ULTRACENTRIFUGATION STUDIES ON THE ELEMENTARY BODIES OF VACCINE VIRUS

I. General Methods and Determination of Particle Size

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The work of Stanley (1) which indicates the protein character of the etiological agent of the first recognized virus disease, tobacco mosaic, has added to the speculation regarding the nature of the elementary bodies associated with certain of the larger viruses. Elementary bodies of vaccinia either represent the virus itself or else are so intimately associated with it as to be inseparable (2–4). These bodies are similar to bacteria and protoplasm in their content of protein, fat, and ash (5); nevertheless, they give no evidence of respiratory activity *in vitro* (6). It was considered of interest to obtain further evidence regarding the size and density of elementary bodies of vaccinia and to ascertain to what extent they respond to osmotic influences.

In the present experiments the behavior of elementary bodies was studied by photographic methods. Sedimenting boundaries of virus particles were obtained by means of the air-driven centrifuge of Bauer and Pickels (7), and the light-absorption method of Svedberg (8) was employed for the measurement of sedimentation rates. Changes in the physical nature of the elementary bodies could thus be followed by the reflected changes in sedimentation rate. In our experiments, as well as in those of previous workers (9–12) who have studied the size or density of vaccine virus by centrifugal methods, the assumption is made that elementary bodies are spherical or approximately spherical. The ultraviolet light photomicrographs of Barnard (13) would indicate that this assumption is justified.

Materials and Methods

Preparation of Diluents for Virus Suspensions.—The pH values of the suspending media were determined generally by the glass electrode method. In some instances, however, colorimetric indicators were employed. Disodium phosphate-citric acid buffers were used to bring all solutions to the desired pH, usually 7.2; in two instances runs were made with dilute buffer solutions having values of 6.2 and 8.0. Because normal rabbit serum, when present in small amounts, is known to increase the stability of elementary body suspensions (14), 2 per cent by volume was used in many of the test solutions, and a 10 per cent concentration was used in one experiment.

Viscosities were measured with an Ostwald viscometer which had been carefully calibrated. The viscosity value determined for dilute buffer solutions, with and without 2 per cent rabbit serum, was the same as that for water to a close approximation. Whenever the value was appreciably different, a correction was made in computing the sedimentation rate in accordance with the equations given below. The densities of the sucrose, glycerol, and urea (all of C.P. grade) solutions were carefully adjusted to the desired values by the ordinary pycnometric methods. The densities of all other buffer solutions with or without rabbit serum were found to be close enough to 1.00 gm. per cc. to make no density corrections necessary.

Preparation of Virus Suspensions.—The CL strain of dermal vaccine virus which has been carried in rabbits by dermal inoculation of elementary bodies for the past 4 years in Dr. Rivers' laboratory at The Rockefeller Institute was used throughout the experiments. Elementary body suspensions were prepared from infectious rabbit dermal pulp by the differential centrifugation technique of Craigie (2). Washed virus particles obtained from groups of four rabbits were taken up in 100 cc. of a 1/50 dilution of standard disodium phosphate-citric acid buffer, pH 7.2, and stored at 5°C. in flasks containing small amounts of ethyl ether. Preliminary experiments indicated that elementary body suspensions prepared according to routine, *i.e.*, three washings in a Swedish "angle centrifuge" followed by horizontal centrifugation at 2500 R.P.M. for 1 hour to remove clumps, gave as consistent results as more thoroughly washed suspensions.

Elementary bodies were generally prepared for ultracentrifugation in the following manner: 4.5 cc. of stock virus suspension were centrifuged for 1 hour in the Swedish angle machine in a flat pyrex tube with an inside width of 4 mm. Supernatant fluid was poured off and the tube was inverted on blotting paper for 1 minute. While the tube was still held in the inverted position, residual drops of fluid were removed by touching them with bits of absorbent paper. The sedimented elementary bodies, which had a volume of about 0.01 cc., were spread about the bottom of the tube with a glass rod; 3 cc. of the test solution were added drop by drop at first, with constant stirring, and then the suspension was vigorously shaken. The suspension, still in the flat tube, was centrifuged

horizontally in a properly cushioned cup for 5 minutes at a speed of 1000 R.P.M. The supernatant elementary body suspension was poured into a test tube and a portion of it used to fill the ultracentrifuge cell. When this procedure was followed, a minimum of 12 to 15 minutes elapsed between the first contact of the elementary bodies with the test solution and the loading of the ultracentrifuge cell. In the latter part of the work it was found desirable to obtain the sedimentation rate immediately after resuspension of the virus particles. The initial centrifugation of the virus in the test solution was then omitted. It was thus possible to load the ultracentrifuge and bring it to proper speed for the first photograph within 5 minutes after the virus had come into contact with the test solution.

Certain stock suspensions of elementary bodies exhibited a varying amount of autoagglutination; slight settling of agglutinated particles occurred in the storage flasks after a week or two at 5°C. This phenomenon was unpredictable. Stock suspensions which were unstable in this respect were sometimes used in the early experiments and often failed on ultracentrifugation to display boundaries sufficiently well defined for accurate interpretation. It was found expedient to use only stock virus which was stable after storage over a period of 2 weeks to a month.

Virus preparations were stained by the Morosow technique (15) and examined microscopically in order to demonstrate the purity and monodispersion of the suspensions. Dark-field observations were also made of unstained preparations.

Certain terms employed in the discussion of experiments should, perhaps, be defined at the outset. Stock suspensions or lots of elementary bodies refer to the pooled washed virus corpuscles obtained from four infected rabbits. The word "specimen" applies to elementary bodies obtained from a particular lot and resuspended in a given test solution. An experiment is considered as a single ultracentrifugation of a specimen. Thus numerous specimens were generally prepared from a given stock suspension of elementary bodies, and several experiments were generally done on each specimen. Other terms will be defined as the need arises.

Theory and Equations

When a small particle suspended in a liquid medium is subjected to a constant centrifugal force, the velocity with which it slowly sediments through the liquid is directly proportional to the net force acting on the particle and inversely proportional to the resistance offered by the viscosity of the fluid. If all forces other than the centrifugal force are negligible, the sedimentation velocity V of a particle located at a distance r from the axis of rotation can be expressed, then, as

$$V = \frac{dr}{dt} = K \frac{\omega^2 r v(\sigma - \rho)}{\eta} \tag{1}$$

where ω = speed of centrifuge in radians per second.
v = volume of particle in cc.
σ = average density of particle in gm. per cc.
ρ = density of liquid medium in gm. per cc.
η = viscosity of medium in poises.
K = a constant whose value depends on the amount and shape of the particle's surface which is subjected to the frictional resistance of the liquid.

