Structural Studies on Avian Myeloblastosis Virus: Rapid Purification and Quantitation (36745)

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(Introduced by W. E. Heston)

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This communication describes a method whereby avian myeloblastosis virus (AMV) was rapidly purified from high-titer viremic plasma [over 10^{12} particles per ml by enzyme assay (1)] by using rate-zonal centrifugation in linear density gradients of Ficoll (2, 3). The use of Ficoll was suggested by studies that employed Ficoll to isolate murine leukemia virus (4), and mammary tumor virus (5, 6), a type B RNA tumor virus. Our procedure for AMV was devised in order to (a) minimize the time of purification, (b) avoid the objection that cellular vesicles and AMV band together in equilibrium centrifugation, (c) assess the association of AMV and 18S and 28S species of RNA (7–9) with a different technique of AMV purification, and (d) avoid exposure of intact AMV to high osmotic pressure from glycerol, salt, or sucrose density gradients which hinder production of the AMV core component during surfactant treatment (10).

AMV isolated by this method had a threefold increase in specific activity of adenosine triphosphatase [ATPase, an enzyme of the viral envelope (11)] during purification, and a high degree of morphological purity by electron microscopy. A linear relationship was found to exist between the turbidity of a purified AMV sample and its protein content and ATPase activity. Thus, an estimate of the concentration of AMV could rapidly be made by simply determining the percentage transmission of a virus preparation. Ribosmal-like RNA was present in AMV purified by this method.

Methods. Viremic blood was obtained from chicks as previously described (10). The cells were sedimented from viremic blood by low-speed centrifugation for 15 min at 5000*g* in the IEC model B-20 centrifuge. The plasma was then centrifuged for 15 min at 10, 000*g* to remove larger cellular fragments and organelles. An appropriate volume of this 10, 000*g* supernatant plasma was placed over 5 to 25% (w/w) linear Ficoll gradients in a 50 m*M* Tris–HCl buffer, 0.1 *M* NaCl, and 1 m*M* EDTA, final pH 7.4 (SET buffer) prepared in tubes for Beckman SW27 buckets with an ISCO density gradient former. (This mechanical method of forming Ficoll gradients for routine use avoids the long time consumed when these gradients are poured by means of gravity flow.) Commercially available Ficoll was used without further purification. The length of centrifugation with the SW27 rotor in the Beckman L265B at 25,000 rpm depended upon the path length of the SW27 buckets. With the 1.59 × 10.16 cm tubes, using 8 ml of plasma, an ω^2t function of 2.1×10^9 (approx 55 min) was used. With the larger 2.54 × 8.89 cm tubes, using 16 ml of plasma, an ω^2t of 2.7×10^9 (approx 72 min) was appropriate. Gradients were fractionated on the ISCO density gradient fractionation system as previously described (10), or light-scattering bands were removed by needle puncture from the side of the gradient tubes. After a 1:2 dilution of the bands in Ficoll with SET buffer, the material was sedimented for 60 min at 40,000*g* in the IEC model B-20 centrifuge. Protein content was determined by the method of Lowry *et al.* (12). ATPase assay was done according to the procedure of Mommaerts *et al.* (13). RNA was extracted by the technique of Kruh (14). Methods of obtaining and preparing samples for electron microscopic examination by the thin section technique

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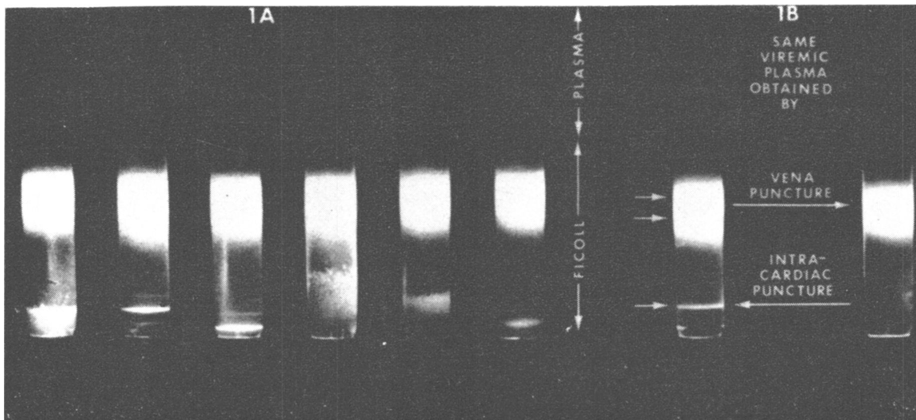


