OBSERVATIONS ON MIXTURES OF ELEMENTARY BODIES OF
VACCINIA AND COATED COLLODION PARTICLES
BY MEANS OF ULTRACENTRIFUGATION
AND ELECTROPHORESIS

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Much emphasis has been placed in recent years on the value of differential centrifugation in obtaining preparations of a number of viruses in a relatively pure state; Stanley has summarized the literature on this subject (1). Workers engaged in the study of vaccine virus were the first to point out the advantages of this method and have used it extensively. Ultracentrifugation and electrophoresis also have been used in the study of viruses, particularly in estimations of their purity and in attempts to separate virus particles from unrelated material. There are certain inherent characteristics of these techniques, however, which from a theoretical point of view should be obvious but which too frequently are disregarded. This disregard for the obvious may result in erroneous conclusions being drawn from accurate experimental data. It seems advisable, therefore, to record at this time certain observations obtained by means of ultracentrifugal and electrophoretic studies on mixtures of elementary bodies of vaccinia and protein-coated collodion particles in order to emphasize some of the limitations of the techniques mentioned.

Materials and Methods

The various materials used and their method of preparation have been described in the preceding publication (2). Mixed suspensions of coated collodion particles and elementary bodies of vaccinia were studied in the Tiselius apparatus by the method previously described (2) and in the analytical ultracentrifuge of Bauer and Pickels (3) by the technique described several years ago (4).

EXPERIMENTAL

Previous experiments have shown that elementary bodies of vaccinia migrate in the Tiselius apparatus at a rate of about 10×10^{-6} cm./sec. per volt/cm. in monovalent buffer solution, 0.01 M pH 7.9 (2, 5), and that

collodion particles coated with the purified heat-stable soluble antigen (S) of vaccinia move at approximately the same rate under similar conditions (2). It has also been demonstrated that collodion particles coated with one of the components of normal rabbit serum move at a different rate under these experimental conditions. Since the collodion particles were prepared by the technique of differential centrifugation employed for the purification of elementary bodies, it could be assumed that average sedimentation rates of the particles and of the virus were similar. One might then expect that mixtures of virus and of collodion covered with protein would give a single sedimenting boundary in the analytical centrifuge and one or two migrating boundaries in the Tiselius cell, depending on whether the collodion bodies had been treated with S antigen or with serum. These expectations were realized as is shown in the protocols of the two experiments which follow.

Experiment 1.—Approximately 0.1 cc. of washed and graded collodion particles was obtained from a portion of the stock suspension by means of angle centrifugation. The sedimented material was freed of excess fluid and suspended in 2 cc. of 0.01 M lithium veronal buffer solution, pH 7.9, containing 45 mg. of purified heat-stable soluble antigen of vaccine virus. The suspension was stored at room temperature for 2 hours and then overnight at 6°C. The collodion particles were removed from the solution of antigen by angle centrifugation and suspended in 15 cc. of buffer solution. After washing, treated particles were resuspended in 15 cc. of buffer solution and then were spun in the horizontal machine at 3000 R.P.M. for 20 minutes. The opalescent supernatant fluid was placed in a cellophane tube and dialyzed against lithium veronal buffer solution for 24 hours at 0°C. Following dialysis the suspension was again spun in the horizontal centrifuge and the supernatant material saved. The fact that the particles in the final preparation were coated with S antigen was demonstrated by their agglutination in the presence of antibody specific for S antigen.

Elementary bodies of vaccinia from 30 cc. of a stock suspension—this represented about 6 mg. of dry material—were concentrated in the angle centrifuge and resuspended in 10 cc. of lithium veronal buffer solution. The suspension was dialyzed for 24 hours in a cellophane bag in the cold against several liters of buffer solution and then centrifuged in the horizontal machine at 3000 R.P.M. for 20 minutes. The opacity of the supernatant material was measured by the method of Gates (6); the nephelometer reading was 1.4 cm. Suspensions of elementary bodies having about this concentration have been shown to display on electrophoresis relatively little streaming at the boundary with currents under 3 milliamperes (2).

