Concentration of Wound Tumor Virus Determined from Absorbance of the RNA of Virus Collected on Filters

Ikuo Kimura and L. M. Black

Provisional Department of Genetics and Development, University of Illinois, Urbana 61801. Present address of first author: Institute for Plant Virus Research, 959 Aoba, Chiba 280, Japan.

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ABSTRACT

KIMURA, I., and L. M. BLACK. 1976. Concentration of wound tumor virus determined from absorbance of the RNA of virus collected on filters. Phytopathology 66:371-378.

Counts of purified wound tumor virus (WTV) particles in an electron microscope were correlated with the ultraviolet absorbance of their extracted RNA. The regression equation for the correlation is $C = (0.935 \pm 0.0146)\ A + (0.012 \pm 0.0049)$ where C = number of virions ($\times 10^{-12}$) per ml and A = absorbance at a wavelength of 258 nm. The purified virus, which has a diameter of ~70 nm, was collected from solution onto Millipore filters with an average pore diameter of 50 nm. The RNA was extracted by placing the filter with its virus deposit in 2 ml of 1 N HCl and maintaining the preparation at 24 to 30 C for 20 to 24 hours. Accurate WTV-RNA readings could be made on virus from 2-g samples of tumors when such filters were used to collect the virus, whereas with samples seven times larger, less than half the WTV-RNA was recovered when virus was collected by centrifugation into a pellet.

Relative concentrations of wound tumor virus (WTV) originally were expressed in terms of the dilution of virus extract from a known weight of tumors or viruliferous vector insects. However, such materials often contain different concentrations of virus depending upon the temperature during plant growth (13), the virus isolate (12, 15), or other factors. Gamez and Black (9, 10) counted WTV particles in the electron microscope to determine absolute concentrations of virions in extracts from tumors and viruliferous insects. Ahmed (1) and Ahmed and Black (2) correlated the concentration of WTV virions, determined by particle counts in the electron microscope, with spectrophotometric measurements of the RNA extracted from them. They determined that an A_{258} of 1.0 corresponded to a WTV concentration of 10^{12} virions per ml.

Particle counts for the determination of virus concentrations are direct and accurate, but they are also complicated and laborious. The concentration of plant viruses in purified preparations commonly has been determined by measurement of the absorbance of a virus suspension at ~260 nm. However, the method is less accurate for large viruses like WTV because such virions scatter so much light. Spectrophotometric measurement of the WTV suspensions has not been satisfactory in our laboratory not only for this reason but also because the absorbance curve of a preparation often changed radically over short periods of time.

This paper reports a reinvestigation of the relation between particle counts of WTV and the ultraviolet (UV) absorbance of its extracted RNA. It also describes a method of collecting WTV from purified preparations onto filters with an average pore size less than the diameter of the virus. This permitted much more accurate spectrophotometric determinations of WTV-RNA from small quantities of virus than were possible previously. Indeed, it is doubtful if several subsequent studies (14, 15, 16) on the WTV genome, and other genomes in this group, could have been carried out successfully without it.

MATERIALS AND METHODS

Wound tumor virus sample.—Root tumors, produced on sweet clover plants infected with WTV isolates which were vectorial (or wild) at the time the research was done, namely VI66 and VI67 (5), were used as sources of virus which was purified by the use of organic solvents (I. Kimura, *unpublished*) and density-gradient centrifugations (7, 8). It should be reiterated that different samples of tumors vary in virus concentration, and that this must be considered when examining the data reported for experiments below. Purified virus was suspended in Gly-Mg solution (0.1 M Glycine; 0.01 M $MgCl_2$, pH 7.0) because the value of the histidine-$MgCl_2$ buffer [(11), page 268] had not yet been discovered when this work was done.

Measurement of wound tumor virus RNA at A_{258} after collection of virus upon a filter.—The apparatus employed for collection of small amounts of virus in these experiments is shown in Fig. 1. Millipore filters with an average pore diameter of 50 nm[i.e., a diameter less than that of the WTV virion (about 70 nm)] were pretreated with 1 N HCl and then with Gly-Mg solution. No other kind of filter was used. The pretreated filters were then emplaced and fixed firmly in the apparatus. A virus