The sedimentation velocity should be a linear function of ρ if K, v, and σ remain constant. It can become zero in a field of force only when $\sigma = \rho$, regardless of the values of K and v.

For the special case of a spherical particle suspended in a liquid consisting of much smaller particles or molecules, K has the value $K = \frac{1}{6\pi a}$, where a is the radius of the particle. This relation is the basis of the well known Stokes' formula as applied to centrifugal fields:

$$V = \frac{dr}{dt} = \frac{2\omega^2 r a^2(\sigma - \rho)}{9\eta} \tag{2}$$

For a slightly oval particle, this equation should be valid to a close approximation if a is taken as the average radius.

The sedimentation velocity in a unit field of force is then

$$\frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{2a^2(\sigma - \rho)}{9\eta} \tag{3}$$

Integrated for a displacement $(r_2 - r_1)$ of the particle during the time interval $(t_2 - t_1)$, the expression becomes

$$\frac{\log_e r_2 - \log_e r_1}{\omega^2(t_2 - t_1)} = \frac{2a^2(\sigma - \rho)}{9\eta} \tag{4}$$

It is customary to describe the behavior of a particle in a field of force by its computed sedimentation constant, S_{w20}, which is the rate at which sedimentation would proceed in a unit field of force if the suspending medium had a density and viscosity equal to those of water at 20°C. However, in order to compare the sedimentation rates of particles of unknown densities in solutions of varying specific gravity, use will be made of a more general term, "corrected sedimentation rate," denoted as S_c and defined by the relation

$$S_c = \frac{\log_e r_2 - \log_e r_1}{t_2 - t_1}\frac{1}{\omega^2}\frac{\eta}{\eta_{w20}} \tag{5}$$

where η_{w20} is the viscosity of water at 20°C.

In accordance with Equation 4, for spherical particles

$$S_c = \frac{2a^2(\sigma - \rho)}{9\eta_{w20}} \tag{6}$$

For dilute buffer solutions, etc., which have densities close to that of water, $S_{w20} = S_c$ to a good approximation.

All sedimentation rates, except where otherwise specified for comparative purposes, will be given in terms of 10^{-11} cm./sec./dyne.

Centrifugation Technique

As soon as possible after a particular sample of elementary bodies had been suspended in the desired medium, a portion of the mixture was placed in the centrifuge cell and the remainder stored in a refrigerator at 5°C. until it was needed for another run. The centrifuge cells used were of two types: One had a hard rubber centerpiece with an aperture 11 mm. in length, and the other had a pontalite[1] centerpiece with an aperture 15 mm. in length. Fresh dry cells were always used except in certain cases where two runs on the same lot of material were made within a short time of each other.

The temperature of the centrifuge rotor was measured by a thermocouple immediately before and after each run. The rise in temperature was seldom more than a few tenths of one degree Centigrade; therefore the mean temperature was used in computing the corrected viscosity of the solution. The speed of the centrifuge was generally adjusted to such a value that seven or eight photographs, spaced at 3 minute intervals, could be taken while the sedimenting boundary traversed about three-quarters of the cell. Speeds ranging from 3600 to 48,000 R.P.M. were used according to the densities and viscosities of the suspending media. The exposure time was usually 15 seconds. All photography was done with ultraviolet light furnished by a mercury arc and filtered through absorption cells of bromine and chlorine gases. The photographs of the sedimentation boundaries were analyzed by means of a continuous recording photomicrometer. The several boundary positions determined for each experiment by this method were used in computing the mean sedimentation rate according to Equation 5.

EXPERIMENTAL

Boundary Measurements.—Sedimentation boundaries produced by the elementary bodies of vaccinia were studied under a variety of experimental conditions. Fig. 1 is a typical photographic record of the sedimentation of Paschen bodies in a dilute buffer solution. The

[1] A new clear, plastic material manufactured by E. I. Du Pont De Nemours and Company.

boundary is seen to become progressively more blurred as it moves through the solution. The blurring, or spread of the boundary, *i.e.*, the distance between the upper and lower edges of the boundary, is better illustrated by the set of curves shown in Fig. 2. These curves represent the photomicrometer tracings of the photographs constituting Fig. 1. Each of these tracings indicates the distribution of the particles at a particular stage of the centrifugation.

Experimental as well as theoretical considerations have shown that only a small proportion of the spread of boundary could have been caused by diffusion effects which usually account for the blurring of boundaries in homogeneous solutions of proteins. The blurring occurred to the same extent when elementary bodies were centrifuged in more viscous solutions of sucrose, glycerol, and urea, instead of buffer solutions. Moreover, the fact that elementary bodies suspended in media of different densities showed no notable variation in the spread of boundaries renders very doubtful any assumption that the particles differ considerably in density. A small amount of the spread depended on the fact that the boundary moved an appreciable distance during the photographic exposure and also on the fact that the photographic definition was not infinitely sharp. These factors, however, remained constant during the course of an experiment; therefore, most of the progressively increasing spread must be interpreted as a centrifugal effect. The spread of boundaries might be explained on the basis of slight variations in the size or shape of the elementary bodies. However, in view of considerations to be discussed below, it appeared probable that part of the spread was caused by the agglutination or partial aggregation of some of the single particles.

