An Improved Adenosine Triphosphatase Assay for Physical Particles of Avian Myeloblastosis Virus (AMV)[1] (34356)

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(Introduced by A. Kellner)

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ATPase activity has been demonstrated in both the plasma of birds infected with avian myeloblastosis virus (AMV) and in media in which infected avian myeloblasts were cultivated *in vitro*. This activity has been reported to be inseparable from the viral particles and to reflect quantitatively the concentration of these particles (3, 6, 9, 10, 13). The properties of this enzyme are similar to those of membrane and "microsomal" ATPase of the leukemic myeloblast (4, 5, 8).

Beaudreau and Becker (1, 2) described an ATPase assay based on the increase in hydrogen-ion concentration resulting from cleavage of the terminal phosphate of adenosine triphosphate. The reaction is followed by measuring the rate of change of absorbance of an appropriate substrate-indicator solution. We report herein a modification of this assay which results in greater precision and reproducibility.

Materials and Methods. Preparation of virus stocks. Virus was obtained from infected myeloblasts of "RIF negative" white leghorn chicks (Spafas, Inc.) which had been inoculated intravenously with about 10^{11} particles of BAI strain A virus at about 1 day of age. Chickens were exsanguinated when microhematocrits revealed maximal leukemia. Heparinized blood was centrifuged and the plasma was quick frozen in liquid nitrogen after addition of 0.01 *M* EDTA and stored at —75°. The buffy coat, containing mostly myeloblasts, was grown in Dulbecco's modification of Eagle's medium containing 50% chick serum (Hyland Laboratories) (1, 7). Tissue culture fluids to be used for adenosine triphosphatase studies were harvested every third day, centrifuged at 150*g* for 10 min, quick frozen in liquid nitrogen and stored at —75°. Plasmas whose physical virus particle concentration had been determined by electron microscopic counts were kindly supplied by Drs. Joseph and Dorothy Beard of Duke University.

Preparation of ^{3}H-labeled virus stocks. Myeloblasts growing in tissue culture were centrifuged and washed once with tissue culture medium. After resuspending the cells in new medium, ^{3}H-labeled uridine (uridine-5 T, ca. 20 Ci/mmole) was added. Fluids were harvested after 48 hr; the cells were removed by centrifugation at 12,100*g* for 10 min at 4°, and small aliquots of the supernatant fluid were frozen in liquid nitrogen and stored at —75°.

Adenosine triphosphatase assay. As described in the text, many modifications of technique were evaluated. The following is the final procedure arrived at:

The buffer solution contained NaCl, 0.05 *M*; $MgCl_2$, 0.05 *M*; Tris(hydroxymethyl)aminomethane hydrochloride (Tris), 0.3 m*M*, pH 7.5; phenol red, 1.41 m*M*.

The substrate solution consisted of 5 m*M* of disodium adenosine triphosphate ($Na_2ATP \cdot 3H_2O$ crystalline, Sigma Chemical Company, St. Louis, Missouri) in the above buffer. The pH of this solution was adjusted with 0.2 *N* NaOH to 7.5. This solution was stored at —75°. Shortly before each experiment, one bottle was thawed at 37° and immediately placed in an ice bath. Fifty μl of sample was added to a total volume of 1.0 ml of the above solution prewarmed at 30°.

We found it necessary at times to vary this

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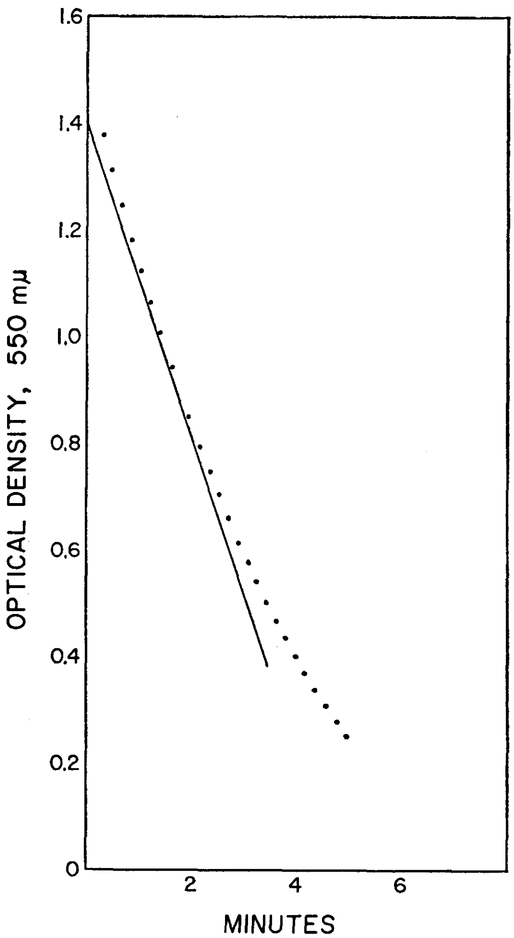

