ELECTRON MICROSCOPIC MEASURE OF VIRUS PARTICLE DISPERSION IN SUSPENSION*

by

D. GORDON SHARP AND M. J. BUCKINGHAM

Department of Surgery and Department of Physics, Duke University School of Medicine and Duke University, Durham, North Carolina (U.S.A.)

An important property of liquid suspensions of virus particles in body fluids, such as blood plasma, or in purified concentrates in buffer solutions is the state of dispersion or aggregation of the individual particles. Existing methods for estimation of the quantitative aspects of this state are rather indirect.

Sedimentation velocity analyses in the ultracentrifuge are capable, mainly, of yielding data on particle size in such suspensions. Information regarding the kind and degree of aggregation existing among the particles comes from the number and magnitude of secondary, more rapidly sedimenting, boundaries present. These are difficult to evaluate accurately[1], and nothing can be done with suspensions containing much less than 10^{12} virus particles per ml.

Virus particle suspensions exhibit light scattering, analyses of which may yield a measure of the weight and number of the scattering units. Light-scattering analysis is a widely used tool in present-day polymer chemistry, and it has been applied to viruses chiefly by OSTER, whose many papers and review[2] provide complete coverage of this method. It has many advantages, prominent among which is the opportunity to observe suspensions continuously by procedures not interfering with the process under study. It gives, however, a result, with aggregated materials, to which the contributions of the various particle clusters must be inferred and not accurately assigned.

The electron microscope, as usually employed, produces pictures in which particle aggregation is so much the product of the methods of preparation for the microscope that analysis could be expected to yield little useful information about the state of dispersion of the particles in the suspension from which they came. This is because preparation of the suspended material for electron micrography, in general, involves drying of the suspension on a suitable collodion or formvar surface, and during the process, forces of surface tension exert their potent influence upon the final arrangement of the particles. Other procedures[3] such as freeze-drying, spraying in microdrops, *etc.*, would also be expected to exert a reorganizing effect on loosely formed aggregates in a particle suspension.

Many of these difficulties in electron micrography can be avoided with a method

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recently developed for counting virus particles[4-6]. This involves sedimentation of the particles from a measured volume of suspension onto an agar surface. In this process the particles come to rest on the surface, and the residual surface fluid and salt pass into the agar. By this means the violent surface forces ordinarily acting during drying and crystallization of salt are absent, and the particles or aggregates remain *in situ* for electron microscopic examination. The chance distribution of particles sedimented from monodisperse preparations and found on the agar can be calculated, and the difference between this and the distribution (singles, pairs, *etc.*) found with actual suspensions of virus is taken to be a measure of the dispersion of the particles in the latter. Such data can be obtained experimentally from suspensions of as few as 10^8 particles per ml. The experiments described in this report were made to test the validity of the simplifying assumptions involved in the theory and to demonstrate the range of usefulness of the method in the study of the dispersion of virus particles in suspension.

MATERIALS AND METHODS

The virus of avian myeloblastic leukosis[6] has been used in these demonstrations. Part of the work has been done with dilute plasma from diseased chicks containing large numbers of these particles[7] and the remainder with virus sedimented from such plasmas and resuspended in various buffer solutions. The sedimentations were made in the angle centrifuge head holding small (1.5-ml) lusteroid tubes in a field of 15,000 $\times$ *g* for one hour at room temperature. No low speed runs were made on the resuspended virus. Some of the materials were sedimented and resuspended a second time under the same conditions.

The virus particles were deposited on the agar surface by sedimentation from suitable dilutions to the plasma or concentrates in wedge-shaped (to minimize convection) ultracentrifuge cells[4]. A layer of 2% hardened agar on the flat cell bottom acted as collector for the sedimented virus. After centrifugation, these agar pieces were removed from the rotor cells, and treated with osmic acid vapor to fix the adhering virus. Pseudoreplicas were then made from the agar surface with collodion*. The virus on the collodion was shadowcast with chromium, and random fields were photographed in the electron microscope at 3,740 $\times$ magnification.

Image diameters were measured on the resulting negatives with a traveling microscope. The total number of virus particles per unit area of the photograph was recorded together with the number of particles in the same area that were free, that is, not touching any other particle. Several different dilutions of a given sample, covering a range of 20-fold, in addition to the initial dilution of the plasma or concentrate, were sometimes made in order to examine the effect of further dilution on aggregation.