As can be seen both in Figs. 1 and 2, the concentration of virus particles below the boundary is decreasing at what appears to be an approximately uniform rate, except, of course, at the bottom of the cell. This decrease has been attributed by Svedberg to the fact that as any given group of particles sediments through the wedge-shaped cell, it is progressively subjected to a slightly higher centrifugal force and at the same time is progressively passing into a wider section of the cell. For an approximately homogeneous group of particles, this decrease in concentration bears a definite relation to the distance

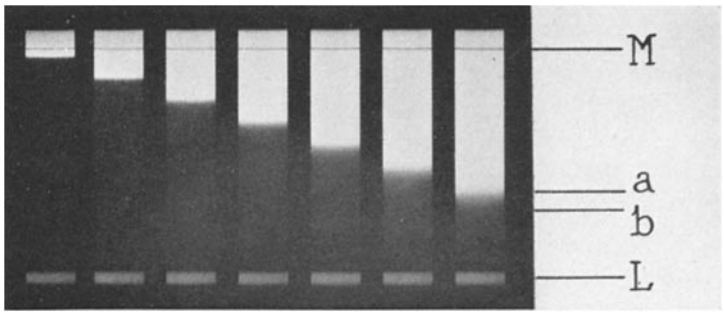

FIG. 1. Sedimentation of elementary bodies of vaccinia in a dilute buffer solution of pH 7.6 containing 2 per cent normal rabbit serum; speed, 4800 R.P.M.; mean centrifugal force, 1670 times gravity; interval between photographs, 3 minutes; exposure time, 15 seconds. *a* and *b* indicate the upper and lower edges respectively of the sedimenting boundary; the distance between constitutes the spread. *M* is the meniscus of the medium and *L* the light intensity standard.

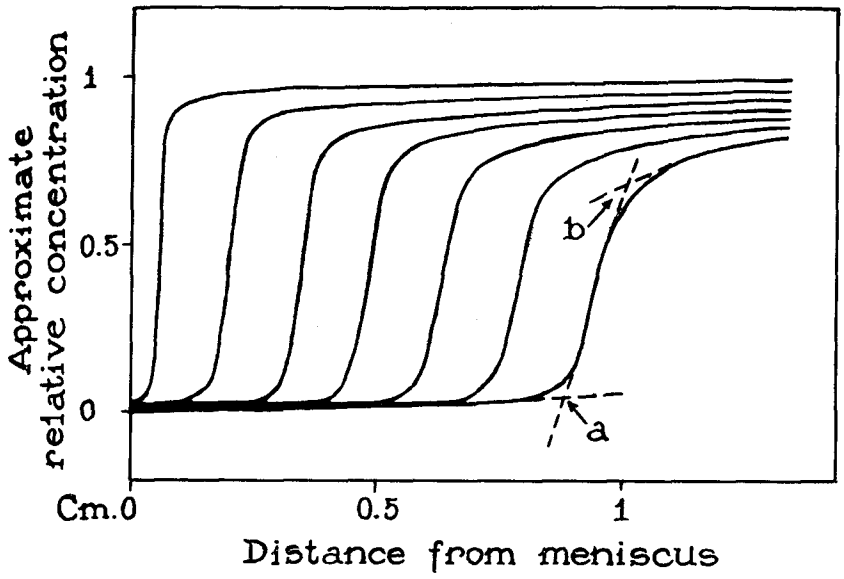

FIG. 2. Sedimentation curves reproduced from the photomicrometer tracings of the photographs shown in Fig. 1. The method of determining the upper and lower edges (*a* and *b*) of the sedimenting boundary is described in the text.

through which the boundary has progressed at any time, and therefore its theoretical value can be computed and used as one indication of the presence or lack of homogeneity.

The upper edge of the boundary, *i.e.*, the slowest moving part, was always more sharply defined than the lower edge; consequently, it has been used for the determination of boundary positions. A method was adopted whereby a tangent was drawn to the distribution curve, obtained by means of the photomicrometer, on either side of the region of highest inflection, as is illustrated in Fig. 2. The intersection of the tangents, represented by point *a* in the figure, indicated the distance, from the meniscus, of the slowest moving vaccine virus particles present in an appreciable concentration. The distance of the lower edge of the boundary from the meniscus was similarly approximated by applying the method of tangents. Point *b* in Fig. 2 indicates the position of the lower edge of the boundary. By repeating the procedure for each of the several curves, the average sedimentation velocities of both the upper and lower edges of the boundary were determined. The difference between these sedimentation rates, expressed in per cent, was employed as a measure of the boundary spread.

The lower edge of a boundary consistently sedimented 12 to 16 per cent more rapidly than the upper edge. This definite amount of spread was characteristic of a sedimenting boundary of elementary bodies, whether the particles were suspended in solutions of dilute buffer alone, or in various concentrations of sucrose, glycerol, or urea.

Sedimentation in Dilute Buffer Solutions.—The results of centrifugation experiments carried out on elementary bodies of vaccinia suspended in dilute buffer solutions having pH values between 6.8 and 7.6, both with and without small amounts of rabbit serum, are summarized in Fig. 3, Chart 1.

Eleven specimens were prepared from seven different stock suspensions of elementary bodies and employed for twenty-five centrifugation experiments extending over a period of 12 months. Several experiments were generally performed on a given specimen. These were done at various intervals after the specimen had been prepared from a particular stock suspension of virus. The suspension time was taken as the interval between the addition of the elementary bodies to the test medium and the first photographic exposure taken during the experiment.

