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Counts of Virus Particles by Sedimentation on Agar and Electron Micrography.* (19782)

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The solution of many problems in virology is dependent on the quantitative correlation of number of virus particles or unit virus mass with the biological attributes of the agent. Estimation of mass or particle number in preparations of purified viruses of uniform shape and size can be made simply and fairly accurately by chemical analysis. In other cases, only direct counts of the particles offer assurance of accuracy. One excellent method for this purpose involves the comparison of a known number of latex particles mixed with virus particles in sprayed droplets(1). In another procedure(2), virus is sedimented on a collodion membrane, and counts are made of the particles in a measured area. Success with the first of these methods, however, is dependent on high concentration of virus and, with both, on the absence of interfering extraneous material such as protein or salt.

Recently, a modification of the sedimentation procedure has been developed which retains the high sensitivity demonstrated in the earlier work and, in addition, gives promise of high accuracy with preparations containing protein, salts, and other material which hinder electron micrography or may have a harmful effect on the virus particles when dried with them. This method consists in the sedimentation of the virus onto an agar surface from which it can be removed by means of a collodion pseudoreplica.† In this process, excess protein can be washed away, and the salt remaining diffuses into the agar. It is evident, however, that the applicability of this process, as well as others involving electron micrography, is entirely dependent on criteria for unequivocal identification of the virus particles because of characteristic morphology or a specific means for relating virus activity to the particles counted. Preliminary studies on the use of agar for examination of the virus particles of erythromyeloblastic leucosis have been described(3). In the present paper are given results obtained with the agar technic applied to the enumeration of influenza virus particles in purified preparations and in a comparison of these findings with those observed with other methods of counting.

Materials and methods. Counts were made by sedimentation of the influenza virus on an agar surface; sedimentation of the particles on a collodion membrane; and by the spray technic. The mechanical equipment employed in the sedimentation experiments consisted of an air-driven ultracentrifuge, ordinarily used for analytical work, the rotor of which was fitted with cells specially made for the study.

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† The pseudoreplica is a film which, when stripped off, brings with it the virus particles.

These cells, described previously(2), have a wedge-shaped cavity 1 cm in radial depth and 1 cm^2 at the bottom. A group of 4 cells of similar size and weight were operated simultaneously. Sedimentation was effected by spinning the rotor at 16,300 X g for 30 minutes.

Agar in 1 to 3% concentration in water or buffer solution, as described later, was poured into petri dishes to a depth of 2 to 3 mm and allowed to harden. Pieces 1 cm square were cut out and, with care to avoid damage to the surface, placed on the bottom of the cells. The cells were then filled with the virus preparation, sealed and spun. At the end of the run the fluid was carefully pipetted off and, if necessary, the agar surface was flooded gently with Ringer's solution several times to remove the supernatant fluid. The agar was taken from the cell and placed on a glass slide for convenience in handling. When the excess fluid had evaporated or diffused into the agar, a small glass dish or capsule carrying small drops of 2% osmic acid on the inner surface was inverted over the agar for about 20 minutes. A solution of 0.5% collodion in amyl acetate was then pipetted over the agar surface and drained off by tilting the slide against a piece of filter paper. The collodion film, after drying for 3 to 5 minutes, was torn with a needle about the edges of the agar block and floated off on a water surface by submerging the slide held obliquely as it passed into the water. Since the virus was on the underside, the film was inverted as it was removed from the water in preparation for shadow casting.

Agar was buffered with sodium acetate-acetic acid in the range of pH 4-6; with phosphate at pH 6 to 8; and with sodium borate-boric acid between pH 8 and 9. Stock solutions of the acetate and phosphate buffers of 1.0 M concentration were diluted 1 part with 4 parts of 0.85% NaCl solution containing 0.4% formaldehyde. The borate solution was of 0.5 M concentration and was diluted appropriately to yield the same final buffer salt concentration as the others in the agar. The melted agar in 3% concentration in distilled water was mixed with an equal volume of this solution and poured into petri plates to harden.