Virus samples were tested for effect of pH on aggregation by sedimenting virus from plasma and resuspending it in the desired buffer. After appropriate time, dilutions of the samples for aggregation analysis were made, in the cases of phosphate and

* For reasons not yet sufficiently well understood to warrant discussion, replicas of the agar surface do not always remove all the virus. This can be checked by removing a second collodion film from the same surface and observing the virus present. The data reported here were taken only from experiments in which second replication of the agar surface yielded an insignificant number of particles.

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veronal buffers, with the same buffer. Histidine buffers interfere with the replication technique on the agar; consequently, the required dilutions were made for analysis with physiological saline solution.

THEORETICAL CONSIDERATIONS

If a population of similar particles of radius r is sedimented uniformly on a plane surface, a certain fraction, f, of the total number, N, of the particles on the area A will be found to be free from the rest (*i.e.* the distance between the center of the particle and that of its nearest neighbor is greater than $2r$). In this preliminary treatment we shall not discuss the condition of the remaining particles—how many are in pairs, triplets, *etc.*

We can easily calculate f for a randomly distributed population; each particle has an equal probability of falling at any point on A, so that the probability of a given particle falling within $2r$ of the center of another given particle is $4\pi r^2/A$. The probability of its *not* falling within this distance is $1-4\pi r^2/A$. Thus the probability, P, of not finding *any* particle within the distance $2r$ of our given one is

$$P = (1 - 4\pi r^2/A)^{(N-1)} \longrightarrow (-4\mu r^2 N/A) \text{ as } N \longrightarrow \infty.$$

The fraction of free particles amongst the total found on the area is then $f = \exp(-4\pi r^2 hn)$, where h is the height of liquid from which the particles were deposited and n is the number of particles per unit-volume of the liquid suspension*.

It is convenient to express this result in a logarithmic form:

$$\text{Log}\,(1/f) = 4\pi r^2 N/A = 4\pi r^2 hn.$$

This is related to the fraction, p of A covered by the particles.

$$p = \pi r^2 N/A \text{ so Log } (1/f) = 4p.$$

We can extend these considerations to calculate f when the original suspension has a certain state of aggregation: Let $F_1, F_2 \ldots F_j \ldots$ be the fraction of particles in the suspension which are single, in pairs, triplets and in clusters of j. Then $\Sigma_j F_j = 1$. Thus the number of clusters of j particles is NF_j/j. If we can make the simplifying assumption that a sedimented cluster of j particles effectively occupies a circular area of radius r_j, we can calculate the fraction of single particles on the area A. We shall write $r_1 = r$.

There are $F_1 N$ single particles from which our given particle, to be free, must not fall within a distance $2r_1 = 2r$, $1/2F_2 N$ pairs not within a distance $r_1 + r_2$, $1/3F_3 N$ triplets not within a distance $r_1 + r_3$, *etc.*

The probability that a given single particle remains free is then

$$P = [1 - 4\pi r_1^2/A]^{F_1 N} \cdot [1 - \pi(r_1 + r_2)^2/A]^{\frac{1}{2}F_2 N} \cdot \ldots \ldots$$
$$\longrightarrow \exp\left[-\pi/A \sum_j (r_1 + r_j)^2 F_j N/j\right] \text{ as } N \longrightarrow \infty.$$

where the summation extends over all clusters. Thus, finally, for a large enough N,

$$P = \exp -\pi \left[\sum_j (r_1 + r_j)^2 F_j/j\right] N/A.$$

The total fraction free is then $f = F_1 P$.

* This is subject to a small correction under actual conditions of sedimentation from a sector-shaped cell. In our cell the number of particles falling on unit area of its bottom is 0.93 hn.

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Taking the logarithm of this equation we have

$$\text{Log}\,(1/f) = \text{Log}\,(1/F_1) + YN/A \quad \text{where} \quad Y = \pi \sum_{j}\sum_{1} (r_1 + r_j)^2 F_j/f$$

If $F_1 = 1$, $F_2 = F_3 \ldots = 0$, we have the case of random distribution and

$$Y = 4\pi r^2, \text{ then } \log\,(1/f) = 4\pi r^2 N/A$$

as obtained above for this special case.

To compare experimental results with the above calculations, the former may be plotted with log $(1/f)$ as a function of N/A. The intercept (extrapolated to $N/A = 0$) on the vertical axis gives log $1/F_1$, where F_1 was the fraction of single particles in the original suspension. If the assumptions made are valid, the plot should be a straight line whose slope is Y. The value of Y depends upon the "effective" radius of clusters, but in the case of complete randomness (no clustering), $Y = 4\pi r^2$.