FIG. 1. Reaction kinetics of tissue culture virus, 4.2 $\times 10^{10}$ particles/ml; recorded with Gilford spectrophotometer with recorder adjusted to a full scale of 2 OD units.

amount depending on the virus concentration in the sample. Thus, for very high virus concentrations we used 5 μl sample in a total volume of 1 ml, and for very low virus concentrations, a 50-μl sample was used in a total volume of 0.15 ml. Mixtures of sample and substrate solution were immediately transferred from a test tube to microcells equilibrated at 30° between thermospacers in the Gilford multiple sample recording spectrophotometer. The change in optical density, at 550 mμ, of each of four different reactions, was then recorded every 2 sec for 3–5 min. It was important to wash cuvettes between assays *in situ* in order to maintain temperature control of the thick-walled microcuvettes. This was accomplished with thin polyethylene tubing connected to a vacuum aspirator, and similar tubing connected to an automatic dispenser containing buffer solution held at 30°.

A standard, consisting of tissue culture fluid of known activity, which had been quick frozen in liquid nitrogen and kept at —75°, was run with all tests. This served as a control for the activity of the substrate solution.

Density gradient centrifugation. Linear density gradients containing 10–40% or 11–65% sucrose in 0.1 *M* NaCl, 0.01 *M* Tris, 0.001 *M* EDTA at pH of 7.3 at 0° were prepared with a Buchler polystaltic pump and mixing assembly. 10–40% sucrose gradients were centrifuged at 2–4° in a SW-39 Spinco rotor for 30 min at 64,361*g* for rate zonal runs; 11–65% sucrose gradients were centrifuged for 4 hr under the same conditions for equilibrium runs.

Results. In the presence of adequate substrate concentrations enzymatic reactions initially follow zero order kinetics, and then, as substrate becomes limiting, approach the kinetics of a first order reaction (11).

Figure 1 summarizes a typical experiment in which 50 μl of an AMV virus stock (4.2 $\times 10^{10}$ particles/ml) was added to the substrate-indicator solution described above and immediately placed in a cuvette of a recording spectrophotometer adjusted to record optical density at 550 mμ. This wavelength represents the absorption maximum of the alkaline form of the pH indicator, phenol red. The decrease in optical density thus serves as a measure of the acidification of the solution due to release of terminal phosphate groups from ATP by ATPase. It was noted that the reaction was initially linear, and then slowed. Estimates of reaction rate were made only from the zero order portion of the curve.

A number of variables were investigated to achieve an assay having optimal sensitivity and reproducibility. Temperature of 30° was approximately optimal, due to the high degree of thermolability of this enzyme. Accur-