The procedures employed for sedimenting the particles on collodion fixed on glass were those previously described(2). Particles were counted by the spray technic as described by Backus and Williams(1). Polystyrene particles mixed with virus were a part of the original sample of Dow latex 580 G for which size and density measurements have been reported(4), and particle number was estimated by weighing samples of the suspension. Bovine serum albumin (Armour) in 0.3% concentration was added to the mixture to outline the drops.

Influenza virus A (PR8 strain) was purified as previously described(5), suspended in Ringer's solution of pH 7.4 and preserved with 0.4% formaldehyde at 0-4°C. All subsequent adjustments of pH of this virus suspension were made with NaOH or HCl and checked with a pH meter with a glass electrode. Measurements of pH were frequently made on the suspensions from which virus was spun, and no significant drift was observed.

Results. Influence of pH. The initial experiments were made to determine the conditions optimum for the sedimentation of the influenza virus on an agar surface and for the quantitative incorporation of the particles in a collodion membrane suitable for electron micrography. In these studies the purified virus in Ringer's solution of pH 7.4 was spun down on the surface of agar prepared in distilled water without the adjustment of pH. It was soon evident that the number of particles observed varied over a considerable range. One factor investigated for possible contribution to these inconsistencies was the pH of the suspension from which the virus was sedimented.

The results of a series of counts through the range of pH 5 to 9 are shown in Fig. 1. The stock suspension containing 4.23 mg of influenza virus per ml was diluted 10,000-fold with Ringer's solution in 5 ml volumes and the pH adjusted to the required value. Two screens were prepared from each agar block and shadow-cast with chromium in the usual way. In Fig. 1, each point is the average of 5 fields in the electron microscope at a magnification of 3,610 X and 25 cm^2 on the negative. All areas in these and the other experi-

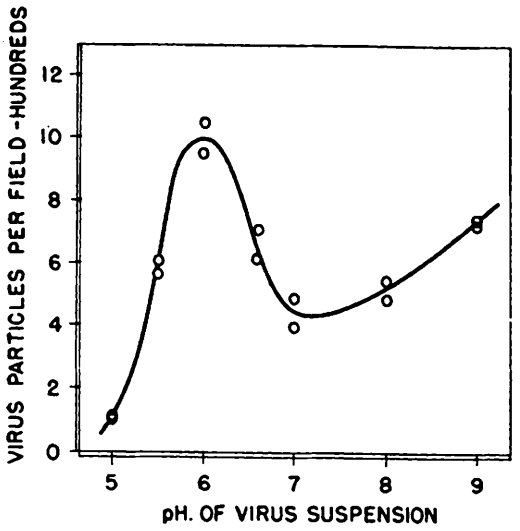

FIG. 1. Relation of pH of influenza virus suspension to number of virus particles sedimented on the agar surface and removed in the pseudoreplica for electron micrography.

ments were chosen by turning the stage controls of the microscope at random, after focusing, and taking the picture where the stage stopped.

A strong dependence of the number of particles on the pH of the virus suspension is clearly evident. Most conspicuous is the maximum at pH 6. At pH 5 and pH 9 there were signs of alteration of the virus particles. The virus was severely aggregated at pH 5. At pH 9 there was no serious aggregation, but the particles in the pictures seemed greatly flattened.

Many of the studies reported here were made by this technic. Later, it was learned that the method could be simplified, and the routine consistency of the results could be improved by adjustment of the pH of the agar rather than that of the suspension of virus particles. Unbuffered saline suspensions of virus quickly take on the pH of the agar block when placed in the cell for sedimentation. An additional refinement of the procedure consisted in the preparation of the agar plates at least a day in advance of the study. Under these conditions, the pH of the agar in the range of 4 to 8 was of little influence on the counts, as seen from the data of the following experiment. A sample of the stock virus suspension was diluted 10,000-fold with 0.85% NaCl solution containing 0.4% formaldehyde with a resulting pH of 5. Portions of this suspension were then sedimented onto agar buffered at various pH values from 4 to 9.