The experiments described here have been designed to test the applicability of the theoretical considerations to the study of virus suspensions under practical working conditions. Some of the experiments were made with suspensions which, though known to contain particles of somewhat variable size, have yielded single sedimenting boundaries in the ultracentrifuge and were consequently essentially free of aggregation. The experiments will show also the kind of information obtainable on subsequently aggregated samples of the same virus preparation. All data consist of observed values of f, the fraction of individual particles on the observed area. In the description of results, use will be made of an index of dispersion D, which is the ratio of the fraction of free particles seen to the fraction predicted for an equally concentrated but unaggregated suspension. From the theory, this may be seen to approach F_1 as N/A approaches zero.

EXPERIMENTS AND RESULTS

In earlier studies[8], calculations made from sedimentation velocity data gave the value 144 mμ as the average diameter of the hydrated virus of myeloblastosis. For the present purpose, it was necessary to measure the diameters of images of the particles sedimented on agar, fixed with OsO_4 vapor, and pictured in electron micrographs. In Fig. 1 there is shown the distribution of virus particle sizes derived from measurements of images of these fixed particles at 3,740 $\times$ electron magnification. The mean particle diameter was 126 mμ. Using this value, the graph of the predicted fraction, f, of free particles from an unaggregated suspension *vs* N/A (expressed as particles per 2" square micrograph) is shown in Fig. 2 for the range of particle concentrations covered in the following experiments.

Electron micrographs have suggested that the virus particles in some plasmas from diseased chicks are almost completely free of aggregation. Fig. 3a shows virus in one such plasma. The results of studies on this plasma in four dilutions are shown in Fig. 4 plotted at $\log_{10} 1/f$ *vs* N/A, in the lower solid line. The dotted line corresponds to $\log_{10} 1/f$ *vs* N/A for no aggregation, as predicted and shown in Fig. 2 for particles of the observed average diameter. Sedimentation of the virus after 24 hours storage of the plasma at 0°–4°C and resuspension in physiological saline resulted in the increased aggregation (Fig. 3b) shown in all three dilutions analyzed for the second line of the figure. Here the line was arbitrarily drawn parallel to the two below. A second sedimentation of the virus sample was carried out 24 hours later resulting in even

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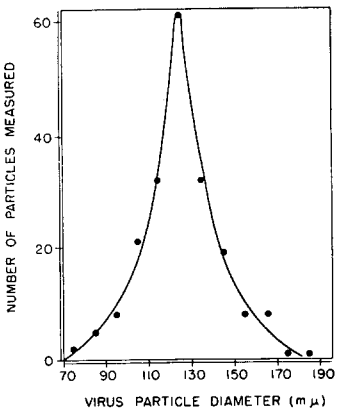

Fig. 1. Distribution of virus particle size as calculated from image measurements (perpendicular to the direction of shadow) on electron micrograph plate taken at 3.740 ×.

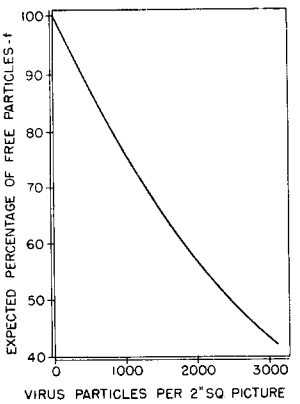

Fig. 2. Relationship between the fraction f of free particles and the number of particles per unit picture area calculated for completely random dispersion. $D = 1.00$.

