CENTRIFUGATION STUDIES ON PNEUMONIA VIRUS OF MICE (PVM)

The Relative Sizes of Free and Combined Virus*

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Pneumonia virus of mice is a latent infectious agent which appears to be strictly pneumotropic. Originally recovered by serial passage of lungs from apparently normal mice (1), this virus has been found to be capable of inducing fatal pneumonia not only in mice but also in cotton rats and hamsters (2). The frequent occurrence of neutralizing antibodies against this agent in serum obtained from members of different mammalian species including man (2) indicates that inapparent infection with it is not only common but also that this virus is widely distributed in nature. For convenience pneumonia virus of mice will be referred to as PVM in this as in previous communications.

The discovery by Mills and Dochez (3, 4) that suspensions of mouse lungs infected with PVM, if adequately heated, cause agglutination of mouse erythrocytes provided a basis for the development of *in vitro* techniques for the study of this virus. The occurrence of hemagglutination with such heated suspensions and of inhibition of hemagglutination by specific immune serum, as reported by Mills and Dochez, was readily confirmed in this laboratory, and methods were developed for obtaining reproducible results with these reactions (5).

Evidence in wide variety but of an indirect nature which indicates that the component responsible for hemagglutination is the virus particle itself has been presented in a preceding paper (5). Moreover, it was shown that the virus possesses the capacity to combine firmly with either mouse or hamster erythro-

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cytes as well as with tissue particles obtained from the lungs of certain mammalian species susceptible to infection with it. The occurrence of such combination was thought to provide an adequate explanation not only for the phenomenon of hemagglutination but also for a number of other unusual properties manifested by this virus (5).

The experimental data presented in this paper were derived entirely from the results of hemagglutination tests with the virus. It was found that the results of infectivity tests with PVM were not sufficiently precise or dependable to be useful for the purposes of this study. In this paper are presented the results of further studies on certain properties of PVM, particularly as shown by analytical experiments utilizing the high speed angle centrifuge. It will be shown that it is possible by special procedures to obtain free virus from infected lung tissues. Free virus, obtained in this manner, is not only infectious but also causes hemagglutination directly, as is shown in the accompanying paper (6). Additional evidence that PVM readily combines with lung tissue particles will be given, and estimates will be made, from the centrifugal analyses, of the particle sizes of free virus and combined virus. It will be shown that combined PVM is, as would be expected, demonstrably larger than free PVM.

Materials and Methods

Virus

Pneumonia virus of mice (PVM), strain 15 (1), was used. The virus was maintained by occasional lung passage in albino Swiss mice approximately 5 weeks of age, as described previously (7). Suspensions were prepared from the lungs of mice 5 to 7 days after intranasal inoculation with PVM. In view of the capacity of PVM to combine with particles of certain tissues, including mouse erythrocytes and lung, virus was obtained in different states by preparing and treating suspensions of infected lungs in various ways.

Preparation of Suspensions

1. Combined PVM.—A 10 per cent suspension of infected lungs in saline buffered at pH 7.6 was made by whirling 50 to 100 cc., 1.5 minutes at room temperature, in a Waring blendor. In certain instances the coarse particles of tissue were sedimented (at 1,000 R.P.M. for 2 minutes) and discarded. In accordance with previous findings (5) the virus in such preparations appears to be in firm combination with tissue particles and is, therefore, designated as combined virus. It is of importance to reiterate that PVM in such preparations, although infectious, is not capable of agglutinating erythrocytes.

2. Heat-Released PVM.—Suspensions of combined virus, prepared as described above, were heated at 70°C. for 30 minutes. As shown previously (5), such heating apparently releases the virus from combination by destroying the combining capacity of the tissue component. Heated suspensions were cleared by centrifugation in an angle head at 8,000 to 10,000 R.P.M. for 10 to 15 minutes. The virus remains in the watery supernate following this procedure and, although it is non-infectious as a result of heating, it is capable of agglutinating RBC or of recombining with fresh lung tissue particles; moreover, the immunological properties characteristic of PVM are retained (4, 5). The virus present in such preparations is designated as heat-released virus.