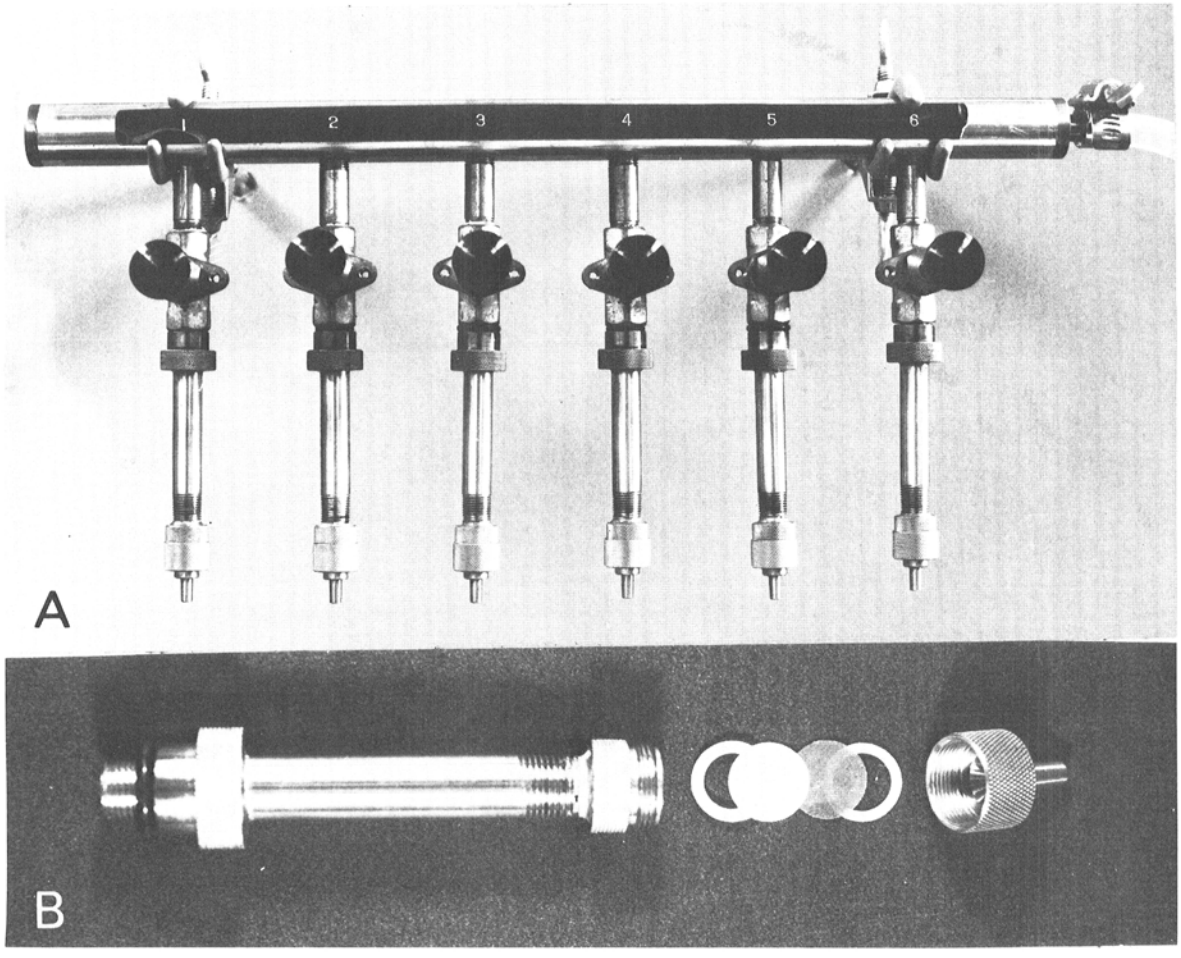

Fig. 1-(A, B). The apparatus employed for collecting wound tumor virus on Millipore filters. **A)** Assembled apparatus. **B)** The parts of an individual collecting unit before assembly. Each unit consisted of a stainless steel Millipore filter holder welded to a metal tube large enough to receive the virus sample before screwing it to the manifold which supplied the air pressure.

sample in 1.0-2.5 ml of suspension was passed through one of the filters under a pressure of 1.4 kg/cm^2 (20 lb/in^2); one or two additional such portions from the same preparation could be added to the same filter if it were desirable to so treat samples of low virus concentration. Afterwards Gly-Mg solution was passed through the filter to wash the virus deposit and the inside of the tube of the apparatus. The virus was not removed from the filter; instead the filter with its deposit of virions was treated with 2 ml of 1 N HCl at 24-30 C for 20-24 hours in a 10-ml glass centrifuge tube which was sealed with parafilm. After the treatments, the denatured protein of the virus was sedimented by centrifugation at low speed and the optical density of the supernatant was measured in a Cary Model 14 spectrophotometer.