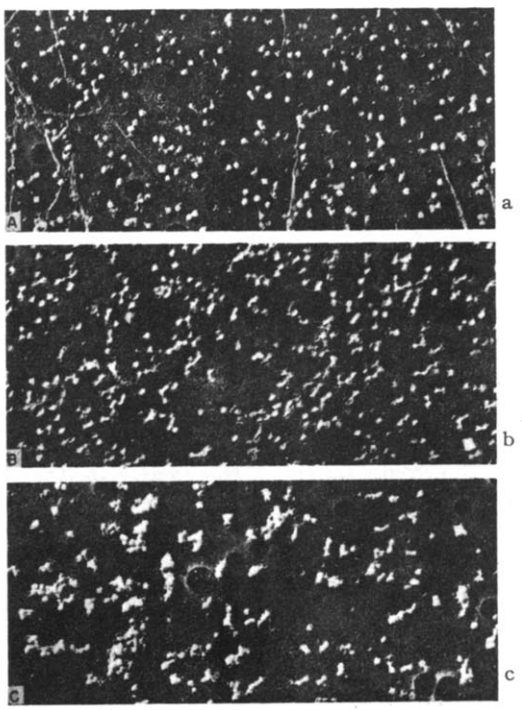

Fig. 3. Electron micrographs of the virus of myeloblastic leukosis obtained with chromium-shadowed pseudoreplicas from agar surfaces (18,000 ×) (a) virus particles from plasma, $D = 0.95$; (b) virus particles sedimented once and resuspended, $D = 0.59$; and (c) virus particles sedimented once and resuspended, $D = 0.18$.

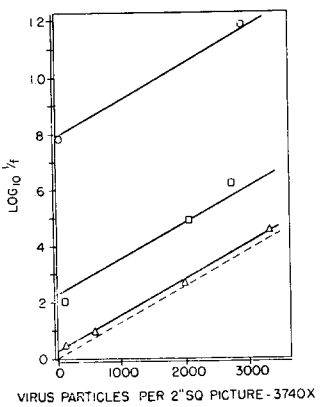

Fig. 4. Dispersion of the particles of avian myeloblastic leukosis virus in various dilutions of plasma (triangles); of the particles sedimented once and resuspended (squares); and of the particles sedimented twice and resuspended (circles). The broken line represents the distribution expected for randomly dispersed particles of the same size. (See Figs. 3a, b and c for electron micrographs from which these data were obtained.)

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further aggregation (Fig. 3c). Here, again, the degree of aggregation determined from the analyses of widely differing dilutions was essentially the same (Fig. 4, top line, also drawn parallel).

These experiments provide evidence that the virus particles may occur in an essentially free, unaggregated state in the blood plasma. They describe quantitatively and pictorially the progressive aggregation which occurs during repeated sedimentation.

Another sample of plasma was divided into two equal parts, the particles were sedimented, and the two pellets were resuspended in (a) phosphate buffer at pH 7.0, $\mu = 0.1$ and (b) veronal buffer pH 8.5, $\mu = 0.1$. The volume of resuspended virus was the same as that of the plasma from which it came. These two samples were kept at 0°–4°C and analyzed at 18 hours, 40 hours and 112 hours by diluting 1–500, 1–1000 and 1–5,000 and counting. Table I shows the values of the dispersion coefficient and demonstrates quantitatively the effects of the two buffers on the dispersion of the virus.

TABLE I

COMPARATIVE STABILITY OF AVIAN MYELOBLASTIC LEUKOSIS VIRUS IN TWO BUFFERS KEPT AT 0°–4° C

The data are given in values of a dispersion coefficient, D, which is the ratio of the number of free particles seen divided by the number predicted for a monodisperse suspension of equal concentration.

	Phosphate buffer $\mu = 0.1$, *pH 7.0*			*Veronal buffer* $\mu = 0.1$, *pH 8.5*		
Virus dilution	1–500	1–1000	1–5000	1–500	1–1000	1–5000
18 hours		1.01			0.63	
40 hours	0.82		0.80	0.22		0.29
112 hours			0.83	ppt.		

Another pH stability study was made with virus prepared in the same way but suspended in 0.1 M histidine hydrochloride adjusted to pH values ranging from 5.1 to 9.1, all within the useful buffering range of this material. The initial state of the sample at pH 7 was such that $D = 0.63$, this being a rather poorly dispersed suspension at the start.

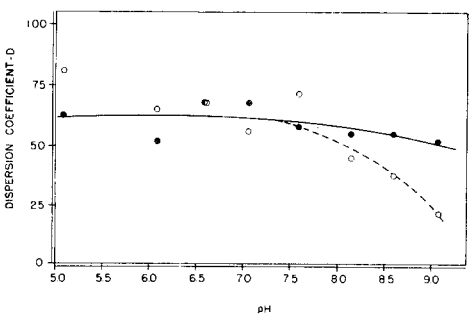

Fig. 5. Dispersion of avian myeloblastic leukosis virus in histidine hydrochloride solution, 0.1 M. Values of D were measured at 18 hours (closed circles) and at 66 hours (open circles).