FIG. 1. Density gradient band photograph. (A) six Ficoll gradients overlaid with separate viremic plasma obtained by intracardiac exsanguination of six different leukemic chickens. (B) Ficoll gradients overlaid with viremic plasma obtained by the intravenous route (B, right tube) followed by subsequent intracardiac exsanguination (B, left tube) of the same leukemic chicken. The double arrows (B, left tube) indicate a representative upper light-scattering band of homogeneous AMV particles (see Fig. 2A). The single arrow (B, left tube), which designates the lower particulate band, consists in part of extraneous material (see Fig. 2B) which was related to intracardiac exsanguination. For all gradient tubes in (A and B) 8 ml of high-titer viremic plasma (over 10^{12} particles/ml by ATPase assay), after centrifugation for 15 min at 10,000 rpm (10,000 *g*), was placed over 5% to 25% (w/w) Ficoll gradients in SET buffer in 5/8 × 4 in. centrifuge tubes. The ω^2t function of the Beckman L265B centrifuge was 2.1×10^9 at 25,000 rpm (approx 55 min) at 0°.

have been described (10).

Results. Figure 1A shows the variation in appearance after centrifugation of six Ficoll gradients which were overlaid with separate viremic plasma from six chickens that were each bled by intracardiac exsanguination. In each gradient tube there was a dense homogeneous light-scattering band at a constant position just beneath the plasma–Ficoll interface. However, there was variation in the location and character of a lower particulate light-scattering band in each gradient.

As shown in Fig. 1B, the presence of the lower band in the Ficoll gradient was related to the process of intracardiac exsanguination. Note that viremic blood obtained by the intravenous technique did not contain the lower particulate band (right tube). The lower particulate band was present when the same chicken underwent subsequent intracardiac exsanguination (left tube).

When a representative upper homogeneous band (Fig. 1B, left tube, double arrows) was withdrawn by fractionation and prepared for electron microscopy, examination of thin sections at low magnification revealed a highly purified population of AMV particles (Fig. 2A). These particles infected neonatal chicks to cause myeloblastic leukemia. A representative lower particulate band (Fig. 1B, left tube, single arrow) upon morphologic examination consisted of an admixture of cellular vesicles and associated AMV (Fig. 2B).

When the 10,000*g* supernate from high-titer viremic plasma underwent centrifugation at 40,000*g* for 60 min, the resuspended pellet resulted in only a single A_{260} peak and a single light-scattering band (buoyant density of 1.16 g/ml) after equilibrium-density centrifugation in 20–50% (w/w) sucrose gradients or 2–40% (w/w) potassium tartrate gradients. However, Figs. 1 and 2 illustrate that with the same type of plasma the use of Ficoll gradients and rate-zonal centrifugation has purified AMV from some material, not separable by conventional equilibrium centrifugation, that appears to derive from the process of intracardiac exsanguination.

Our experience with intracardiac exsan-

TABLE I. Comparison of Specific Activity of Adenosine Triphosphatase of Pellets During Purification of Avian Myeloblastosis Virus from Viremic Plasma.[a]

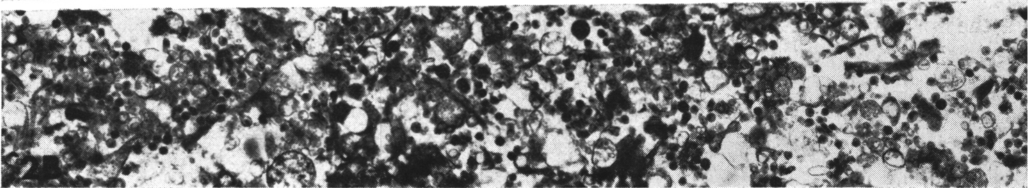
	Viremic plasma			Material from lower band of Ficoll gradient (diluted 1:9 with buffer, then 40,000g for 60 min)	Purified AMV from upper band of Ficoll gradient (diluted 1:2 with buffer, then 40,0000g for 60 min)
	(40,000g for 60 min)	(10,000g for 15 min)	After 10,000g for 15 min (40,000g for 60 min)		
Adenosine triphosphatase (μmoles P_i released/min/mg protein)	6.1	3.7	9.4	2.8	17.3

[a] Increase in specific activity of adenosine triphosphatase during AMV purification. Viremic plasma was divided into aliquots and, at the various stages indicated above, samples were set aside as pellets for determination of protein content and ATPase activity.

guination of more than 1000 leukemic chickens has been that the amount of light-scattering in the lower particulate band increased in proportion to the difficulty of intracardiac exsanguination. In no case has a lower band been observed in Ficoll gradients which were overlaid with viremic plasma obtained by intravenous exsanguination. The homogeneous upper band has had a constant position just below the plasma–Ficoll interface, and its intensity is related to the ATPase titer of the viremic plasma. This upper band is proportionately faint when viremic plasma in the middle range of 10^{11} particles/ml is used.