Virion counts in an electron microscope (Hitachi Model HU-11A).—The method of counting WTV virions was that described by Gamez and Black (10) and was based on the work of Backus and Williams (3) and Watson (18). The same samples were used for A_{258} measurements of WTV-RNA and for virion counts.

RESULTS

Effect of sucrose on ultraviolet absorbance by wound tumor virus.—Black (4, page 247) reported that reagent grade sucrose commonly contains colloidal material. All of several brands of reagent-grade sucrose when dissolved in solution and ultracentrifuged yielded pellets of the colloidal material, but highly purified sucrose from the U.S. Bureau of Standards did not. Because sucrose was used to prepare gradient columns for the purification of WTV, the possible influence of this colloidal material on analytical procedures was examined. In comparison with a number of brands of sucrose, Analar sucrose (BDH Chemicals Ltd., Poole, England), contained the smallest amount of the colloidal contaminant. Sucrose solutions were prepared which contained 100, 200, 300, 400, 500, and 600 g sucrose per liter. These were centrifuged at 27,000 rpm in a Spinco Rotor No. 30 for 3 hours. After centrifugation, each tube contained a pellet which looked like a pellet of highly purified virus. The pellets in the tubes were obviously smallest in the samples from the

TABLE 1. A_{283} values obtained by acid hydrolysis of pellets formed by colloidal contaminant when solutions of reagent-grade sucrose were ultracentrifuged[a]

Sucrose concentrations centrifuged (g/liter)	Total A_{283} after heating	A_{283} per 100 g sucrose centrifuged
100	0.95	0.95
200	3.45	1.72
300	7.20	2.40
400	9.45	2.33
500	9.20	1.84
600	5.50	0.91

[a]Sixty ml of each sucrose solution was centrifuged at 27,000 rpm in a Spinco No. 30 rotor for 3 hours. Each pellet was resuspended in 1 N HCl and kept at 30 C for 24 hours. It was then heated at 100 C for 1 hour.

highest and lowest sugar concentrations, largest in the two samples from solutions of 300 or 400 g/liter, and of intermediate size in the remaining two (Table 1). Each pellet, with the part of the tube supporting it, was cut out and put into a 10-ml glass centrifuge tube with 2 ml of 1 N HCl. The pellet was treated at 30 C for 24 hours and afterwards it was treated at 100 C for 1 hour. After the treatment at 100 C, marked absorbance was obtained at 283 nm (Fig. 2). The absorbances (Table 1) appeared to be roughly proportional to the observed size of the pellets. If the sample of resuspended colloidal contaminant in Gly-Mg solution was dialyzed against about 300 volumes of that solution for 20 hours at 4 C before the treatment with 1 N HCl at 100 C, there was no UV peak at 283 nm (curve 5 of Fig. 2). The substance, which if heated at 100 C for 1 hour produced a peak at 283 nm, appeared to have been removed by dialysis prior to heat treatment.

The filter procedure for collecting small quantities of purified wound tumor virus.—If filters were to be used for collecting purified virus, the RNA of which was to be quantitated by determining its absorbance at 258 nm, it was important that the filter itself not contribute to the absorbance. The same consideration applied to the eluate, the 1 N HCl, and the Gly-Mg solution. In one experiment, the virus with the filter, the eluate, and a sample of the Gly-Mg solution were each treated at 30 C for 24 hours after making the concentration of HCl in the treated solution 1 N. Following centrifugation to pellet virus protein, the supernatant from each, and the 1 N HCl itself, were measured for UV absorbance. As the results show in Fig. 3, a typical RNA curve was obtained only from the sample containing the virus. A second experiment was carried out in which the virus sample was suspended in a sucrose solution (300 g/liter). After the virus sample had been collected on the filter, it was washed by passing Gly-Mg solution through the filter and then virus and filter were placed in the 1 N HCl solution. As the results show in Fig. 4, the virus contained in sucrose solution yielded a clean RNA absorbance curve, without artifactual absorbance from the sucrose.