The suspension of collodion particles, which was slightly more opalescent than the virus suspension, was diluted with dialysate until a nephelometer reading of 1.4 was obtained. Then 9 cc. of the suspension of coated collodion particles were mixed with an equal volume of suspended elementary bodies. Studies by means of ultracentrifugation and electrophoresis were carried out on the mixture, while ultracentrifugal studies were also made on each of the component suspensions. The results are recorded in Figs. 1 to 5.

Each of the three suspensions, described in the above experiment, exhibited only one sedimenting boundary on ultracentrifugation, and, although each showed a different amount of spreading, the average sedimentation rate of the particles was in every instance approximately the same, *i.e.*, 53×10^{-11} cm./sec./dyne. The sedimenting boundary of elementary bodies (Fig. 1) was, as previously observed (4), relatively sharp and well defined. The boundary formed by the collodion particles (Fig. 2) was not so well defined, becoming progressively more diffuse as it migrated. The boundary obtained with the mixture (Fig. 3) showed an amount of spread intermediate between those of the boundaries associated with the individual components. These differences in spread of boundaries, as well as the coincidence of sedimentation rates, are clearly presented in Fig. 4 which represents a group of corresponding microphotometer curves obtained from the sedimentation photographs of the three suspensions. Each set of such curves which showed the distribution of the particles at a particular stage of the centrifugation had the general characteristics of that illustrated in Fig. 4, *i.e.*, the midpoints of the three boundaries were at the same distance from the meniscus, with the relative concentration of the mixture at any level having a value intermediate between those of the two components. It is thus apparent from the curves shown in Fig. 4 that no appreciable agglomeration of collodion particles and elementary bodies occurred in the mixture.

A portion of the mixture of virus and collodion treated with S antigen, studied by electrophoresis, was found to have a single discrete boundary that moved at a rate of 10.5×10^{-5} cm./sec. per volt/cm. (Fig. 5).

Experiment 2.—A suspension of collodion particles treated with a 10 per cent solution of normal rabbit serum and subsequently washed was obtained by a technique corresponding to that employed in Experiment 1 for preparing collodion coated with S antigen. A concentrated suspension of elementary bodies similar to the one previously used was prepared and mixed with an equal amount of the suspension of collodion particles. The opacity of the two component suspensions was of the same degree, as determined by nephelometer readings. Ultracentrifugal and electrophoretic studies were made on the mixture and the results of the latter are portrayed in Fig. 6.

Ultracentrifugal studies on the mixture described in Experiment 2 gave results similar to those obtained in Experiment 1, *i.e.*, only one boundary was present. Electrophoretic observations, on the other hand, showed two distinct migrating boundaries (Fig. 6). The collodion particles coated with a component of normal serum moved at a rate of 13×10^{-5} cm./sec. per volt/cm., while the elementary bodies traveled at their usual speed of

10×10^{-5} cm./sec. per volt/cm. Differences in staining characteristics of coated collodion particles and elementary bodies (2), as demonstrated by the use of Morosow's technique, permitted an identification of the constituents of the two boundaries; collodion particles coated with rabbit serum protein took a tan color in contrast to the jet black displayed by elementary bodies. Parts *a* and *b* of Fig. 7 illustrate, respectively, the microscopic appearance of elementary bodies and of mixtures of elementary bodies and serum-coated collodion particles stained in the usual manner. It may be pointed out, however, that collodion particles coated with protein can be stained so deeply that they become almost indistinguishable from elementary bodies; part *c* of Fig. 7 represents the results of such a stain applied to collodion particles coated with S antigen, and is included to illustrate the relative homogeneity of the suspensions of graded collodion particles.

FIG. 1. Photographic record of a series of sedimenting boundaries obtained in the ultracentrifuge with elementary bodies of vaccinia. Light from the 334 m μ region of the spectrum was employed; speed of ultracentrifuge was 4200 R.P.M.; intervals between photographs were 3 minutes.

FIG. 2. Sedimentation photographs of collodion particles which had been graded by the procedure used for purifying vaccine virus. Photographs were taken under the same experimental conditions as those in Fig. 1.

FIG. 3. Photographs of a series of sedimenting boundaries displayed by a mixture of elementary bodies and collodion particles. Optical density of the two components was equal. Photographs taken under same experimental conditions as those in Fig. 1.