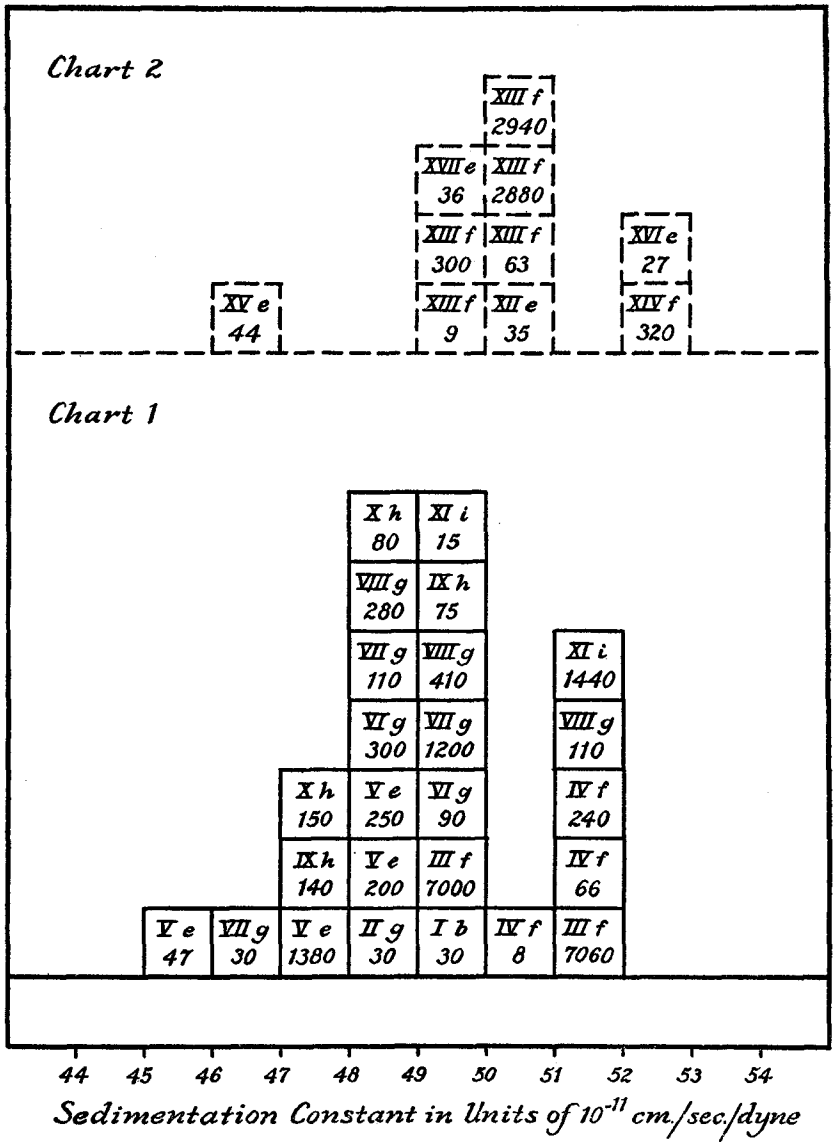

FIG. 3. *Charts 1 and 2.*—Distribution of the sedimentation constants of elementary bodies of vaccinia in media with densities and viscosities near those of water. Each experiment is indicated as a small square. The letter inside the square refers to the lot of stock virus from which the particular specimen was prepared. The Roman numeral refers to the specimen number, while the Arabic numeral indicates the suspension time in minutes. The suspending medium used in Chart 1 was dilute buffer of approximately neutral pH; specimens IV, V, VI, VII, and X contained 2 per cent rabbit serum. The media in Chart 2 had varying salt and serum concentrations and covered a wider pH range.

It is apparent that the distribution of twenty-five sedimentation constants, graphically represented in Fig. 3, Chart 1, approximates a normal probability distribution. Each value is based on measurements taken of the upper and better defined edge of the boundary. No preferential distribution was shown by these sedimentation constants with respect to different stock suspensions of virus, different specimens, time after suspension, or with respect to the presence or absence of 2 per cent rabbit serum.

The mean sedimentation constant from these twenty-five experiments was computed to be 49.1 x 10^{-11} cm./sec./dyne, with a probable deviation of 0.5 per cent. The highest and lowest rates of uniform sedimentation recorded in dilute buffer were 51.7 and 45.3. Over 90 per cent of the boundaries settled at rates between 47 and 52, only two of them falling below this range. The correct value for the most probable sedimentation constant of the slower moving elementary bodies of vaccinia in a standard buffer solution must be close to 49.1 x 10^{-11} cm./sec./dyne, and this value is used as the basis of the interpretations to follow.

Sedimentation in Other Media with Densities Near That of Water.— From the experiments just described, dilute buffer solution of about neutral pH and with 2 per cent rabbit serum was found to be a desirable suspending fluid. Nevertheless, a study of the behavior of elementary bodies in other solutions was indicated. The distribution of the sedimentation rates measured in ten experiments with solutions of about the density of water but differing from those used in the preceding section is indicated by the series of dotted squares in Fig. 3, Chart 2.

The virus material for the six specimens employed was taken from two of the lots which were used for studies with dilute buffer solutions. Specimen XII was a suspension of virus in a dilute buffer-serum solution, pH 7.2, to which had been added 0.4 per cent NaCl, while in specimen XIII the salt content was increased to 0.8 per cent. 10 per cent, by volume, of rabbit serum was incorporated in dilute buffer for the suspending medium of specimen XV. Specimen XIV was a resuspended sample of specimen IV (Fig. 3, Chart 1) which had been sedimented completely to the bottom of the centrifuge cell and then spun at a speed of 720 R.P.S. for about 15 minutes. Specimens XVI and XVIII, containing 2 per cent rabbit serum in dilute buffer, had final pH values of 6.2 and 8 respectively.

None of these variations in the conditions of experiment, *i.e.*, subjection to high centrifugal forces or moderate changes in the salt, serum, or pH of the suspending media, produced any change in sedimentation rate, as evidenced by Fig. 3, Chart 2, which might be regarded as significant. However, an interesting observation was made in connection with the specimen which had been suspended in an 0.8 per cent NaCl buffer solution. Within only 9 minutes after preparation the concentration of elementary bodies constituting the primary sedimentation boundary had dropped to a very low value. That the great majority of the virus particles had agglutinated was evidenced by a progressive increase of particle concentration below the sedimenting boundary at every stage of the centrifugation, without visible evidence of discrete multiple boundaries. The marked tendency of elementary bodies to agglutinate in physiological salt solution is well known (16); however, macroscopic agglutination is not generally apparent in such a short time. The concentration of single particles continually decreased in several experiments with this specimen over a period of 48 hours until barely a trace of a primary boundary could be detected. However, another experiment performed on the same specimen only 1 hour later (*i.e.*, suspension time, 49 hours) showed a higher concentration of single particles. This, perhaps, indicated a spontaneous partial reversal of agglutination. Such partial reversal of agglutination of elementary bodies has not been considered previously, mainly because the existing methods were inadequate for its detection unless the effect were very marked.

Multiple Boundaries.—In addition to the primary sedimentation boundary, more rapidly moving boundaries were recorded in several instances. In no case did the presence of such boundaries appear to influence the sedimentation rate of the principal boundary. An example of multiple boundaries is illustrated by the photographs in Fig. 4 and the corresponding sedimentation curves in Fig. 5. A second boundary is clearly defined, and there are distinct traces of even a third. In Fig. 5 the three boundaries are indicated, in order, as 1, 2, 3. As the figure shows, the transition in concentration of material from one boundary to the next is rather gradual, and the main boundary continues to be better defined than the others. The method of measurement which has already been outlined was success-

FIG. 4. Multiple boundaries in a sedimenting suspension of elementary bodies of vaccinia.