Table I indicates the specific activity of ATPase (an enzyme of the viral envelope) during the various stages in this method of AMV purification. Nearly a threefold increase in specific activity of ATPase was achieved when a pellet derived directly from viremic plasma (6.1) was compared with a pellet derived from the upper band in the Ficoll gradient (17.3). Centrifugation of the viremic plasma for 15 min at 10,000g prior to overlaying it on Ficoll gradients removed material of low specific ATPase activity (3.7). Use of the Ficoll gradient nearly doubled the specific ATPase activity of the viral material in the upper band when it was compared to a 40,000g pellet of the same viremic plasma (after the 10,000g sedimentation). The morphologically homogeneous upper band of AMV in the Ficoll gradient had a much higher specific activity of ATPase (17.3) than the morphologically heterogeneous lower band (2.8).

The relationship between ATPase activity and the particle number (1), coupled with the morphologic purity of the AMV, suggested that an approximate quantitation of purified virus might be obtained by simple measurement of turbidity by percentage transmission (% T) on a spectrophotometer. In addition, when applied to the lower band, this procedure permitted a biochemical comparison of the morphologically homogeneous upper band material with the more heterogeneous material in the lower band from the Ficoll gradients. Accordingly, the upper and lower bands were withdrawn by fractionation of a series of the Ficoll gradient tubes, the fractions containing the upper and lower bands were pooled separately, diluted 1:2 and 1:9, respectively, with SET buffer, and centrifuged for 60 min at 40,000g in the model B-20 centrifuge. The pellets were resuspended in SET buffer, dispersed by hand homogenization (25 strokes) in Potter-Elvehjem tissue grinders (clearance 0.004 to 0.006 in.) and diluted with SET buffer to random percentage transmission values between 10 and 80% T as read at 540 mμ on the Beckman DU-2 spectrophotometer. The percentage transmission of each sample of diluted material at 4° was read as soon as condensation no longer formed on the optical surface of the cuvette. Samples were then



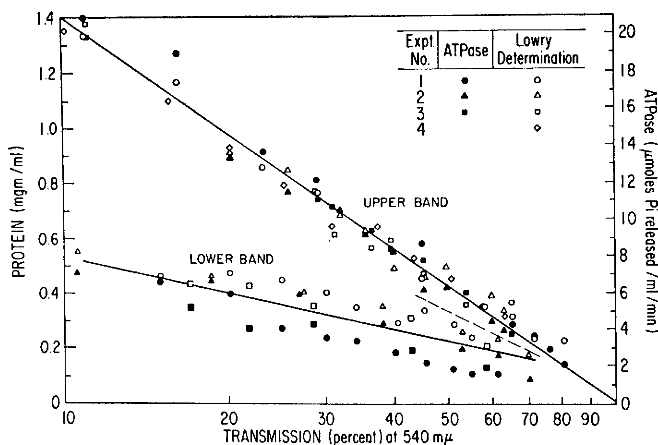


FIG. 3. A graph of the relationship between viral turbidity [percentage transmission (% T)], protein content, and ATPase activity which compares the upper and lower bands in Ficoll gradients from several different preparations of viremic plasma. The particle count can be derived from the reported relationship between ATPase activity and particle number (1) in which 0.021 fragments, and associated AMV. 12,600×.

quick frozen in dry ice–acetone. This procedure was repeated with several more separate preparations of viremic plasma from leukemic chicks that were also bled by intracardiac exsanguination. Assays for ATPase activity (13) and protein content by the method of Lowry *et al.* (12) were then performed.

Figure 3 compares the correlation of turbidity, protein concentration, and ATPase activity between the material of the upper and lower bands. Notice the steeper slope of the line that characterized the samples from the upper bands which, morphologically, consisted of a uniform population of AMV particles. The more structurally heterogeneous material from the lower bands resulted in a more scattered series of points. The ATPase activity was less in respect to the protein content for each percent transmission sample from the lower bands. Because ATPase specifically reflects AMV concentration, Fig. 3 provides suggestive biochemical evidence, in agreement with the morphological observations, that the lower bands contained nonviral material.