3. Free PVM.—The lungs of infected mice were perfused with saline prior to their removal

to wash out erythrocytes capable of combining with the virus. The perfused lungs were then cut or sliced into small pieces either immediately or after varying periods of storage at −70°C. Subsequent treatment was carried out at 4°C. The lung pieces were suspended in 9 cc. of cold buffered saline per gm. of lung tissue, shaken lightly for 10 minutes, and then centrifuged in an angle head at 12,000 R.P.M. for 30 minutes. The centrifuge employed was described previously by Pickels (8); the rotor diameter is 20 cm.; the tube diameter is 1.27 cm.; and the tube length is 8.90 cm. The virus remaining in the supernate of such preparations is both infectious and capable of causing hemagglutination directly (6). It is designated and will be referred to as free virus. The reasons for thinking that PVM prepared in this manner is not combined with tissue particles will be apparent from the findings of the present study and from evidence presented in the accompanying paper (6).

4. Heated Free PVM.—Suspensions of free virus, prepared as described above, were heated at 70°C for 30 minutes and cleared by centrifugation as in procedure 2. The virus present in the supernatant fluids obtained following this treatment is designated as heated free virus. In such preparations the property of infectiousness is lost as a result of heating but the capacity to produce hemagglutination is retained.

5. Artificially Combined PVM.—Suspensions of heat-released virus, prepared by procedure 2, were mixed with an equal volume of 20 per cent saline suspension of normal mouse lungs which had been ground in a Waring blendor. The mixture was held for 2 hours at 27°C. and overnight in a refrigerator. That the heat-released virus present in this mixture actually entered into combination with the normal lung tissue particles was indicated by the finding that the mixture failed to cause hemagglutination. However, aliquots of the mixture which were heated at 70°C. for 30 minutes agglutinated mouse RBC in a titer which closely approximated that of the heat-released PVM included in the mixture. The virus present in this mixture is designated as artificially combined virus.

In suspensions prepared by procedures 1 and 3, described above, the virus remained infectious whereas in all of the heated suspensions the property of infectiousness was destroyed. Wherever possible, the virus titer of each preparation was determined by tests both for infectivity and hemagglutination.

Infectivity Tests

The technique of virus titrations in mice utilizing serial tenfold dilutions was identical with that used in previous studies on PVM (7). Virus titration end points were calculated by the 50 per cent maximum score method previously employed (7).

Hemagglutination Tests

The technique of hemagglutination tests with mouse RBC and the procedure for the estimation of end points were identical, respectively, with those previously described (5).

Centrifugal Analysis

Suspensions of PVM prepared by each of the methods described above were spun in an air-driven centrifuge of the vacuum type (9). A synthetic density gradient of sucrose was added to the virus suspensions in order to counteract convection disturbances (10). The method was similar to that used previously in studies on influenza viruses (11).

The sucrose gradient was prepared in the following manner: To each of 4 equal aliquots of virus suspension was added an equal amount of diluent consisting in 1 instance of distilled water and in 3 instances of sucrose in distilled water. The amount of sucrose in the diluents was so adjusted that the resulting mixtures contained sucrose in concentrations of 0, 3, 6, and 9 per cent, respectively. Volumes of 2.25 cc. of each mixture, in the order given above, were

successively allowed to feed by gravity from a syringe barrel through a long needle reaching almost to the bottom of a lusteroid centrifuge tube which measured 1.27 by 8.90 cm. When all 4 samples had been delivered, a few bubbles of air were gently forced through the needle to effect slight mixing between adjacent layers of different densities. Thus, while the concentration of virus remained uniform throughout the fluid column, the concentration of sucrose increased from a negligible amount at the top to approximately 9 per cent at the bottom of the tube.

In the rotor used for the centrifugation of these preparations the meniscus of fluid was 5.3 cm. from the axis of rotation and the tubes were inclined at an angle of 35° to the axis. After centrifugation 8 equal (1.0 cc.) samples were removed successively from each tube by means of a sampling apparatus (12), and the pellet of sediment was resuspended in the 9th and final sample which included also any small amount of remaining fluid in excess of 1.0 cc. With each preparation of virus several tubes were usually centrifuged simultaneously. In certain instances samples were tested individually; frequently corresponding samples from several tubes which had been centrifuged together were pooled and then tested. Tubes, the contents of which had been prepared in an identical manner but had not been centrifuged, were, in certain experiments, also sampled in like fashion and tested as controls. In other experiments multiple control tests were carried out with aliquots from each of the 4 starting mixtures containing virus and the various diluents.