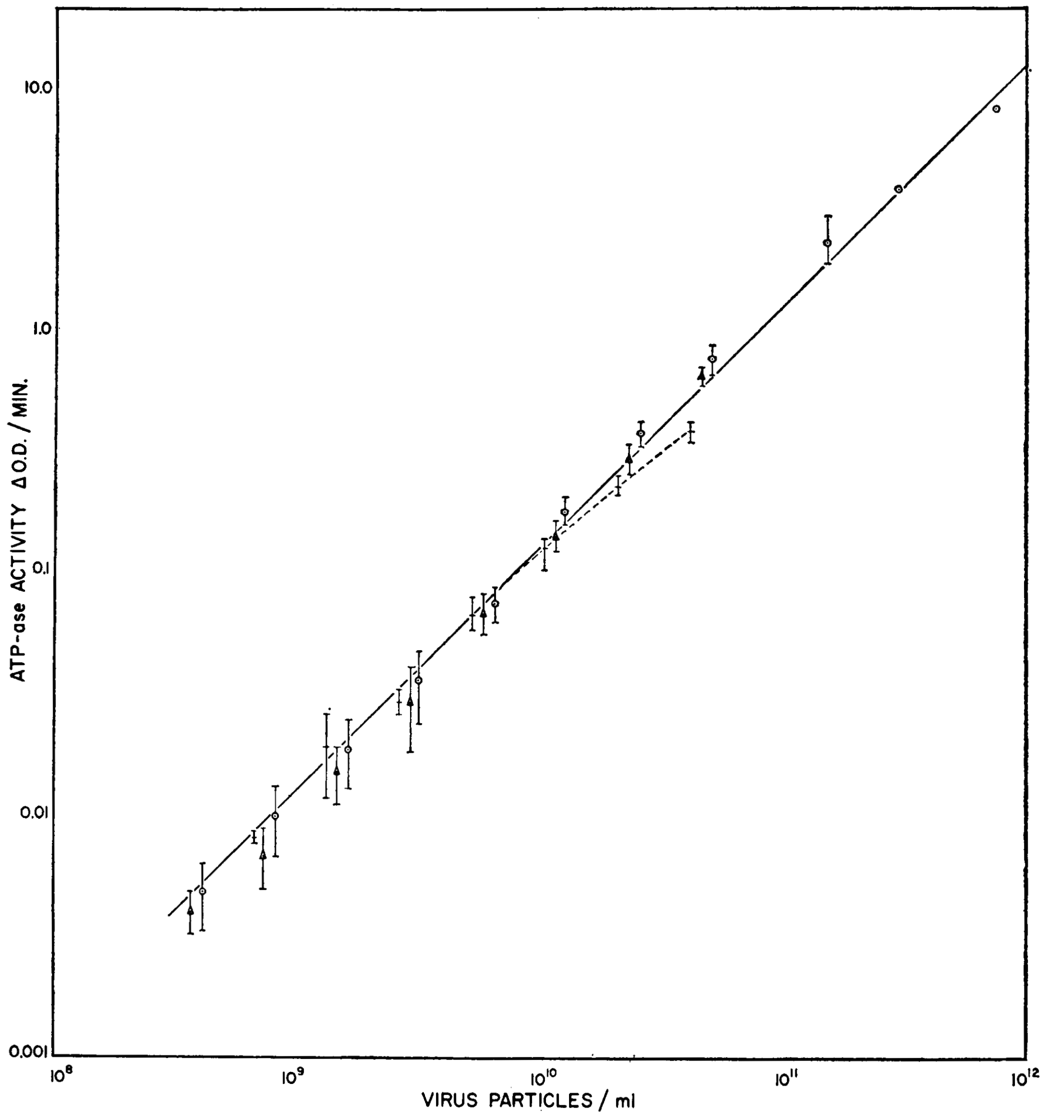

FIG. 2. The relationship between adenosine triphosphatase activity and virus particle concentration; reactions contained 1 ml of substrate solution and 50 μl of virus samples. Plasma No. 2 (Δ); Plasma No. 3 (⊙); and tissue culture fluid (+); (—), the theoretical curve based on the average of all the data obtained for plasmas 2 and 3.

ate control of temperature was necessary. With virus stocks of average concentration, a substrate concentration of 5 m*M* was optimal; higher concentrations were inhibitory.

Beaudreau and Becker (2) employed a bicarbonate buffer for this assay. This buffer must be carefully protected against loss of CO_2 to the atmosphere. For this reason the use of Tris buffer at various concentrations was investigated. It was found that 0.3 m*M* Tris (pH 7.5, 30°) provided optimal sensitivity and reproducibility. Results obtained with this buffer concentration were similar to those Beaudreau obtained with the bicarbonate buffer when it was optimally handled. The results of the above experiments are reflected in the method which was finally adopted and is described in the section on "Methods."

Figure 2 summarizes three independent experiments relating reaction rate to virus particle concentration. Plasma samples were obtained from Dr. Beard of Duke University. These plasmas had been found to have particle concentrations of 4.7 $\times$ 10^{11}/ml. (plasma no. 2) and 10.3 $\times$ 10^{11}/ml (plasma no. 3) as observed by electron microscopic counts; tissue culture fluid containing virus was from our own laboratory. Serial twofold dilutions were made with substrate buffer and 25 samples of each were tested for adenosine triphosphatase activity. For greater sensitivity, the lower dilutions were tested with the recorder set at a full scale of 3.0 units. The higher dilutions were tested with progressive scale expansions up to a full scale of 0.25 OD units.