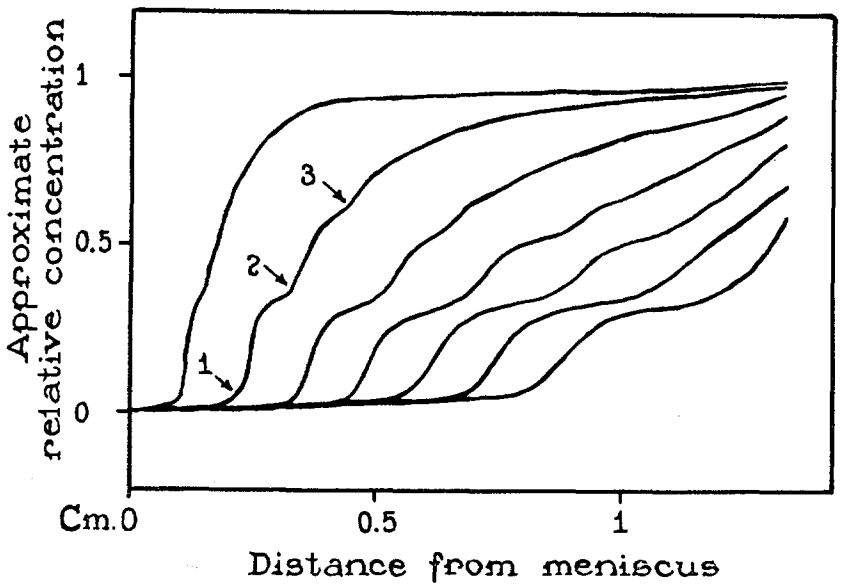

FIG. 5. Sedimentation curves reproduced from the photomicrometer tracings of the photographs shown in Fig. 4. The upper edges of the primary, secondary, and tertiary boundaries are indicated on the second curve.

fully applied in a number of cases to approximate the sedimentation rates of these more rapidly moving particle groups.

Secondary boundaries were evident in at least twenty-five of the 153 experiments performed in connection with the present studies. Definite tertiary boundaries were apparent in nine of these, and in one instance a fourth boundary could be detected. The concentration of particles constituting a primary boundary was in all instances higher than the concentration of those constituting the accompanying secondary boundary. The secondary in turn represented a higher concentration than the tertiary boundary. Thirteen secondary boundaries were

TABLE I

Characteristics of Secondary Boundaries

Virus lot	Specimen	Suspension time	Suspending medium	Approximate per cent of total virus in 1st and 2nd boundaries		Ratio of sedimentation rates S_2/S_1
				S_1	S_2	
		min.		*per cent*	*per cent*	
g	VII	30	Dilute buffer	70	15	1.52
g	VII	110	" "	45	25	1.47
g	VII	1200	" "	30	20	1.43
e	XII	35	0.4 per cent NaCl + buffer	60	20	1.46
e	XVI	27	Dilute buffer	50	20	1.47
g	XVIII	190	13 per cent sucrose	50	30	1.57
g	XVIII	260	" " " "	60	25	1.52
g	XVIII	1380	" " " "	30	25	1.56
i	XIX	7	Dilute buffer*	20	15	1.51
i	XX	180	" " *	45	15	1.50
i	XX	1380	" " *	35	15	1.57
f	XXI	10	" " *	60	15	1.37
f	XXI	55	" " *	60	20	1.45

* Virus sedimented from urea solution and resuspended in dilute buffer solution.

sufficiently well defined to permit approximate measurements of their sedimentation rates. These are compared with the corresponding principal boundary rates in Table I. On the average the secondaries sedimented about 1.49 times as fast as the primary boundaries. The highest and lowest ratios recorded were 1.57 and 1.37 respectively. 70 per cent of the ratios lay between 1.43 and 1.51. A few measurements indicated a sedimentation rate for the tertiary boundaries of approximately 1.85 times that of the primary.

Little definite correlation has been established between the conditions of experiment and the appearance of multiple boundaries.

Only half of the lots of virus displayed the phenomenon, although it is probable that any lot was potentially capable of doing so.

The first specimen prepared from lot *b* showed a secondary boundary when centrifuged in a dilute buffer solution. A number of subsequent preparations obtained from the same lot and suspended in sucrose solutions all showed irregular sedimentations of a nature to be discussed below. Lot *e* displayed only primary boundaries during a number of experiments extending over a period of several weeks. However, when the pH value of a dilute buffer suspending medium was lowered to 6.2, a double boundary appeared. Another experiment performed a few hours later on the same lot suspended in a similar solution having a pH of 8 showed no evidence of the phenomenon. Several more experiments were performed on this same lot using glycerol solutions as suspending media; only primary boundaries were in evidence. However, a few days later another suspension of the same lot *e* in a 0.4 per cent NaCl buffer solution displayed a secondary boundary. It is worthy of note, in this connection, that a double boundary was produced by suspending the virus particles in a 0.4 per cent NaCl solution, whereas raising the salt content to 0.8 per cent in the suspending medium of specimen XIII resulted in an agglutination of most of the particles without the evidence of multiple boundaries. Lot *g* likewise showed only primary boundaries over a period of several weeks when suspended in dilute buffer and in various concentrations of glycerol. Then it began to show multiple boundaries consistently when suspended in an identical buffer solution and also when suspended in various concentrations of glycerol and sucrose.

The only other multiple boundaries recorded could be classed as falling under a special set of conditions. In five separate instances elementary bodies were centrifuged from various solutions of sucrose, glycerol, and urea and resuspended in a dilute buffer-rabbit serum solution. In every instance resuspension was followed by the appearance of secondary boundaries. The effect was most pronounced with resuspensions from urea solutions and least pronounced with resuspensions from sucrose solutions. Specimens XIX, XX, and XXI, which are listed in Table I, fall into this classification.

In only one instance did the presence or absence of secondary boundaries fail to be consistent throughout a series of experiments performed on a particular specimen. A sample of a specimen resuspended from a 33 per cent glycerol solution showed both primary and secondary boundaries after 6 minutes, but only the primary boundary was present in the sample run after 1 hour. Both boundaries were noted again after 1 hour and 50 minutes. The concentration of the single particles increased from about 55 per cent of the total concentration in the first experiment to 75 per cent in the last experiment. In general, however, a given specimen showed a decrease in the concentration of the principal boundary on long standing (see Table I, specimens VII, XVIII, and XX). This was associated with microscopic, and in some instances macroscopic, evidence of

agglutination of elementary bodies. Nevertheless, instances such as the one just mentioned were recorded of spontaneous small increases in the concentration of the single particles several hours after suspension. This probably represents dispersion of small aggregates of elementary bodies.