FIG. 4. Reproduction of microphotometer tracings of a set of corresponding photographs (third in each series) from Figs. 1, 2, and 3. The curves illustrate the identity of the three sedimentation rates. In addition, they show that the spreading of the boundary of the mixture is intermediate between those displayed by the two components of the mixture.

FIG. 5. Photographic illustration of the single moving boundary obtained in the Tiselius apparatus with a mixture of elementary bodies and collodion particles coated with the heat-stable soluble antigen of vaccinia. 0.01 M lithium veronal buffer solution, at pH 7.9, was employed in all electrophoretic experiments; current was 2.5 milliamperes; picture taken after 1 hour.

FIG. 6. Photographic illustration of the two moving boundaries displayed by a mixture of elementary bodies and collodion particles coated with a component of normal rabbit serum. Conditions were the same as those employed in experiment illustrated in Fig. 5.

FIG. 7. Photomicrographs illustrating (a) elementary bodies, (b) a mixture of elementary bodies and collodion particles coated with a constituent of rabbit serum, and (c) collodion particles coated with heat-stable soluble antigen of vaccinia. Elementary bodies stain black. Collodion coated with serum stains light brown (b), while collodion particles coated with antigen and overstained appear black (c). Morosow's silver stain. $\times 1000$.

DISCUSSION

The results of our experiments demonstrate several facts that should be apparent from a theoretical point of view, namely, that the technique of differential centrifugation selects particles with approximately the same rate of sedimentation, and that a mixture of different types of particles which have been selected by the same procedure presents a single sedimenting boundary in the analytical ultracentrifuge. Furthermore, the different types of particles constituting a mixture and being indistinguishable by ultracentrifugation methods, may or may not be distinguishable by electrophoretic techniques, depending on the character of the surface of the particles.

The homogeneity of a group of discrete particles, *e.g.*, virus particles or bodies, might be immediately evident on ultracentrifugation if their concentration were high in comparison with that of other associated particles of a similar size. Such a condition, however, is often impossible to realize, particularly if at some stage of the work the virus-laden tissues have been ground or emulsified. The variations in size of particles in ungraded suspensions of collodion approach those found in suspensions of ground tissue, *i.e.*, over a relatively wide range, particles of many different sizes are present in appreciable concentration. Therefore, procedures designed to select particles of a given range of size will, even though frequently repeated, continue to provide particles with slight difference in rates of sedimentation. The spread of the boundary displayed in the analytical centrifuge by such a preparation, *viz.*, one containing particles of the same constitution but of different sizes or shapes, will necessarily be appreciable. It may be pointed out that suspensions containing particles of like kind and size, too large to be appreciably affected by diffusion in the centrifuge cell, frequently sediment with a boundary that exhibits considerable spread because of the varying degree of aggregation of the primary particles (4, 7). Marked spreading of the boundary may also occur in a mixture of unlike particles which have the same size but slightly different densities. Thus, the interpretation of spread of boundary obtained in the analytical centrifuge with biological preparations is often difficult. It happens that elementary bodies of vaccinia, obtained by the method employed by us, are not associated with an appreciable amount of other material existing in the form of particles of similar sedimentation rate. Hence it is possible to purify the virus bodies by means of differential centrifugation and with them to obtain in the ultracentrifuge a sedimenting boundary which has only a relatively small amount of spread (4).

From what has been said and from the data presented, the desirability of applying, whenever possible, a method similar to that developed for estimating the purity of preparations of vaccine virus (8) should be apparent. This method depends on a comparison of the number of infective units in a given preparation with the number of elementary bodies estimated from the dry weight of the material. In other words, ultracentrifugal and electrophoretic data may be misleading at times in regard to the purity of virus preparations.

SUMMARY

It has been shown experimentally that mixtures of two types of particles, namely, elementary bodies of vaccinia and collodion particles coated with protein, sediment with a single boundary in the analytical centrifuge. Such mixtures have been shown to develop one or two boundaries on electrophoresis in the Tiselius apparatus, depending on the type of coating on the surface of the collodion particles. When covered with the heat-stable soluble antigen of vaccinia, collodion particles migrate in the electrical field at the same rate as elementary bodies. On the other hand, if they are coated with a component of normal rabbit serum, they migrate at a different rate.

The estimation of purity of preparations of virus by means of data obtained by ultracentrifugation and electrophoresis is discussed.

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