Centrifuged samples of free virus (procedure 3) and identical uncentrifuged control preparations were tested directly for hemagglutination as well as for virus activity in mice. When suspensions of combined virus (procedure 1) or artificially combined virus (procedure 5) were centrifuged and sampled, the corresponding samples from several tubes were pooled. Pools of combined virus were tested for infectiousness in mice. The artificially combined virus used was, of course, non-infectious because of the preliminary heating of the virus contained in it. With both combined virus and artificially combined virus aliquots of each pool were heated at 70°C. for 30 minutes and cleared by centrifugation in the angle head at 8,000 to 10,000 R.P.M. for 10 to 15 minutes. Heated portions of the uncentrifuged mixtures used for controls were prepared similarly. As none of the unheated samples of either preparation of combined virus caused hemagglutination directly, the effect of centrifugation, as measured by the hemagglutination technique, was determined in each instance from the results of tests with heated aliquots. The centrifuged samples of heat-released virus (procedure 2) and corresponding uncentrifuged control suspensions were tested directly for hemagglutination without additional heating.

All samples were tested on the same day on which they were centrifuged. In certain experiments repetitive tests for hemagglutination were carried out a day or two later, and the specimens were stored at 4°C. during the interval.

Determinations of Density and Viscosity

The densities of the starting mixtures were determined by the pyknometer method and their viscosities were measured with a viscometer of the glass capillary type.

Presentation of Data

As pointed out above, in each of the different preparations of PVM, infected mouse lung and buffered saline were mixed initially in the proportions of 1 gm. of lung to 9.0 cc. of diluent. Titrations of virus activity, either hemagglutination or infectiousness, were carried out by serial dilution techniques. Throughout this investigation, and in conformity with previous studies (2, 5, 7), end points are expressed in terms of the final dilution of the infected mouse lung itself. As would be expected, the virus titers obtained were influenced by the method employed in the preparation of the suspension.

When multiple hemagglutination titrations of a preparation were carried out, as was usually the case, the final titer recorded was the geometric mean of all the individual titration end points. In each experiment the geometric mean hemagglutination titer of corresponding uncentrifuged preparations was determined similarly. The difference between the geometric mean titer obtained with each successive sample taken from the centrifuged specimens and the geometric mean titer of the control specimens was also determined.

The observed difference in the hemagglutination titer of the centrifuged sample from that of the control was plotted on a logarithmic scale against the position (*i.e.*, the number) of the sample in the centrifuge tube. The percentage of virus activity (*i.e.*, the hemagglutination) present in the centrifuged sample as compared with the control was calculated and was plotted also against the position of the sample in the centrifuge tube. It should be emphasized that the values shown represent the degree of difference between the experimental and control end points. On the logarithmic scale the greater the deviation of a point from zero (*i.e.*, no difference in titer), the greater the difference between the control and experimental titration end points. Similarly, on the percentage scale, the greater the deviation of a point from 100 per cent (*i.e.*, a titer identical to that of the control), the greater was the difference in demonstrable virus activity in the control and experimental samples. Because the amount of virus present in individual suspensions varied considerably, the experimental results were expressed in the manner described above in order to facilitate a direct comparison between the results obtained with different preparations of virus under similar conditions.

EXPERIMENTAL

Suspensions of PVM which had been prepared according to the several procedures described above were subjected to gravitational fields of different intensities for various periods of time. The suspensions which were studied contained, respectively, combined virus, artificially combined virus, heat-released virus, free virus, and heated free virus.

The titer of virus which was present at various levels in the centrifuged specimens was measured by its capacity to cause hemagglutination according to the methods described above. The differences between the experimental and control titration end points obtained with each suspension were plotted as outlined in the preceding section and are presented graphically. Smooth curves have been drawn through the experimentally determined points shown on the logarithmic scales as well as through the calculated values shown on the percentage scales. It is of importance to point out that the calculated values shown on the percentage scales were derived directly from the experimental points shown on the logarithmic scales and not from the curves drawn through the latter points.