Effects of temperature and period of 1.0 N HCl treatment of wound tumor virus (WTV) for measuring A_{258} of wound tumor virus RNA.—Experiments were carried out to determine suitable conditions for complete release of RNA from WTV collected on the filters. The effects of temperature and period of treatment with 1 N HCl were tested for quantitative release of the WTV-RNA. As shown in Table 2, treatments with 1 N HCl for 24 hours at 24, 27, or 30 C gave essentially the same results, but treatment for 24 hours at 20 C gave a significantly lower absorbance. At temperatures of 24, 27, and 30 C sufficient periods of treatment to attain maximum absorbance were 24, 20, and 18 hours, respectively (Table 3). It was assumed that maximum absorbance corresponded to complete recovery of the

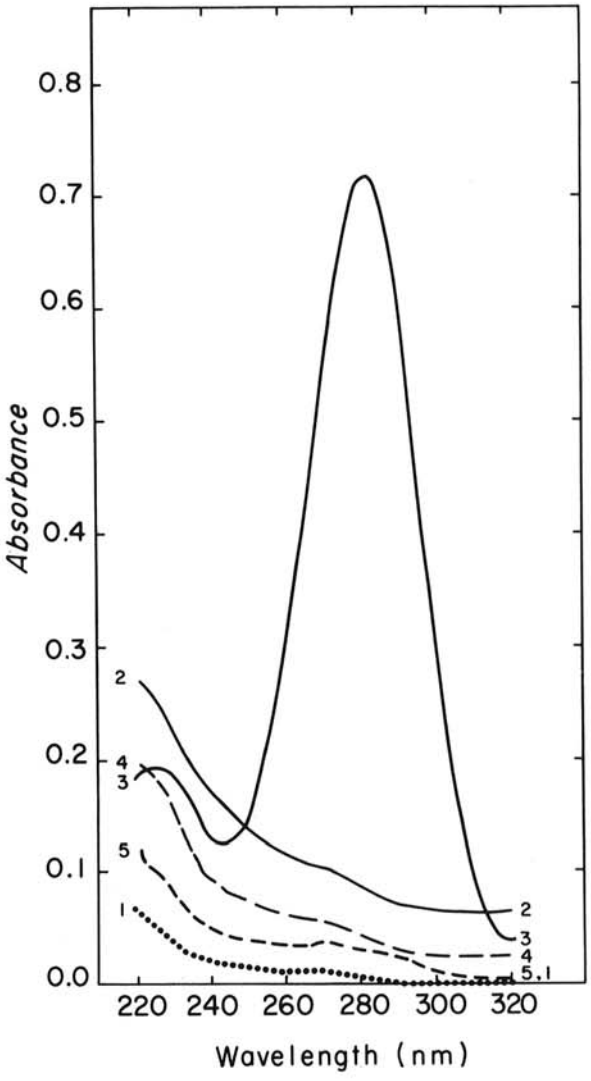

Fig. 2. Ultraviolet-absorbance curves of preparations from pellets obtained from a sucrose solution (300 g/liter) by centrifugation and subsequently treated in various ways. Legend: 1) 1 N HCl (control). 2) Resuspended pellet after heating at 30 C for 24 hours but before heating at 100 C for 1 hour. 3) Resuspended pellet after heating at 100 C for 1 hour. Note that absorbance peak is at 283 nm. 4) Resuspended sample dialyzed but not heated. 5) Sample dialyzed and then heated.