The data from all three experiments fell on a single linear curve having the theoretically expected slope. Such an ideal relationship can be obtained with this assay only when accurately recorded reaction rates are employed, due to the diphasic nature of the reaction kinetics.

It has been pointed out (12) that a serum component in tissue culture fluids containing virus may inhibit adenosine triphosphatase activity. The effect of this inhibitor is indicated by the broken line in Fig. 2. This effect can be avoided by tenfold dilution of tissue culture virus samples before assay.

Sensitivity of adenosine triphosphatase assay. In order to determine the limit of sensitivity of the assay, experiments were designed at lower virus concentrations. These experiments utilized a total reaction volume of 150 μl, in microcells, with 50 μl of sample. As shown in Fig. 3, the reaction rate became nonlinear below 1 $\times$ 10^8 particles/ml. This degree of sensitivity could not be achieved, however, when normal chicken plasma was employed as diluent. In this case, linear reaction rates extended only to about 10^9 particles/ml.

Precision of adenosine triphosphatase assay. The standard deviations of the experimental points are shown on Figs 2 and 3. Between 5 $\times$ 10^{10} and 5 $\times$ 10^8 virus particles/ml, the standard deviation averages about $\pm 5\%$; with lower virus concentration this error rises to as high as ± 25–30%.

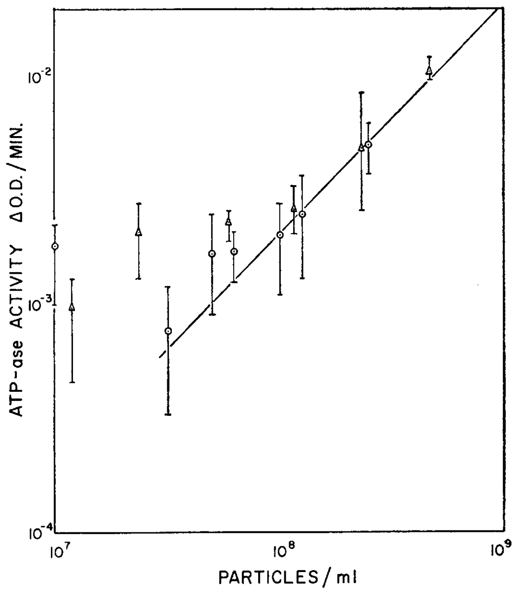

FIG. 3. The relationship between adenosine triphosphatase activity and virus concentration at high dilutions; reactions contained 0.1 ml of substrate solution and 50 μl of virus sample. Symbols are those employed for Fig. 2.

In order to determine the reproducibility of this assay over a period of months, frozen aliquots of virus in tissue culture fluid containing 4.2 $\times$ 10^{10} particles/ml were tested over a period of 6 months using three different batches of substrate. Initially, 25 assays were carried out on a single aliquot. This yielded an average reaction rate of 0.376 $\pm$ 0.015 ($\pm$2 SD) OD units/min. Twenty-five subsequent assays gave an average reaction rate of 0.35 $\pm$ 0.03 OD/min. The detailed results are illustrated in Fig. 4. It may be concluded that the viral enzyme and substrate were stable when stored at —75° for several months; and that there was little or no additional variation due to time of testing.

Stability of viral enzyme to freezing and thawing. To determine for practical purposes the stability of the viral enzyme, samples of infected tissue culture fluid were distributed to a number of glass and autoclavable polyeth-