Fig. 5 shows the changes that took place in the samples as demonstrated by the studies made after 18 and 66 hours of storage at 0°–4° C, respectively. In this case it was necessary to make the dilutions (to bring N/A into the counting range) with 0.9% NaCl solution because the histidine interferes with the replication process.

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DISCUSSION

It was the purpose of the present work to devise a procedure suitable for measurement of the state of dispersion or aggregation of virus particles in suspension and to establish an index useful in the numerical expression of the result. The principal condition essential for such a procedure is dependent, basically, on a method for the direct determination of the relations between the individual particles and particle aggregates as they exist in suspension. This has been accomplished by sedimentation of the particles, in appropriate concentrations, onto a smooth, flat surface from which they could be removed quantitatively without change in the relations established as the particles settled on the surface. The surface employed was agar, which has the ability to absorb simultaneously both residual water and salt to prevent disarrangement of the particle pattern by drying effects, and the vehicle for removing the particles was collodion which, on solidifying, maintained the relative positions of the particles for subsequent electron micrographic examinations. Experiment showed that the pattern of the particles deposited from unaggregated preparations coincided with that predicted statistically and suggested that the ratio of free particles observed to the number predicted for random dispersion could be employed as an index, D, of dispersion in aggregated suspensions.

The fact that the data obtained with some of the plasmas examined (Fig. 4) agree so well in both slope and intercept with the values predicted for random dispersion may be taken as evidence that, (1) the assumptions made in the derivation of P are valid; (2) the virus actually sedimented was very nearly monodisperse throughout the series of dilutions studied; and (3) no selective removal of virus from the agar by the pseudoreplica is evident. The analyses of aggregated material show that the degree of aggregation found at the lowest dilutions is not changed when $20\times$ further dilution is made. The plots of log $(1/f)$ *vs* N/A are approximately parallel for heavily aggregated material thus making possible the calculation of D from a single dilution anywhere in the statistically significant range.

Precision attainable through repeated observations of the dispersion of a given sample can be judged from the data in the mid-pH range of the experiment of Fig. 5. Between pH 6.2 and 7.6, the values obtained in eight determinations of D ranged from 0.52 to 0.71 or *ca.* 16% about a mean value of 0.62. No systematic variations are apparent among these points.

That the procedure is practically applicable under relatively simple conditions has been amply demonstrated in the experiments cited. Access to data obtained by this means is not only useful but critical in the interpretations of many kinds of experimental findings. The problem of the state of dispersion of virus particles in suspension is one of frequent recurrence in interpretations of the behavior or activity of the individual entities in a total population. This is of particular importance in investigations involving bioassay of virus activity based on titration. The factors determining host response to these agents and the patterns of response, with few exceptions, are as yet poorly understood. It is a frequent practice to ascribe unexpected or obscure aberrations to the possibility of particle aggregation. This is the more important with the recent development of tissue culture methods for the detection of single foci of infection.

Several years ago when the ultracentrifuge was the chief instrument supplying

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physical data on the concentration and purity of virus in purified suspensions, it was often seen that partially purified materials exhibited single sedimenting boundaries while further purification resulted in multiple boundaries. This has been attributed to progressive aggregation of the virus particles. The procedure described here provides the means for direct analysis, as seen in Fig. 4, of the contribution of aggregation to the formation of multiple boundaries in specific experiments. In the present studies for example, the plasma ($D = 0.95$), having only 5% aggregated particles, would doubtless show only a single boundary. First cycle material ($D = 0.59$) would have to be concentrated almost 2 times in order to display the same sized primary boundary, and secondary boundaries due to pairs, triplets, *etc.* would be only 41% as large. The third cycle material ($D = 0.18$) would not be recognizable in the sedimentation-velocity study as a single substance because of overlapping boundaries of the various orders of aggregation. Nevertheless, the twice-sedimented virus has been washed free of most of the plasma, and is therefore several hundred times purer than the starting plasma on a nitrogen basis. Inasmuch as purification of animal viruses generally involves methods which may result in aggregation of the particles, some quantitative estimate of the degree of aggregation is essential to interpretation of data bearing on purity of the final product. It has been mentioned that the phenomenon of light scattering may be employed as a measure of aggregation. Methods based on this property have suffered from the lack of standardization which may be accomplished with the present method.