The behavior of various preparations of PVM under the influence of intense gravitational fields is illustrated by the results shown in Figs. 1 to 4. It can be seen that when centrifugation at a selected speed is continued for an appropriate length of time, the sedimentation curves exhibit the following characteristics: The concentration of virus near the top of the fluid column is very low, even negligible in some instances. At a certain level in the tube the concentration rises rather sharply. Below this level the virus concentration rises

gradually or is more or less uniform, *i.e.* plateau-like, except for the bottom sample, which shows a several fold increase over the starting concentration due to deposition of the virus. Within the limits of precision of the hemagglutination test, the increase in the concentration of the bottom or 9th sample accounts for the decreases in concentration of the other 8 samples. That these characteristics arise directly from the action of centrifugal force, and not through any action of the sucrose diluent on the virus, was indicated by the failure of control experiments to show any significant difference between the stability of the virus in diluents with and without sucrose.

The relatively sharp increase in the concentration of the virus observed in the upper half of the tube appears to be indicative of a *sedimentation boundary* and suggests that a large group of virus particles are sedimenting at nearly the same rate and, therefore, are of nearly the same size. Furthermore, these represent the smallest virus particles present in appreciable concentration. These particles may then be referred to as the principal particles possessing the capacity to cause hemagglutination in the particular preparation under study, as contrasted with other particles possessing similar properties but sedimenting at a much faster rate, as would, for example, large aggregates of particles. That the sharp rise in concentration actually represents a group of sedimenting principal particles, and hence a real boundary, was verified in several instances by the experimental observation that the displacement of the boundary (taken as the midpoint of the sharp rise in concentration) from the meniscus of the fluid increased with, and was approximately proportional to, the amount of centrifugation. This is illustrated by the three curves shown in Fig. 2. Hence, from the displacement of a boundary under controlled conditions, in accordance with principles already described (10), it is possible to compute the approximate average sedimentation constant of the principal virus particles in the particular preparation under study. Previous studies with hemocyanin (10) and with influenza viruses (11) have indicated that sedimentation constants, determined as in the present instance with the angle centrifuge, usually agree within about 25 per cent with values obtained in the sectoral cell of the more precise optical ultracentrifuge of Svedberg.

Because of intrinsic limitations of the methods, the boundaries are not so sharp nor the plateau regions so flat as they would be if ideal experimental conditions could be realized and, therefore, it can be said with reasonable certainty only that the great majority of the principal virus particles in a particular preparation have sedimentation constants within a few per cent of the average value computed for that preparation. Some of the apparent boundary spreading is due to the following factors: the coarseness of the sampling, the small amount of remixing which characterizes sedimentation in inclined tubes (10), and the limited precision of the hemagglutination test itself. However, an indeterminate portion of the observed boundary spreading is probably due also

to an actual inhomogeneity of sedimentation rate, which may be associated with various degrees of aggregation between principal virus particles or between virus and lung tissue particles.

The presence in these preparations of virus particles which sediment at rates higher than that of the principal virus particles is evidenced by the pronounced departure in some cases from uniformity of concentration in the plateau region (see curve II of Fig. 2) and in other cases by the low concentration of the plateau region (see Fig. 4). A certain amount of progressive decrease in concentration is to be expected under the experimental conditions employed because of the the non-uniformity of the centrifugal field. The actual concentration which should occur in the plateau region with perfectly homogeneous particles can only be estimated roughly when sedimentation occurs in inclined tubes. This should be of the order of 65 per cent of the starting concentration when the boundary has sedimented about one-quarter of the length of the fluid column, such as is the case with most of the curves shown in Figs. 1 to 4. As is illustrated by these curves, the principal virus particles usually accounted for about 50 to 75 per cent of the total virus demonstrable by the hemagglutination technique. In the single experiment with very high concentrations of sucrose in the diluent (see Fig. 4), only about 25 per cent of the total virus was accounted for by the principal virus particles. There is not yet sufficient experimental evidence to warrant the conclusion that there was any consistent and significant difference in the relative homogeneity of the particles in the various preparations of PVM which were tested.

Previous studies (5) have indicated that in ground mouse lung suspensions PVM is firmly combined with lung tissue particles from which it can be released by adequate heating. In the light of these observations it would be expected that combined virus should consist of particles demonstrably larger than those of heat-released virus. To test this possibility directly, suspensions containing separately combined PVM, heat-released PVM, and artificially combined PVM were examined in the high speed centrifuge.