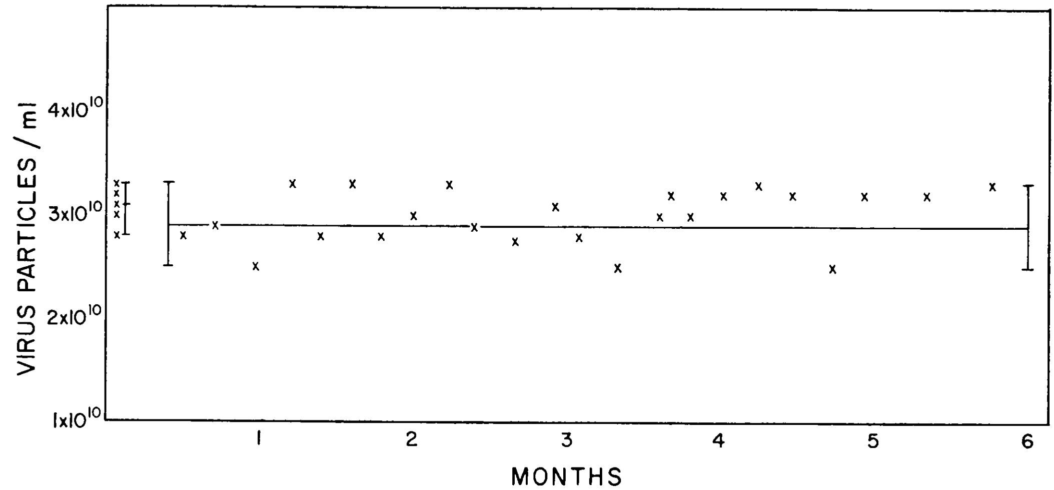

FIG. 4. Reproducibility of the adenosine triphosphatase assay; a tissue culture virus stock containing 4.2×10^{10} particles/ml was tested 25 times on the same day and then over a period of 6 months. Aliquots were stored at—75°.

ylene tubes in 1-ml amounts, and repeatedly frozen and thawed. Freezing was carried out by placing the samples in a —75° deep freeze, and thawing was carried out rapidly in a 37° water bath. Both freezing and thawing were considerably slower in the plastic tubes due to their insulating properties. The results summarized in Fig. 5 revealed no significant loss of activity with as many as six successive freezings and thawings in both types of tubes.

Specificity of adenosine triphosphatase test. It has been previously clearly established (6, 10, and 14) that the adenosine triphosphatase test was quite specific for virus particles in plasma, there being no measurable activity in uninfected avian plasmas. Furthermore, the enzyme activity was associated with virus particles throughout a wide variety of physical separation techniques. It is for these reasons that the adenosine triphosphatase assay can reliably be employed to estimate concentrations of avian myeloblastosis virus in plasma.

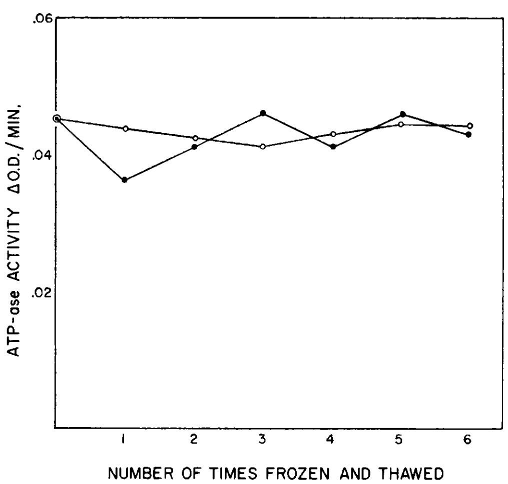

FIG. 5. Stability of viral adenosine triphosphatase activity to repeated freezing and thawing. Glass tubes (○); plastic tubes (●).

The specificity of the adenosine triphosphatase assay for virus contained in tissue culture fluids has not been investigated. Some cultures show appreciable proportions of dead or injured cells, especially as a result of some experimental manipulations. It was, therefore, important to determine whether such cultures might liberate nonvirion associated adenosine triphosphatase into the supernatant fluids.

Rate zonal and equilibrium density gradient centrifugation through sucrose gradients was employed to examine this question. Virus infected ^{3}H-uridine labeled tissue culture fluids were placed on top of 10–40%, and 11–65%, sucrose gradients and centrifuged for 0.5 and 4 hr respectively at 64,361g in the SW-39 Spinco rotor. Gradients were analyzed by collecting drops from the bottom of