In Fig. 2 each point is the average of the counts of 5 fields chosen at random from a screen of the various preparations indicated. It is evident that the values from pH 5 to 8 were essentially identical; much lower counts were obtained at pH 9, and at pH 4 the values were somewhat erratic. The number of par-

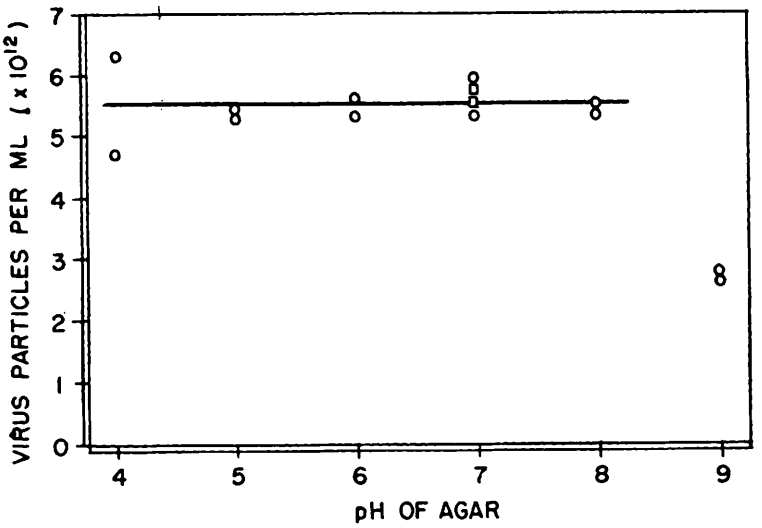

FIG. 2. Relation of pH of agar block to number of virus particles sedimented on the agar surface and removed in the pseudoreplica for electron micrography.

TABLE I. Virus Particle Counts on 25 Cm^2 Negatives of Purified Influenza A (PR8 Strain) Diluted with Ringer's Solution of pH 6 and Sedimented Directly onto an Agar Surface.

Run	Cell	Micrograph field A	B	C	D	E	
1	1	718	774	630	686	668	
	1	612	650	776	608	816	
	2	794	750	862	874	856	Mean 728
	2	770	798	798	764	692	$\sigma = \pm 13.7\%$
	3	920	774	882	702	836	$\epsilon = \pm 2.2\%$
	3	698	668	798	820	677	
	4	626	636	620	818	480	
	4	512	744	688	662	660	
2	1	626	766	704	682	716	
	1	790	656	698	736	650	
	2	894	598	896	744	756	Mean 748
	2	954	892	958	756	922	$\sigma = \pm 16.6\%$
	3	508	758	550	534	558	$\epsilon = \pm 3.6\%$
	3	750	560	844	838	734	
	4	952	784	822	874	858	
	4	732	810	570	704	756	

TABLE II. Comparison of Particle Counts of Dow 580G Latex and of Purified Influenza Virus A (PR8 Strain) Obtained by the Various Methods.

Material	Method	Particle No.
Latex	Weighing	6.2×10^{10}
	Sedimentation on collodion	6.1×10^{10}
Virus	Spray	5.2×10^{12}
	Sedimentation on collodion	5.1×10^{12}
	" " agar	5.5×10^{12}
	Nitrogen content	8.9×10^{12}

ticles per ml of the stock suspension, calculated as the average of the data of Fig. 2 from pH 4 to 8, was 5.5×10^{12}. This is not significantly different from the data of the following studies.

Precision of the results. The variations observed in repeated examinations of a given preparation are shown in Table I. For this experiment, the stock virus preparation was diluted 1 to 10,000, as before, with Ringer's solution adjusted to pH 6. Two sedimentation runs were made at 16,300 X g for 30 minutes with 4 cells each and employing unbuffered agar. A pair of films was prepared from the agar block of each cell, and 5 exposures were made with each film. The data of Table I show the individual variations encountered and indicate that values of considerable consistency can be obtained with the number of counts practical for routine studies.