Multiple boundaries were not always equally well defined. Thus, in a few instances they were more sharply differentiated than those in Fig. 4, while in many cases they were barely distinguishable. In these latter cases it was debatable whether to attribute the character of the photomicrometer tracings to the presence of multiple boundaries or to the presence of aggregates composed of various numbers of elementary bodies, all of which were sedimenting more rapidly than the particles constituting the primary boundary. Specimens that had multiple boundaries generally gave evidence, in the photomicrometer curves, of a considerable amount of aggregated material which settled more rapidly than any of those particles constituting a boundary. Microscopic examination of stained smears and dark-field examination of specimens showing multiple boundaries have confirmed the presence of aggregated groups containing various numbers of elementary bodies.

Irregular Boundary Movements.—The groups of sedimentation rates presented in Fig. 3, Charts 1 and 2, were based, with only one exception, on boundaries moving at uniform rates through the suspending media. A photographic record of sedimentation obtained with specimen II indicated a non-uniform sedimentation velocity; the boundary alternately speeded up and slowed down throughout the centrifugation. The average sedimentation rate for this specimen was found to be far above that of any other specimen included in Fig. 3, Chart 1, although the shape of the photomicrometer curve at any stage of the centrifugation was not greatly different from the others. On the other hand, when the sedimentation velocity was determined from only those two consecutive curves which would give a minimum value, an approximately normal value was obtained. It appeared, therefore, that the boundary was behaving in an approximately normal manner only when it was moving slowest. To reduce experimental error in the approximation of such minimum rates, the sedimentation velocity was taken as the mean of the determinations on two pairs of curves. The sedimentation rate given in Fig. 3,

Chart 1 for the one experiment with specimen II is a minimum rate determined as just described.

Irregular boundaries similar to the one just described occurred in thirty-three experiments in which various concentrations of sucrose were employed. Their appearance was confined to four of the ten stock suspensions of elementary bodies used. These four lots of virus were investigated only in dilute buffer and in sucrose solutions. They were quite consistent in their abnormal behavior, although in some instances the boundary appeared almost normal. In every case

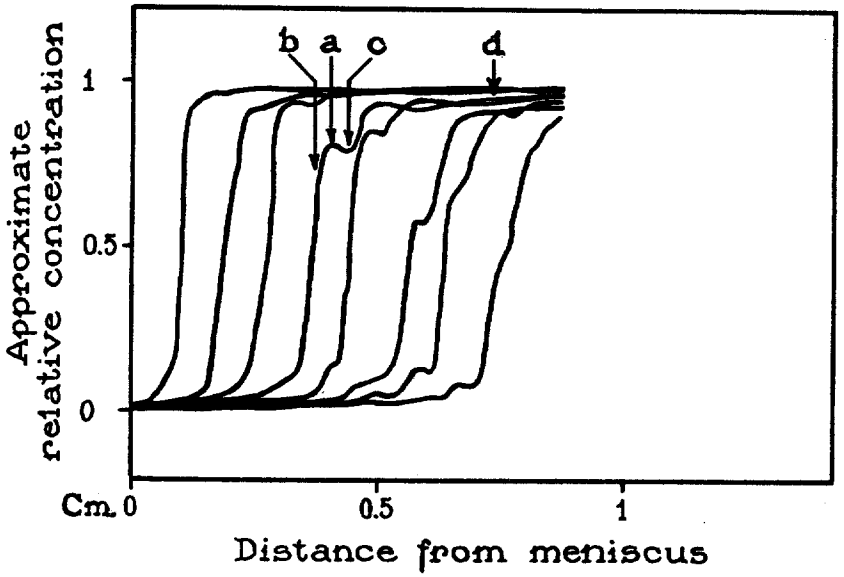

FIG. 6. Irregular sedimentation of elementary bodies. See text, page 597, for interpretation of sedimentation curves.

the minimum rate approximated the normal rate as determined with more stable suspensions. As an example of abnormal behavior, the average rate of sedimentation for a particular boundary was computed to be about 1.6 times its minimum rate, which was approximately normal. Its maximum rate, as determined from three of the photomicrometer curves, was 2.2 times the normal rate.

Many of these irregular boundaries were readily identified by the characteristic striated appearance of their photographs. A typical example is illustrated by the series of photomicrometer tracings in Fig. 6. The irregular spacing and broken appearance of the distribu-

tion curves are at once evident and distinguish them from the curves shown in Figs. 2 and 5.

A peculiar feature was observed on several occasions in specimens with irregular boundaries. The concentration of particles at some particular level (*a*, Fig. 6) in the cell was actually greater than the concentration of material at levels just above *b* and below *c*. This condition does not persist, probably, in part at least, because the slightly heavier layer tends to displace the less concentrated layers below it. This phenomenon never occurs in the course of a normal sedimentation, even of complex mixtures, and it can be explained plausibly only by assuming at least one of three possibilities: (1) Some of the particles which had been at the level *c* had experienced a spontaneous increase or decrease in their sedimentation rate; (2) Some of the material corresponding to level *a* had suffered a decrease in sedimentation rate; (3) Or some of the particles corresponding to level *b* had increased their settling rate. The last of these possibilities seems the most probable. A spontaneous increase in the sedimentation velocity of particles near the boundary could result from agglutination of elementary bodies which would account for these negative concentration gradients.

Still another distinguishing feature connected with irregular boundaries was the piling up of particles in the regions below the boundary; thus the regular and progressive decrease in concentration that occurred during the centrifugation of homogeneous material was absent. For instance, in Fig. 6 the concentration curves are close together near the level *d*, and some of them cross each other, indicating an actual increase in the concentration of material in some regions at various stages of the centrifugation. (Compare with Figs. 2 and 5.) This abnormality can be explained best by postulating a more rapid sedimentation, on the average, for those particle groups in the region of the boundary than was experienced by the mass of particles constituting the suspension.

It was found, as has already been mentioned, that irregular boundaries of this type could generally be avoided by a more careful selection of the lots of stock virus.