Another very fruitful application of aggregation analysis is the study of the influence of the composition of the suspending medium on dispersion. This is illustrated by the comparison of aggregation rates in two different buffers (Table I). It is clear that the virus is more stable in phosphate buffer at pH 7 than in veronal buffer at pH 8.5; progressive decrease in D values occurred with the virus in veronal, while with phosphate at pH 7, D remained constant for 72 hours after a small initial drop. Statement of these conditions in terms of D factors and time is clear and quantitative, affording a means of studying the kinetics of the aggregation reaction under various conditions.

The more extensive experiments (Fig. 5) designed to show the stability of the virus suspension as a function of pH in histidine buffer showed a range of stability extending from pH 6.2 to 7.6. The degree of dispersion of this virus suspension at pH 7.1 was initially such that $D = 0.63$. Slight evidence of further aggregation might be deduced from the 18 hour points at pH 8.2, 8.6 and 9.1. This effect is pronounced at 66 hours as shown by the dotted line. Although stability in this medium is greater than that shown at pH 8.5 in Table I for veronal, it is clear that some instability exists above pH 7.6 in both media.

At pH 5.1 the interpretation is not clear. Although visible turbidity could be seen in this sample, it showed good dispersion when diluted for analysis. Had it been possible to make the dilution with the same buffer, redispersion might not have occurred. This result does indicate that the type of aggregation induced at this acid reaction is substantially different from that at alkaline pH. All samples were diluted with the material for micrography.

A potential field for exploration is the quantitative study of the precipitin reaction with specific immune serums. The procedure has the very great advantage in the need for exceedingly minute amounts of material and the means for quantitative estimation of the degree of reaction, which might be useful in work with serums of low precipitin

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content. An example of results obtained in the electron microscopic study of the reaction between the myeloblastic leukosis virus and immune serum from the chicken has already been described.

SUMMARY

A method is described whereby information regarding the degree of dispersion of a virus particle suspension may be derived from electron micrographs of the virus particles. It involves sedimentation of the particles on an agar-receiving surface and subsequent replication for electron microscopy. The pictures constitute a two-dimensional pattern from which the three-dimensional particle relationships in the liquid suspension can be derived by counting the number of free and of aggregated particles in a given area. From a consideration of the probability of chance superposition of particles when they are sedimented, a measure of dispersion "*D*", is obtained for aggregated suspensions which has been found to be substantially independent of the number of particles present in the picture. Examples are given of how "*D*" varies under some conditions commonly experienced in the study of purified virus suspensions.

RÉSUMÉ

Les auteurs décrivent une méthode renseignant sur le degré de dispersion d'une suspension de particules de virus à partir de l'examen au microscope électronique des particules de virus. Cette méthode utilise la sédimentation des particules sur une surface réceptrice d'agar et l'obtention ultérieure de répliques destinées à la microscopie électronique. Les images constituent une représentation à deux dimensions à partir de laquelle les rapports entre particules à trois dimensions dans le liquide de suspension peuvent être déterminés par comptage du nombre des particules libres et des particules en aggrégats dans une surface donnée. En tenant compte de la probabilité de la superposition de particules quand elles sont sédimentées, une mesure de la dispersion "*D*" est obtenue pour les suspensions d'aggrégats. Cette valeur est pratiquement indépendante du nombre de particules présentes dans l'image. Les auteurs donnent des exemples de variations de "*D*" dans diverses conditions qu'on rencontre communément dans l'étude de suspensions de virus purifiés.

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben, welche es ermöglicht, Auskünfte über den Dispersionsgrad einer Viruspartikelaufschlemmung aus Elektronenmikrographien der Viruspartikel zu gewinnen. Die Methode involviert die Ablagerung der Partikel auf einer Agaroberfläche und die nachfolgende Replikation für Elektronenmikrographie. Die Bilder zeigen eine zweidimensionale Distribution, aus welcher die dreidimensionalen Partikeldaten der flüssigen Aufschlemmung festgestellt werden können, indem die freien und aggregierten Partikel auf einer bestimmten Oberfläche gezählt werden. Durch Wahrscheinlichkeitsberechnungen der zufälligen Superposition von abgelagerten Partikeln wird ein Mass "*D*" der Dispersion für aggregierte Aufschlemmungen erhalten; es wurde festgestellt, dass dieses Mass im Wesentlichen von der Anzahl der auf dem Bilde anwesenden Partikeln unabhängig ist. Es werden Beispiele für die Schwankungen von "*D*" unter bestimmten, beim Studium gereinigter Virusaufschlemmungen vorkommenden Bedingungen angeführt.

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