In view of recurrent reports of small amounts of ribosomal-like RNA (about 5% of total RNA exists as 18 and 28S species) in AMV purified by other various methods (7–9), and recently ribosomes themselves (E. deHarven, Int. Symp. Comparative Leukemia Research, 5th, Padova, Italy; and J. Riman, personal communication), in preparations of murine leukemia virus and AMV, the material in the upper homogeneous AMV bands and the material found in the lower particulate bands were compared in regard to their respective content of ribosomal-like species of RNA. Figure 4 shows a comparative profile of the RNA species found in these samples which differ widely in morphological homogeneity by electron microsopic examination. The structurally more heterogeneous material (lower band) had less high molecular weight RNA (60–70S) than the morphologically homogeneous sample (upper band). The amount of 18 and 28S RNA was

FIG. 2. Thin section electron micrographs from an upper homogeneous band (double arrows) and a lower particulate band (single arrow) in Fig. 1B, left tube. After isolation using the ISCO density gradient fractionation system, each band was centrifuged into a pellet and prepared for electron microscopy by conventional methods. The upper band (A) consisted of a homogeneous population of AMV particles; the lower band (B) consisted of an admixture of cellular vesicles, fragments, and associated AMV. 12,600×.

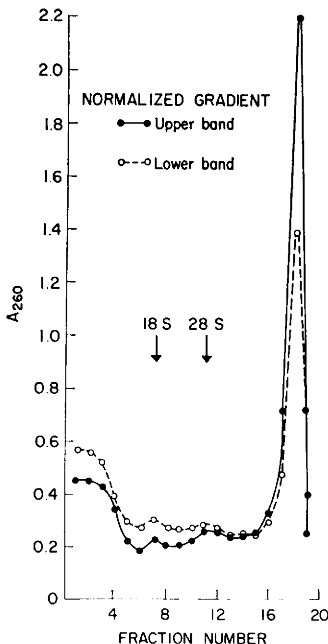


FIG. 4. Sucrose gradients of RNA isolated from upper and lower light-scattering bands of Ficoll gradients. Material in each band was collected and RNA was isolated as described in Methods section. RNA was dissolved in 0.5 ml of 0.01 *M* Tris–HCl (pH 7.4) and put on a linear gradient of 15% to 30% (w/v) sucrose in 0.01 *M* Tris–HCl (pH 7.4) and 0.1 *M* NaCl. The gradients were centrifuged for 24 hr at 25,000 rpm in an SW27 rotor at 15°. Fractions of 1 ml were collected by use of the ISCO density gradient fractionation system. For quantitation, fractions were read on the Cary Model 16 spectrophotometer. Curves for the upper and lower band material were normalized so that the same total optical density was plotted for each. Arrows mark the position in the gradient where ribosomal RNA isolated from chick myeloblasts was found under these conditions; (—) upper band; (- - -) lower band.

nearly the same. If these species of ribosomal-like RNA were related to contamination from cellular fragments and vesicles, the material in the lower bands would be expected to have a greater proportion of these species of RNA than the upper bands which were morphologically free of cellular contamination.

Discussion. This method of purification of AMV from viremic plasma has several advantages. The procedure takes only several hours, and it appears to remove from viremic plasma a class of contaminating cell fragments associated with AMV that is a consequence of intracardiac exsanguination of leukemic chickens. A thorough electron microscopic assessment of purified material at low magnification indicates a high degree of morphologic purity. Unfortunately, no convenient and accurate *in vivo* or *in vitro* system of assay for infectivity has been established for AMV. Consequently, the extent of purification by the Ficoll method can not be given using the criterion of viral infectivity. However, a nearly threefold increase in ATPase activity per milligram of protein is achieved during the process of AMV purification by this method. The proportionality among viral turbidity, ATPase activity, and protein content permits a speedy adjustment of virus concentration. The presence of a proportional amount of ribosomal-like RNA in AMV of high morphologic purity as in material of a much less homogeneous character suggests that these species of RNA are associated with the viral structure, and are not related to cellular contamination of viral preparations. Only AMV obtained from the upper band material by this method of purification was suitable for satisfactory recovery of the core component of AMV after treatment of intact virions with nonionic surfactants (10). The yield of AMV, in terms of protein content as measured by percent transmission, has been about 1 mg of AMV protein for every 4 ml of high-titer viremic plasma purified by this method.

Summary. A method of purification, which employs Ficoll gradients and rate-zonal centrifugation, is described which rapidly separates avian myeloblastosis virus in high-titer viremic plasma from material that is associated with the process of intracardiac exsanguination of leukemic chickens. A nearly threefold increase in specific activity of adenosine triphosphatase (ATPase, an enzyme of the viral envelope) is achieved during purification. Ribosomal-like species of RNA of 18S and 28S are found in AMV purified by this procedure. In addition, a simple method based on viral turbidity is available for approximate quantitation of this

purified AMV in terms of viral protein and ATPase activity.

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