In only one recorded instance was the primary boundary replaced or accompanied by an appreciable amount of ultraviolet light-absorbing material which sedimented slower than the normal rate:—

During the course of 1 day, three experiments were performed on a specimen of lot *g* which had been suspended in a 59 per cent glycerol solution. The primary boundaries appeared normal, but were accompanied by more rapidly moving

boundaries and a considerable quantity of large aggregates which rapidly sedimented out of the field. The sedimentation rate, corrected for viscosity, was consistently about 28 (17). However, when the same specimen was again centrifuged the following day, the peculiar sedimentation record illustrated in Fig. 7 was obtained. There was no well defined boundary present during the early stages of the centrifugation, but one did appear later which could be measured approximately. Its rate was estimated to be about 20 during the sedimentation between levels *a* and *b*, as compared to 28, the normal in this medium. A bound-

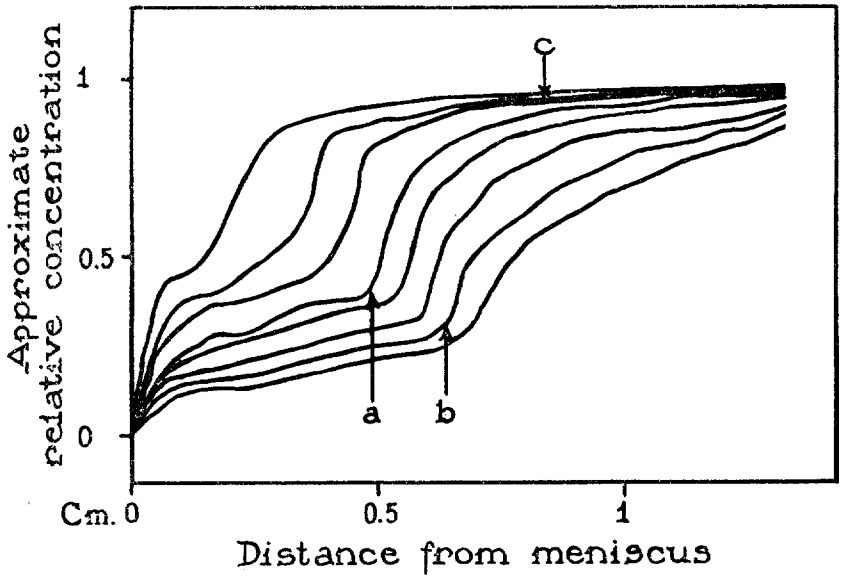

FIG. 7. Sedimentation curves for the single specimen of elementary bodies whose sedimentation rate was appreciably lower than normal. See text, page 599, for discussion.

ary moving at this rate could not have progressed from the meniscus to the indicated positions during the respective time intervals of centrifugation. It must, therefore, have originated at some stage during the centrifugation and consequently could not represent a group of particles which had remained unchanged since the beginning of the experiment. It is to be noted that in Fig. 7 the first three curves lie close together at point *c*, as did the curves at point *d* in Fig. 6. This again indicates abnormal concentrations in the lower portions of the medium. A similar set of experiments with a specimen taken from virus lot *c* did not show any of the abnormalities observed with the specimen just discussed.

Estimation of Particle Size.—The most probable sedimentation constant for the slower moving single virus particles present in an appreciable concentration has been computed to be 49.1 x 10^{-11} cm./sec./dyne. From the results reviewed elsewhere (17) the particle density has been determined as 1.16 gm. per cc. From these values, the mean diameter of the elementary bodies of the CL dermal strain of vaccine virus was estimated at 236 mμ, on the reasonable assumption of an approximately spherical shape. Allowing for the possibility that the observed 14 per cent spread in the sedimentation boundary might be due to a variation in the size of the elementary bodies, the average particle diameter was computed to lie within the range 236 mμ to 252 mμ.

DISCUSSION

The Nature of Primary Boundaries.—Studies on the elementary bodies of the CL dermal strain of vaccine virus by means of the analytical ultracentrifuge indicate that under the standard conditions of experiment, virus particles obtained from a properly prepared and selected material generally sediment with the formation of a single boundary. Such a boundary is not sharply demarcated but always exhibits a definite amount of blurring or spread. The major portion of this spread apparently is accounted for by slight differences in the size or shape of the particles or by a slight degree of agglutination among some of the particles; perhaps all three factors play a part. The spread of the boundaries generally lies between 12 and 16 per cent. Sedimentation rates calculated from the movement of these primary boundaries were consistent for different lots of virus suspended in various solutions having approximately the same density and viscosity as water when the pH values of the solutions lay between 6.2 and 8. In these media the mean sedimentation constant of the slowest moving virus particles present in an appreciable concentration was found to be 49.1 x 10^{-11} cm./sec./dyne, with a probable deviation of 0.5 per cent.

Characteristics of Multiple Boundaries.—Under conditions not yet strictly defined, multiple boundaries of elementary bodies of vaccinia may appear on centrifugation. Lowered pH values (6.2), medium concentrations of salt (0.4 per cent NaCl), and relatively long storage of stock suspensions appear to favor the phenomenon. Some lots of

material are more apt than others to show the effect, although it may not invariably recur when several centrifugations are made on the same lot. Multiple boundaries were consistently produced when Paschen bodies which had been stored in a concentrated solution of urea for a number of hours were resuspended in a dilute buffer solution.

Virus particles constituting the primary boundary were always present in a higher concentration than those constituting an accompanying secondary boundary; an analogous rule also governed the content of the secondary boundaries with respect to that of the tertiaries. This fact suggests that the production of each of the more rapidly moving particle groups depends on, and is in some way proportional to, the concentration of particles constituting the slower moving boundaries.

According to Stokes' law, if the masses of two identical spherical particles were combined to form a larger spherical particle having the same density, its sedimentation velocity in a gravitational field of force would be 1.58 times the velocity of the two original particles. Three particles so combined would increase their rate by a factor 2.07. In either case the ratio could be anything between unity and the value cited if the larger particle formed had some shape other than spherical. For example, two non-rigid particles could conceivably be drawn into a close enough combination by the action of surface forces to increase their potential sedimentation rate by a factor closely approaching 1.58. It is interesting to note in this connection that secondary boundaries of Paschen bodies generally sediment about 1.50 times as rapidly as their primary boundaries; moreover, the tertiary boundaries usually move about 1.85 times more rapidly than the primary. The possibility that multiple boundaries are dependent on the close combination of 2, 3, or 4 elementary bodies is consistent with many microscopic examinations made on stained and dark-field preparations of these suspensions of vaccine virus.