The results of typical centrifugal experiments, carried out as described above, are presented graphically in Figs. 1 and 2. It can be seen that the heat-released virus required at least 10 times more centrifugation (the amount of centrifugation being proportional to the product of time and the square of the speed) than the combined virus to produce a comparable degree of sedimentation. It can be seen, also, that artificially combined virus (*i.e.*, a mixture of heat-released virus and a suspension of normal mouse lungs) behaved in the centrifuge almost exactly as did the naturally combined virus present in suspensions of infected mouse lungs. These results indicate that either naturally or artificially combined PVM is distinctly larger than heat-released PVM. Other points of particular interest are the relative sharpness of the boundaries and the substantial height of the plateau regions shown in Fig. 1. These indicate that approxi-

mately 50 per cent of the combined virus, and hence of the tissue components which entered into combination with the virus, are composed of relatively uniform particles.

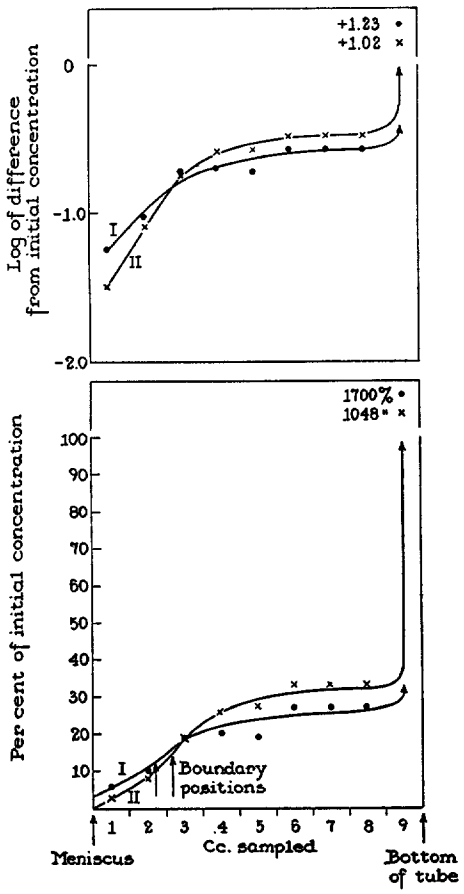

FIG. 1. Sedimentation of combined PVM and artificially combined PVM. Centrifugation at 9,000 R.P.M. for 15 minutes (equivalent to 14,000 R.P.M. for 6 minutes). Curve I, combined virus. Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-3.98}$. Curve II, artificially combined virus. Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-2.99}$.

As a result of the development of a special technique, described under Materials and Methods, by means of which free virus could be obtained directly from infected mouse lungs, it became possible to determine whether virus particles, which had not been in combination with tissue particles, were similar in size to heat-released virus particles which had been so combined. Suspensions containing free PVM, which were infectious and gave hemagglutination on

direct test, as well as such suspensions following heating (heated free PVM), were examined in the high speed centrifuge.

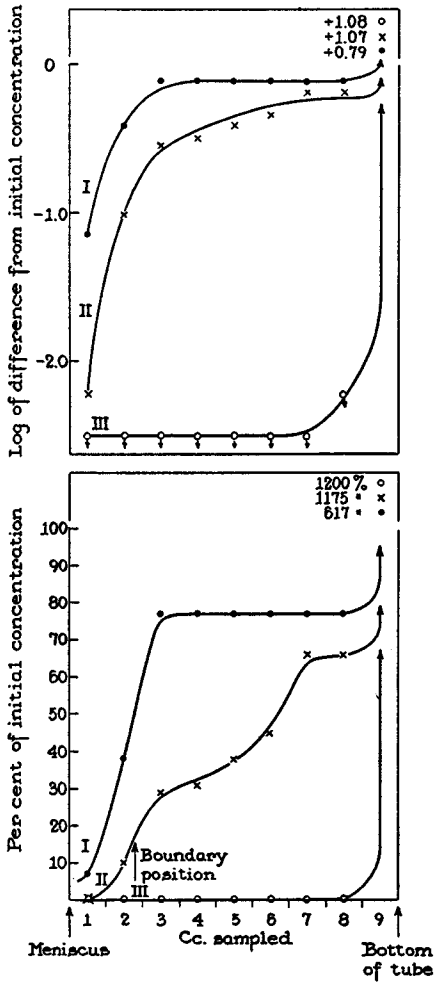

FIG. 2. Sedimentation of heat-released PVM. Centrifugation: Curve I, 14,400 R.P.M. for 60 minutes; Curve II, 14,400 R.P.M. for 120 minutes; Curve III, 30,000 R.P.M. for 120 minutes (equivalent to 14,400 R.P.M. for 540 minutes). Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-3.52}$.