Accuracy of the counts. The results obtained by sedimentation of the virus on agar have been compared with those seen with the spray procedure and on sedimentation of the virus on collodion-on-glass. The numbers of particles enumerated by the 3 methods are given in Table II.

A mixture was made containing 0.1 ml of the stock virus suspension, 0.45 ml H_2O, 0.75 ml of a 0.3% bovine serum albumin, and 1.2 ml of a water suspension of latex particles. This mixture resulted in a virus dilution of 1-25 and a salt content sufficiently low to yield clear micro-drop patterns when sprayed on collodion and subsequently shadowcast with chromium. Ten drops were photographed.

The number of latex particles per ml of stock suspension obtained by weighing was checked by sedimentation on collodion. Sedimentation gave a value in good agreement with that obtained by weighing (Table II). The value 6.2×10^{10}, derived by weighing, was used, together with the ratio of virus particles to polystyrene balls in the drop photographs, to calculate the virus content of the original stock from spray data with the mixture. It is seen that the results for virus count, obtained by both types of sedimentation technics, gave values in essential agreement with those yielded by the spray procedure. A somewhat higher, though still comparable, value resulted from calculations of particle number from the nitrogen content of the virus preparation.

Discussion. The data on the counting of influenza virus particles, such as those of Table II, show that essentially identical values were obtained with both the spray method and the procedures involving sedimentation. Only a limited number of factors would be expected to interfere with the accuracy of the spray technic, though absolute values are dependent on the accurate determination of the number of latex particles in the reference suspension and on thorough dispersion of both latex and virus particles in the test mixture.

A principal problem in counting with the sedimentation procedure is the control of the factors influencing the quantitative adherence of the particles to the receiving surface and transfer to the electron microscope. It is evident, however, as previously reported(2)

and as found here, that conditions can be readily established for the counting of the influenza virus particles by sedimentation with the same results as those obtained by the spray method.

While the sedimentation technics are complex and tedious, the advantages which they offer, particularly through the use of the agar receiving surface, greatly broaden the field possible for investigation. The range of particle content optimum for accuracy with the sedimentation procedure is approximately 1,000-fold less than that needed for the spray technic. This far greater range brings within reach many tissue extracts and fluids and eliminates the need for either concentration or purification in those cases in which the virus can be readily recognized.

The special value of the agar surface resides in its applicability to preparations containing salt and, in common with the collodion surface, protein. Some viruses, for example those of Newcastle disease and erythromyeloblastic leucosis of fowls, are so distorted and unnatural when dried from preparations containing salt that recognition and counting of the particles are impossible. The advantages of the use of agar and of fixation with osmic acid in the electron micrography of these agents have been described(3). In that work it was observed that salt diffuses rapidly into the agar leaving no evidence of it detectable in the collodion film poured on the agar surface. In the present experiments there has been evidence that some protein may also diffuse into the agar block. Fixation of the virus with osmic acid while on the agar appeared to make the virus impervious to the effects of water during the immersion necessary for floating the membrane.

The applicability of sedimentation on agar to body fluids containing virus has been well illustrated in the case of the studies(3) on the erythromyeloblastic leucosis virus in the plasma from diseased fowls. More recently, preliminary investigations have indicated the feasibility of quantitative assay of influenza virus in chorioallantoic fluid from embryos diseased with this agent.

Summary. A method has been devised for counting virus particles in electron micrographs of the virus sedimented on an agar surface and removed quantitatively in a collodion pseudoreplica. With influenza virus A (PR8 strain) as test virus, the accuracy of the counts, under conditions practical for routine studies, was the same as that observed with the spray technic or by sedimentation of the agent on collodion. In common with the latter method, sedimentation on agar is applicable to materials of relatively low virus content and, since salt diffuses into the agar, to viruses harmed either by drying in the presence of salt or by salt-free water. The usefulness of this procedure is limited to the study of virus particles which can be recognized either because of characteristic morphology or of other properties that can be related quantitatively to virus behavior.

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