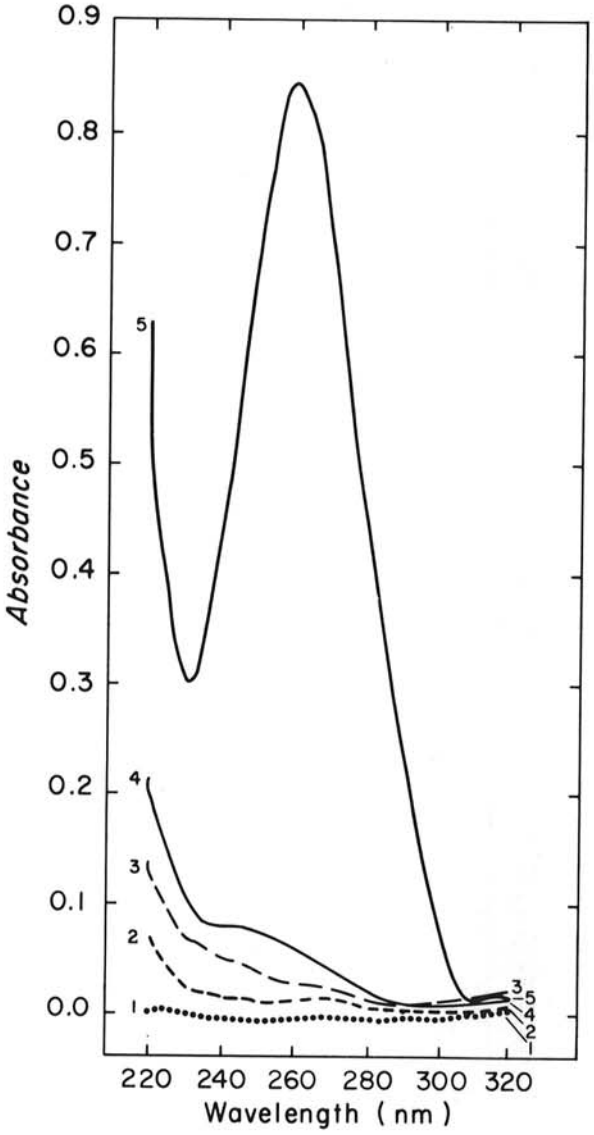

Fig. 3. Ultraviolet-absorbance curves of samples taken during the filter procedure (treatments were with 1 N HCl at 30 C for 24 hours). 1) 1 N HCl (control). 2) Filter alone in 2 ml of 1 N HCl. 3) Gly-Mg solution (2 ml) (0.1 M glycine, 0.01 M $MgCl_2$, pH 7.0) passed through a filter. 4) The eluate. 5) The filter with collected wound tumor virus particles.

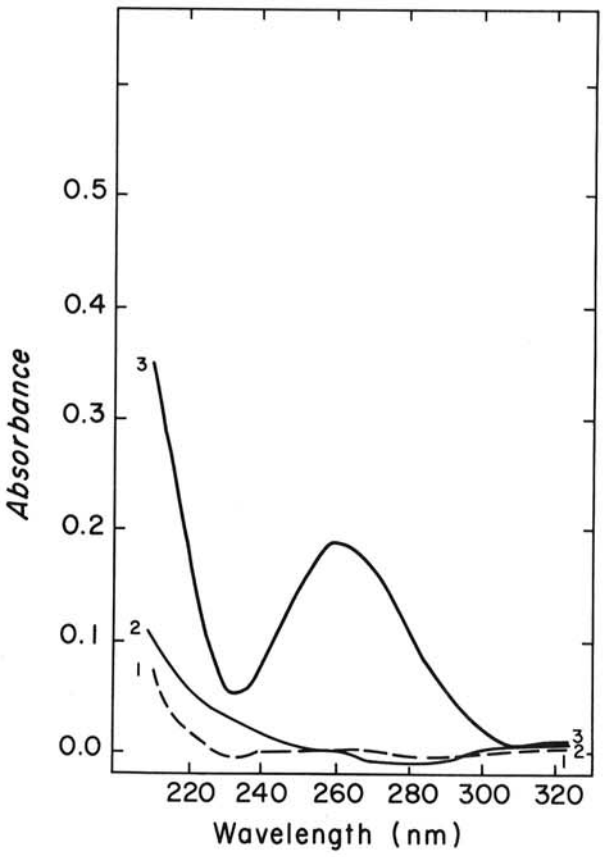

Fig. 4. Ultraviolet-absorbance curve of wound tumor virus (WTV)-RNA and controls for a virus sample containing sucrose (treatments were with 1 N HCl at 30 C for 24 hours). 1) A filter only. 2) A filter after 2 ml of a sucrose solution (300 g/liter) had been passed through it and after it had been washed three times with 2 ml of Gly-Mg solution (0.1 M glycine, 0.01 M $MgCl_2$, pH 7.0). 3) A filter which had been used to filter out purified virus from 2 ml of a solution also containing 300 g sucrose per liter. The filter was then washed three times with 2 ml of Gly-Mg solution before treatment with 1 N HCl.

RNA, and therefore that the data indicate a considerable latitude in satisfactory combinations of temperature and duration of treatment. Most experiments were carried out at 30 C for 20-24 hours.

Measurement of wound tumor virus (WTV) concentration by collection of virions on filters followed by WTV-RNA determinations.—Three experiments were performed to test the quantitative relationship between the amount of virus and the A_{258} value for WTV-RNA in a number of samples. In the first experiment, 3-ml samples of a purified virus suspension, undiluted, diluted one-half, and diluted one-third were passed through filters. After treatment of the collected virus on the filters with 1 N HCl, the UV-absorbance curves of the WTV-RNA were obtained and the A_{258} values for the three different WTV dilutions tabulated (Table 4). In a second experiment, 1, 2, and 3 ml of the undiluted virus suspension from the first experiment were used to measure the A_{258} values of the WTV-RNA. In a third experiment 6-, 5-, and 3-ml quantities of a different virus preparation were similarly tested. In all cases the A_{258} values corresponded well with the known relative quantities of virus (Table 4).