The results of these experiments are shown graphically in Fig. 3. It will be seen that the sedimentation of the principal virus particles of free PVM was closely similar to that of heat-released PVM (see Fig. 2) under the same experimental conditions. It should also be noted that much more centrifugation was

required to produce comparable sedimentation of free PVM than was necessary with the combined virus (see Fig. 1). Although the sedimentation curve obtained with the free virus is somewhat different from that obtained with the

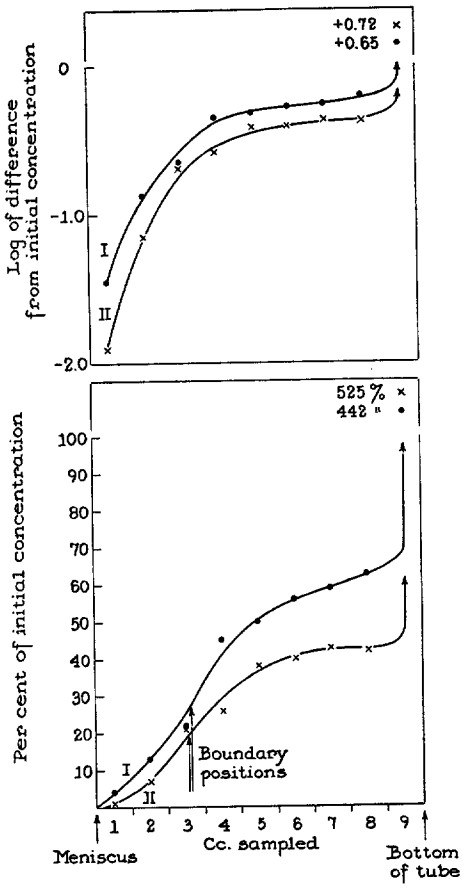

FIG. 3. Sedimentation of free PVM and heated free PVM. Centrifugation at 14,400 R.P.M. for 120 minutes. Curve I, free virus. Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-3.37}$. Curve II, heated free virus. Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-3.22}$.

heat-released virus under the same conditions, it does not appear that there is any significant difference in the average sedimentation constants computed for the respective principal virus particles. It will be observed (see Fig. 3) that the behavior in the centrifuge of free virus which had been heated at 70°C. was closely similar to that of heat-released virus as well as to that of free PVM itself under comparable conditions of centrifugation.

These results indicate that heating at 70°C. for 30 minutes does not alter the physical state of the virus to an extent detectable under the experimental con-

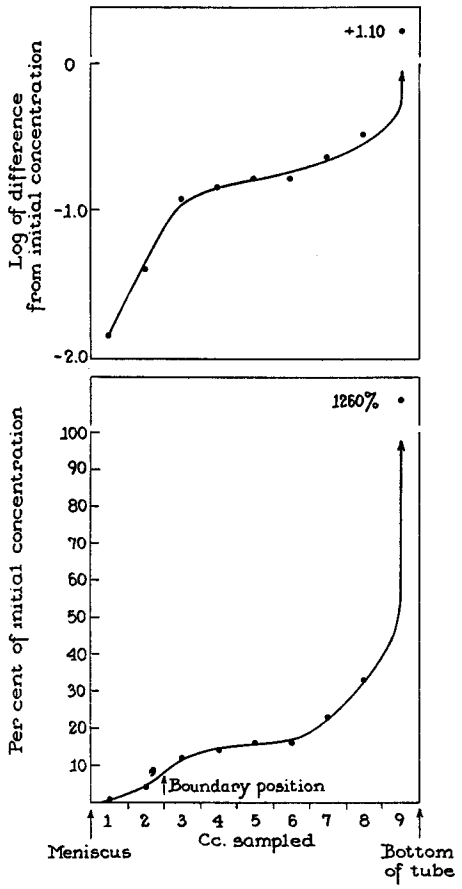

FIG. 4. Sedimentation of heat-released PVM in a medium with the specific gravity increased to 1.079 by the addition of sucrose. Centrifugation at 30,000 R.P.M. for 120 minutes (equivalent to 14,400 R.P.M. for 540 minutes). Titer of uncentrifuged control (reciprocal of initial concentration), $10^{-3.90}$.

ditions employed. Moreover, they suggest that heat-released virus is physically either similar to or identical with free PVM.

