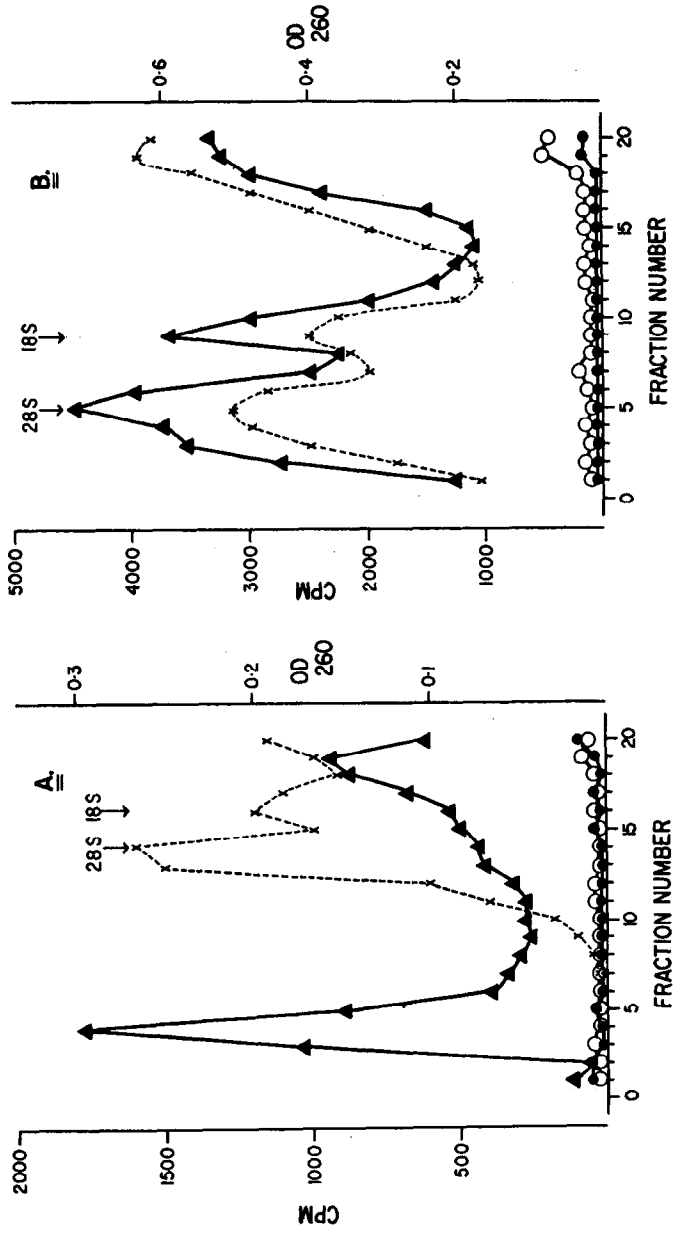

Figure 2. Velocity sedimentation of P^{32}-labeled RNA in 0.15 - 0.6 M sucrose prepared in NTE. (A) RNA from purified virions: SW39 rotor; 36 Krpm; 150 min; 4° C. (B) Ribosomal RNA from infected cells: SW39 rotor; 36 Krpm; 330 min; 4° C. Symbols for radioactivity as in Figure 1, (x---x) represent OD_{260} readings in each fraction.

resolution of virus-specific RNA in such a profile would be improbable. The distribution of H^3 and C^{14} in both profiles suggest minimal contamination of either preparation with protein and lipids.

The average base composition of the two virion-derived components and ribosomal RNA are presented in Table I. The incongruity between values obtained for the cellular species and both viral fractions suggest non-homology. These results also suggest that the primary structure of either viral fraction is in accord with a model for single stranded RNA. This is substantiated by their sensitivity to RNA-ase in the presence of 0.3 M salt. Less than 5% of acid-insoluble radioactivity to RNA-ase remained after 30 min. incubation of these P^{32}-labeled fractions with RNA-ase (5 ug/ml) in 2X NTE, conditions under which double-stranded RNA from poliovirus and the MS2 phage are not degraded (Baltimore, 1966; Billeter et al. 1966).

The disparity in base composition between the two viral fractions is also of interest. It may suggest independent origins for each component or degradation of a single moiety with "region-specific" sequences. These questions may be answered when further knowledge of viral replication is obtained. If precursor(s) of virion-specific RNA can be identified, then it is probable that these findings may become even more meaningful.

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