If a virus sample, collected on a filter, was first treated

TABLE 2. Effect of temperature during treatment with 1 N HCl for 24 hours on the release of RNA from wound tumor virus (WTV) collected on filters

Expt. no.	Temperature (C)	A_{258}[a]	A_{258}/A_{235}
1	20	0.775	2.67
		0.745	2.81
	27	0.890	2.92
		0.905	2.59
	30	0.895	2.83
2	24	0.360	2.05
		0.355	2.03
	27	0.355	2.02
	30	0.360	2.00
		0.365	2.02

[a]In experiment 1 each aliquot of purified WTV was derived from 4.5 g of tumors and was contained in 1.8 ml before filtration; in experiment 2 the corresponding quantities were 5.8 g and 2.5 ml.

TABLE 3. Effect of the period of treatment with 1 N HCl at different temperatures on A_{258} measurements of wound tumor virus (WTV) RNA released from purified virus collected on filters

Expt. no.	Treatment temperature (C)	Hours of treatment	A_{258}[a]	A_{258}/A_{235}
1	24	12	0.540	2.40
		18	0.580	2.42
		24	0.620	2.58
2	27	16	0.290	2.10
		20	0.295	2.07
		24	0.305	2.14
3	30	3	0.510	2.26
		6	0.670	2.82
		12	0.700	2.91
		18	0.830	2.82
		24	0.800	2.90

[a]In experiment 1 each aliquot of purified WTV was derived from 2.5 g of tumors and was contained in 2 ml before filtration; in experiments 2 and 3 the corresponding quantities were 2.1 g in 2 ml and 2.6 g in 1.5 ml, respectively.

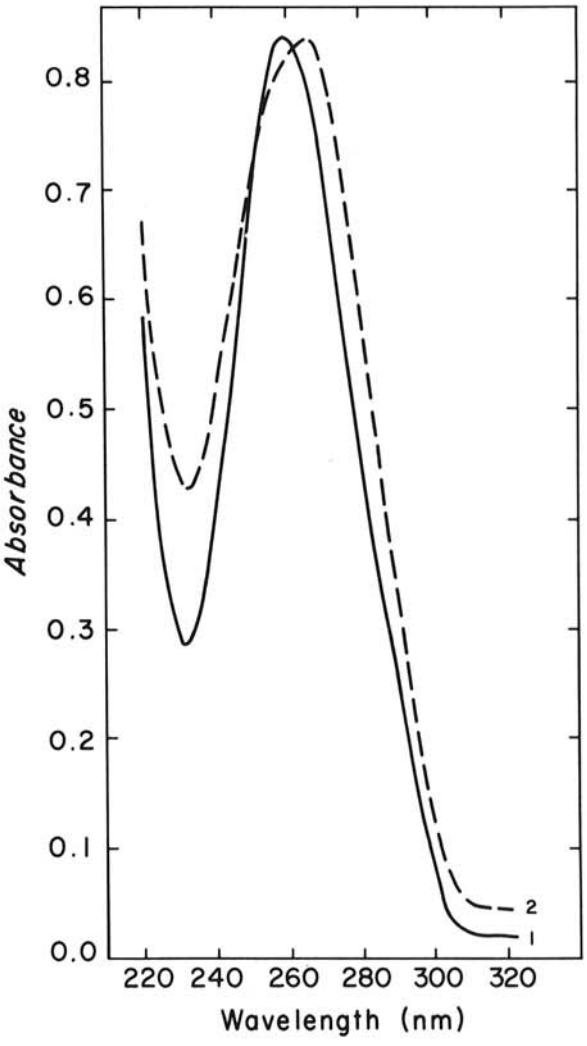

Fig. 5. Ultraviolet-absorbance curves of wound tumor virus (WTV)-RNA preparation: after it was heated at 30 C for 24 hours (curve 1), and after additional heating at 100 C for 1 hour (curve 2).

with 1 N HCl at 30 C for 24 hours, and if the supernatant was then given an additional treatment at 100 C for 1 hour before being allowed to cool slowly, the height of the peak did not change but it shifted from 258 nm to 265 nm (Fig. 5).

Because the A_{258} values for WTV-RNA corresponded with the various purified virus quantities involved, it was decided to use the method for making quantitative determinations of purified WTV.