Several experiments of the types illustrated in Figs. 1, 2, and 3 were performed, some being carried out at ice box temperatures. In all instances the results obtained were in essential agreement with those illustrated. From the measured boundary displacements and application of principals already described (10), the sedimentation constant either of free PVM or heat-released

PVM was computed to be of the order of 120 S, while that of combined PVM was found to be of the order of 1700 S.

In order to gain some idea of the probable density of the heat-released virus particles, one experiment was performed in which the average density of the suspending medium was increased by incorporating greater quantities of sucrose. The concentration of sucrose was graded from approximately 22 per cent at the top to a maximum of 25 per cent at the bottom of the centrifuge tube so as to retain the advantages of a density gradient.

The results of the experiment are shown graphically in Fig. 4. It is evident from a comparison of the results obtained in this experiment with those shown in Fig. 2 that 4.5 times more centrifugation was required to displace the boundary an approximately equal distance from the meniscus in the presence of concentrated sucrose then in the presence of dilute sucrose.

Since sedimentation rate is directly proportional to: (1) the average centrifugal force at the boundary; (2) the difference between the density of the sedimenting particles and the average density of the suspending medium at the boundary; and (3) the reciprocal of the average viscosity of the medium at the boundary, the particle density can be determined if other quantities are known and if the physical characteristics of the particles can be assumed to be unaffected by sucrose. In the present experiments the average densities of the medium in the region of the boundaries were found to be 1.013 gm. per cc. and 1.079 gm. per cc. with the weak and strong sucrose solutions, respectively. Average viscosities were 0.0100 poise and 0.0185 poise, respectively. On the assumption of unchanged physical characteristics, the density of the hydrated virus particles was computed to be approximately 1.13 gm. per cc.

DISCUSSION

By means of a technique described in the present communication, it is possible to obtain preparations of free PVM (*i.e.*, virus which is not combined with tissue particles) which cause hemagglutination even when unheated. Additional evidence that the virus in such preparations is in fact free or uncombined and that it is infectious is presented and discussed in the accompanying paper (6). By utilizing the high speed angle centrifuge (9) and methods previously described (5, 10–12), it has been possible with such preparations to study certain physical characteristics of the virus itself. Computations made from the experimental data obtained indicate that the principal hydrated virus particles of naturally free or heat-released PVM have an average sedimentation constant of the order of 120 S, a density of the order of 1.13 gm. per cc., and hence, in accordance with Stoke's law, a size of the order of 40 millimicrons if spherical shape is assumed. Moreover, they indicate that these physical characteristics are not appreciably altered by heating preparations at 70° C. for 30 minutes.

From evidence presented earlier (5) it was concluded that PVM possesses the

peculiar capacity to enter into firm combination with mouse or hamster erythrocytes as well as with lung tissue particles from certain susceptible animal species. The occurrence of combination between the virus and tissue particles served to account for some unusual properties of the virus, particularly the following: PVM in ground suspensions of infected mouse lungs is infectious but is incapable of causing hemagglutination, PVM in such suspensions does not combine with antibody *in vitro*, and PVM in such suspensions appears to exist in particles larger in size than the particles which show characteristic properties of the virus following appropriate heating. It was postulated that appropriate heating destroys the combining capacity of the tissue component in such combinations, thereby releasing the virus which, although rendered non-infectious as a result of heating, nevertheless retains the following distinguishing characteristics: the capacity to recombine with particles of lung tissue or appropriate erythrocytes, the capacity to unite with specific antibody *in vitro*, and the capacity to stimulate in animals the development of active immunity and specific neutralizing antibodies against PVM.

The results of the studies described in the present paper provide more quantitative evidence in support of the conclusions and hypotheses developed from earlier work (5). By following identical experimental procedures it has been shown that particles of combined PVM present in suspensions of ground infected mouse lungs are indeed larger than those of the free or heat-released virus. Computations based on the experimental results obtained in this study indicate that the principal hydrated particles of combined virus have an average sedimentation constant of approximately 1700 S and, hence, on the assumption of spherical shape and an average density comparable to that of free virus, a diameter of the order of 140 millimicrons. This latter value is in agreement with the estimated size of 100 to 150 millimicrons obtained for this virus earlier (1) by the ultrafiltration technique. Because of the method of preparation of suspensions employed in the earlier study, it is evident in retrospect that combined virus was used. It can be shown readily that the 3.5-fold difference in the particle diameters of free and combined PVM, respectively, is indicative of a difference between them in terms of either volume or weight of at least 42-fold.