Interpretation of Irregular Boundaries.—A few stock suspensions of elementary bodies consistently displayed a non-uniform sedimentation rate during the course of an individual experiment. In every instance in which the average sedimentation rate was found to be abnormally high, it was observed also that variations in the actual

rate occurred during the course of the experiment. This could not be accounted for by experimental error alone. In such an instance the lowest momentary rate recorded over a short period of the total centrifugation time was more nearly characteristic of a normal rate. To be fully aware of such misleading behavior on the part of the sedimenting particles, or to detect the presence of multiple boundaries, it is necessary to record the distribution of the particles at frequent intervals during the centrifugation. For meeting such requirements no simpler technique appears to offer the advantages of the Svedberg photographic method.

A study of the photomicrometer curves representing the irregular boundaries recorded has led to the conclusion that their intermittent acceleration is most probably caused by an autoagglutination of elementary bodies in the region of the boundary itself. The reason for the exaggerated tendency of certain virus suspensions to agglutinate, even with storage alone, is not known. However, if this observation is accepted, then it may be postulated that in the region of the primary boundary centrifugation disturbs some existing statistical equilibrium between single and aggregated particles, permitting accelerated agglutination. Stock virus suspensions which did not give macroscopic evidence of autoagglutination after being stored for several weeks did not demonstrate the phenomenon of irregular sedimentation.

In only one of 153 experiments was there evidence of an appreciable quantity of material that sedimented at significantly slower rates than the normal for the suspending medium employed. Although a possible splitting of the elementary bodies into smaller fragments, or a partial dissolution, must be considered here, an equally acceptable explanation for the retarded sedimentation of the observed boundary might be based on a change in the size or shape of the particles.

Comparison with Previous Investigations.—Previous efforts to assign a definite numerical value to the diameter of vaccine virus have resulted in considerable variation. The methods used for these determinations have fallen into three general groups; namely, centrifugation, filtration, and direct measurement by photomicrography employing ultraviolet light. Earlier experiments have been adequately commented upon by other authors; in this presentation only

a correlation between the more recent results and the present observations will be undertaken.

The use of the analytical centrifuge eliminates two important sources of error which may have entered into previous centrifugation experiments. These are firstly, the difficulties in determining small differences in the amount of virus present when the agent is demonstrated by the infective titer of the material, and secondly, the effect of convection currents in the medium during centrifugation. Further possible sources of error with simpler methods of analysis are apparent from the occasional peculiar behavior of the elementary bodies used in the present studies.

Whatever the relative rôle played by these factors, various workers have used techniques of a certain general type and have obtained centrifugation data that were in many respects reproducible. Thus Bechhold and Schlesinger (10) considered the density of vaccine virus to be 1.10 gm. per cc. and on this basis estimated the diameter of the particle as 210 mμ to 230 mμ. Elford and Andrewes (11) determined the density of vaccine bodies as 1.18 gm. per cc., and by applying this figure to their centrifugation data, they estimated the size of the particle as 170 mμ to 180 mμ. However, they cautioned that this must be regarded as a minimum value. Moreover, these latter workers recalculated the results of Bechhold and Schlesinger, using the value 1.18 gm. per cc. for the density and found that with this figure the size would be 160 mμ to 180 mμ. The sedimentation constant of vaccine virus in broth calculated from the data of these authors (11) would be of the order of 31×10^{-11} which is significantly lower than results with the air-driven centrifuge would indicate. McIntosh and Selbie (12) employed a slightly different centrifugation technique from their predecessors (11); they assumed the density of the vaccine particle to be 1.25 gm. per cc. and estimated the diameter to be 99 mμ to 240 mμ. During the course of the present work a brief report (18) appeared, assigning a sedimentation constant of 54×10^{-11} cm./sec./dyne to elementary bodies of vaccine suspended in dilute buffer solution.

The determination of particle size by means of filtration through graded collodion membranes (19) has proved a valuable laboratory procedure. The diameter of vaccine virus has been determined by

this technique. Elford and Andrewes (20) estimated the size as 125 mμ to 175 mμ, and Païc and coworkers (21) placed the diameter at 140 mμ to 160 mμ. Earlier filtration experiments had indicated a slightly higher value, around 200 mμ (10). Experiments of this type are conducted in the following manner: Virus suspensions are passed through membranes of varying pore size and the end point is taken as the largest pore size that completely withholds the virus. The simple analogy of sand passing through a wire mesh is not applicable here for it is a well recognized fact that the membrane pores must be considerably larger than virus particles to permit their passage. Because of this, a correction factor has been applied to the end point for estimating the size of the particles. The present work suggests that the correction factor in current use may tend to underestimate the size of vaccine bodies.

Barnard has used his photomicrographs taken with ultraviolet light to calculate the size of particles of a neurotesticular strain of vaccine virus. He estimates the diameter of the virus as 160 mμ to 170 mμ according to the publication of Elford and Andrewes (11) and also in a personal communication. There is no obvious explanation for this difference between his results and ours.

CONCLUSION

Ultracentrifugal studies on the CL dermal strain of vaccine virus indicate the following characteristics of the elementary bodies:—

1. A stable suspension of Paschen bodies in a dilute buffer solution of pH 6.2 to 8 sediments with the formation of a characteristic primary boundary which consistently shows a spread of approximately 14 per cent.

2. The principal sedimentation boundary is accompanied frequently by one or several more rapidly moving boundaries which probably are produced by groups of agglutinated elementary bodies consisting of two or more particles.

3. Occasionally the principal boundary may exhibit an irregular or peculiar behavior, a fact which necessitates a careful selection of material and the performance of many experiments for accurate interpretation of results.

4. The sedimentation constant of the slowest moving particles

forming the principal boundary is computed to be 49.1 x 10^{-11} cm./sec./dyne. On the basis of this sedimentation rate, the average diameter of the smallest virus particles in appreciable amounts is estimated at 236 mμ. If the boundary spread is due principally to slight differences in particle size, then the largest single elementary bodies are approximately 252 mμ in diameter.

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