Comparison of A_{258} values of wound tumor virus RNA determined on virus collected by filter and by centrifugal sedimentation into a pellet.—One aliquot of a purified virus sample was centrifuged at 30,000 rpm for 90 minutes in a No. 40 Spinco rotor. The $S_{w,20}$ of WTV is about 510 and it should have been completely sedimented in about 40 minutes. The pellet, with the part of the tube supporting it, was cut out and treated with 1 N HCl. The A_{258} value was measured as described in the text and compared with the corresponding value for a 1-ml sample of the same purified virus preparation after collection of the virus on a filter. In three such experiments determinations of WTV-RNA from pellets obtained by ultracentrifugation were only 30, 36, and 43% of the amounts obtained by the filter method (Table 5) even though three to seven times as much material was used to make the determinations on the virus collected by

TABLE 4. Effect of virus concentration and quantity of virus solution on A_{258} measurements of wound tumor virus (WTV) RNA released from purified virus collected on filters

Expt. no.	Quantity of virus		A_{258}[a]	A_{258}/A_{235}
1	Three ml of diluted virus solution A. Dilutions:	1/1	0.740	2.36
		1/2	0.400	2.22
		1/3	0.270	1.84
2	Volumes of virus solution A in ml	3	0.750	2.68
		2	0.490	2.22
		1	0.270	1.93
3	Volumes of virus solution B in ml	6	1.175	2.93
		5	0.955	2.77
		3	0.545	2.32

[a]One milliliter of undiluted solution A and B contained WTV purified from 2.25 and 1.82 g of tumors, respectively. Treatment with 1 N HCl was for 20 hours at 27 C in experiment 1, 24 hours at 27 C in experiment 2, and 24 hours at 30 C in experiment 3.

TABLE 5. Measurement of purified wound tumor virus collected on filters compared with control samples from the same purified preparations collected by centrifugation into pellets

Purified virus from tumor sample	Method of virus collection	Grams of tumors represented in virus fraction so collected	A_{258} calculated for 10 g of tumors	(%)
1	Filter	2	1.900	100
	Pellet	14	0.689	36
2	Filter	3.5	2.200	100
	Pellet	10.5	0.655	30
3	Filter	2	2.000	100
	Pellet	14	0.860	43

centrifugal sedimentation. It is obvious that the latter quantities were not large enough to approach the accuracy obtained by the filter method. For work with small quantities the latter method has proved to be a valuable technique.

Quantitative relationship between virion counts and A_{258} values of acid hydrolysates of virion RNA.—The virion concentration of each of nine separately purified virus suspensions from different batches of tumors was determined by particle count in the electron microscope. Statistical examination of the counts showed that their variance, for some unknown reason, was less than one would expect from Poisson's theory. The absorbance of hydrolysate was measured on one virus sample from each of six preparations and on three samples of different quantity from each of the three remaining preparations. All 15 virus samples used for hydrolysis were collected by the filter method. The results (Fig. 6) showed a linear relationship between the A_{258} value of the WTV-RNA hydrolysate and the WTV concentration as determined by the number of virions counted in the electron microscope. The regression equation relating the two measures was:

$$C = (0.935 \pm 0.0146)A + (0.012 \pm 0.0049) \text{ (Equation C)}$$

where C = number of virions ($\times 10^{-12}$)/ml and A = absorbance at λ 258 nm. The 15 experimentally determined points and equation C are plotted in Fig. 6. Absorbance values obtained in the same manner as ours readily can be converted to virion concentration from the graph or calculated from equation C. For A_{258} values between 0.25 and 0.585 the 95% confidence limit is less than $\pm$ 2% of the estimated value. The average limit for the line is ~2.3% whereas the coefficient of variation for the nine counted virion concentrations, each of which was determined by counting particles in 10 droplets, averaged 7.7%, a limit more than three times greater. This indicates that use of the regression correlation is preferable to particle counting, even though the former requires much less work.

It should be noted that the standard error shows the slope of the experimentally derived regression line to depart slightly, but significantly, from unity. This may be due to some unknown systematic error inherent in the method, and indicates that it would be better to use the experimentally determined regression than to assume a slope of unity when converting the absorbance values to virion concentrations. Incidentally, the intercept of the line of Fig. 6 is 0.012 ± 0.0049 which is significantly ($P = 0.05$) different from zero.