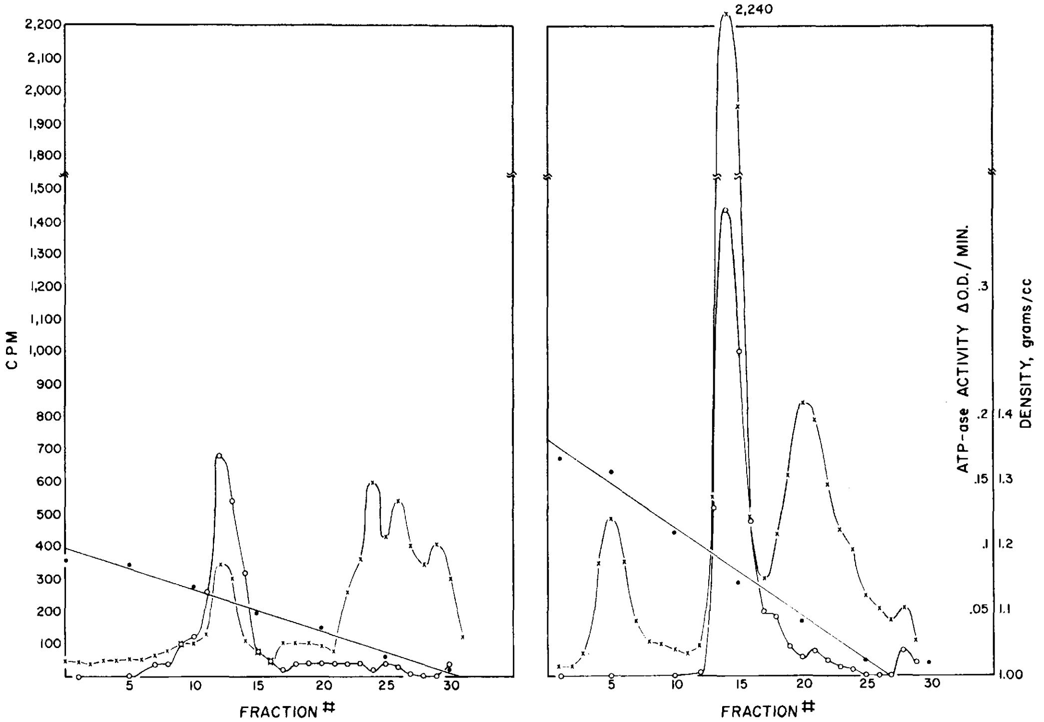

FIG. 6. Rate zonal (left figure) and equilibrium sucrose density gradient centrifugation (right figure) of tritium-labeled avian myeloblastosis virus. Radioactivity (×); adenosine triphosphatase activity OD/min (○).

pierced tubes. Fractions were analyzed for adenosine triphosphatase and for acid precipitable radioactivity as previously described (12). As shown by the results presented in Fig. 6, there was no appreciable adenosine triphosphatase activity located in regions other than that of the virus peak. It may thus be concluded that in tissue culture fluids the major fraction of the adenosine triphosphatase activity sediments like virus particles, and is, therefore, most probably virion associated.

Discussion. The cells and virus of avian myeloblastosis provide a unique system for investigation of host cell–virus relationships in leukemia. The cells are easily maintained for long periods of time, and liberate virus particles in large quantities. There are few other tumor viruses which can be produced in such large quantity *in vitro*; none have such an accurate and rapid bioassay for quantitative studies. For these reasons we considered it of interest to improve the adenosine triphosphatase assay technique for this virus. The introduction of constant temperature, a more stable buffer system, and the use of the Gilford multiple sample recording spectrophotometer resulted in a linear relation between virus concentration and enzyme activity. These modifications have also resulted in a high degree of day-to-day reproducibility of the assay. Variations in enzyme activity of a frozen standard did not significantly exceed those obtained in repetitive assays on any 1 day.

As presently employed, the assay can reliably detect as little as 1×10^8 physical virus particles/ml. This is about a hundredfold lower than the concentrations normally present in infected materials. Since the assay is carried out with only 50 μl of virus material this sensitivity corresponds to detection of approximately 5×10^6 physical particles. This is approximately the same degree of sensitivity achieved by hemagglutination assays. It was, however, found that this degree of sensitivity was not obtained when dilutions were made in normal chicken plasma. A linear reaction was observed in this case only down to about 10^9 particles/ml. This may reflect the intrinsic buffering capacity of plasma. Thus concentrations of virus in chicken plasma below 10^9 particles/ml can probably not be measured by this technique.

Summary. The adenosine triphosphatase (ATPase) assay for avian myeloblastosis virus (AMV) was modified to produce greater sensitivity and reproducibility. With the new assay, and virus cultivated *in vitro*, the relationship between adenosine triphosphatase activity and virus concentation was linear between 1×10^{12} and 1×10^8 particles/ml. This assay was, however, tenfold less sensitive when virus in avian plasma was assayed. Rate zonal and equilibrium density gradient centrifugation experiments confirmed the specificity of the reaction.

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