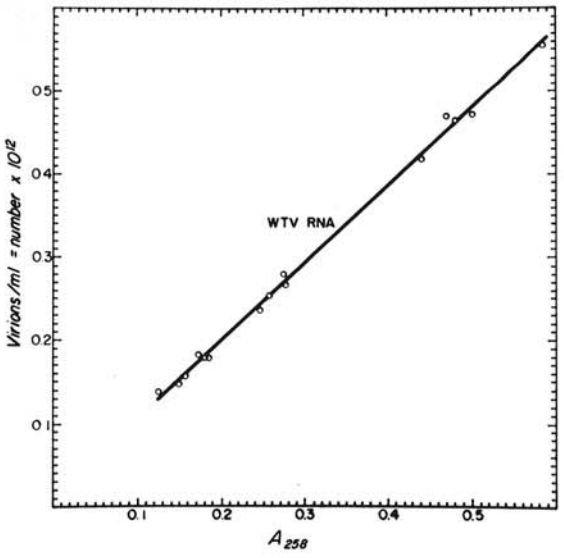

Fig. 6. The regression of wound tumor virions per ml, as determined from particle counts of droplet residues in the electron microscope, is plotted against absorbance at a wavelength of 258 nm. The line covers only the actual range of absorbances for acid hydrolysates of WTV-RNA and of counts of virions determined experimentally. Within these limits, absorbances of small quantities of WTV-RNA hydrolysates can be converted graphically to virion concentrations provided the procedures described in the text are followed. The line is calculated from equation C of the text and precise calculations of virion concentrations and of 95% confidence limits can be calculated from equation C.

DISCUSSION

Although the straight line of Fig. 6 appears closely to approximate Beer's Law, it should be emphasized that it is not presumed to be a test of the validity of Beer's Law by means of the hydrolysates and virion counts. Strictly, the use of the regression should be limited to the conversion of absorbances between 0.125 and 0.585 obtained by the same procedures. According to equation C an A_{258} of 1.0 is equivalent to a virion concentration of 0.95×10^{12} virions/ml. This is comparable to the independent determination of 10^{12} virions/ml by Ahmed and Black (2) who did not collect virus samples on filters and consequently had to use for their measurements, much larger samples from which virus was collected by centrifugation into a pellet.

The RNA of WTV has an AU/GC ratio of 1.58 (6). If the absorbance of the hydrolysate of 1 mg of WTV-RNA in 1 ml of 1 N HCl is taken as 30, the concentration of virion genomes equivalent to an A_{258} value of 1.0 can be calculated from Avogadro's number as follows:

$$\frac{A_{258} \times \text{Avogadro's number}}{\text{MW of WTV genome} \times 10^3 \times 30}$$

or:

$$\frac{1 \times 6.02 \times 10^{23}}{16.7 \times 10^6 \times 10^3 \times 30} = 1.2 \times 10^{12} \text{ virions/ml.}$$

In this calculation the molecular weight of the genome is that estimated from electrophoresis of the genome segments in Loening's buffer (17). The corresponding concentration of 0.95×10^{12} virions/ml derived from equation C, is in satisfying agreement with these independent estimates.

It should be emphasized that the work establishing the linear relation of Fig. 6 was done with isolates VI66 and VI67 when they were vectorial (wild). Subsequently, the relationship was used to determine total amounts of virus (active plus inactive), in combination with infectivity assays, to estimate absolute specific infectivities of WTV at various stages of purification (14).

The filter method of collecting virus avoids the important sources of errors of convection and stirback during sedimentation of virus in angle head rotors. It also avoids loss of virus through its resuspension before the pellet can be removed from the centrifuge tube. All of these errors become more serious the smaller the quantity of virus that is collected. Moreover, the filter method permits the deposition of one virus sample on top of another on the same filter if individually filtered volumes of a sample contain too low a concentration of virus to permit accurate measurement. Little or no virus is lost by passage through the filter; filtration of purified WTV preparations, infective at dilutions of at least 10^{-5}, yield filtrates that are noninfective.

Because the A_{258} values for hydrolysate corresponded closely to the number of virions determined by particle counts, the wild WTV virions from the various purified preparations appeared to have a constant RNA content. It is now known that molecular weight equivalents of the genomes of subvectorial and exvectorial WTV isolates vary (15). However, where the molecular weight equivalents of the genomes of subvectorial and exvectorial isolates are known (15), it should be possible to use the straight line of Fig. 6, with an appropriate correction, to obtain useful estimates of concentrations of such defective isolates. Similarly, it is likely that the concentrations of other viruses in the WTV group can be estimated from Fig. 6 by applying an appropriate correction, once the molecular weights of the other genomes are determined (16).

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