It should be emphasized that the computed size of the particles of combined PVM is very probably a minimal estimate. In computing the size of the particles of combined virus it was necessary to assume a value for the density of the tissue constituent in the combination, and the value obtained for the virus itself was used. It seems improbable that tissue constituents should have a density greater than that of the virus. If the density of the tissue component in combination with PVM is less than that of the virus itself, then the minimal size of the particles of combined virus is somewhat greater than 140 millimicrons. Moreover, if the shape is not spherical as assumed, the length

of the particles is greater than the value computed. Although it has been possible to compute the approximate minimal size of particles of combined PVM in ground suspensions, it should be pointed out that this virus is capable of combining even with intact cells, as for example, with appropriate erythrocytes. It is apparent that the relative size of tissue particles or of cell fragments in a suspension of infected lungs is to some extent dependent upon the preparative technique employed and, consequently, the virus in such a suspension may be combined with particles which vary in size over an extremely wide range.

The concept that PVM can be released from combination with tissue particles by appropriate heating and still retain specific distinguishing characteristics (5), received additional verification from the results of the present studies. Following heating of combined virus at 70°C. for 30 minutes, the non-infectious particles, which still can be identified as PVM, are of the same order of size (*i.e.*, approximately 40 millimicrons) as particles of infectious free virus or of free virus which has been similarly heated. This estimate is only slightly greater than the approximation of the size of heat-released PVM suggested earlier (5) on the basis of preliminary centrifugation studies and a higher assumed density value.

Further demonstration that firm combination between PVM and lung tissue particles does in fact occur, was obtained in centrifugation studies with a mixture containing heat-released PVM and a suspension of ground normal mouse lungs. That the virus combines with particles of normal lung tissue (5) was evidenced by the failure of such a mixture to cause hemagglutintion; and by the reappearance of this property, in undiminished titer, following heating at 70°C. for 30 minutes. The behavior in the centrifuge of such artificially combined PVM was closely similar to that of naturally combined virus present in ground suspensions of infected mouse lungs and its minimal particle size was computed to be of the same order as that of naturally combined virus (*i.e.*, approximately 140 millimicrons).

The experimental data and the estimated particle sizes of free PVM and combined PVM presented in this paper were obtained from determinations of virus activity carried out *in vitro* by the hemagglutination technique. Obviously, it is not possible to obtain measurements of infectiousness with heated preparations of PVM, but it is reasonable to expect that titrations of virus activity with unheated preparations of free PVM and combined PVM should be carried out *in vivo* as well as *in vitro*.

Such *in vivo* determinations were carried out, but the results obtained were inadequate for analysis and, as a consequence, have not been presented graphically. It was found that virus was demonstrable *in vivo* in centrifuged samples of free and combined PVM, but the infectivity titers were almost invariably lower than the corresponding hemagglutination titers. Moreover, uncentri-

fuged control specimens, which were held under similar conditions and were tested following the interval required for centrifugation and sampling, usually had considerably lower infectivity titers than were obtained with the same suspensions immediately after they had been prepared, whereas their hemagglutination titers remained unaltered. Although the results obtained *in vivo* were erratic and difficult to evaluate quantitatively, it may be stated that no definite evidence was obtained of a physical separation between the component responsible for hemagglutination and that responsible for infectiousness.

The relatively low infectivity titers obtained with suspensions of PVM, as well as the marked instability of the property of infectiousness with this virus, are, even under the most favorable circumstances, serious handicaps in attempts at precise measurements of virus activity *in vivo* (1, 5–7). In the light of evidence presented previously (1, 5, 7), as well as that given in the accompanying paper (6), it seems reasonable to conclude that with this virus the conditions, manipulations, and time required to carry out experiments in the centrifuge had adverse effects upon the unstable property of infectiousness but did not have any appreciable effect upon the much more stable capacity to cause hemagglutination.

In carrying out quantitative measurements of the physical characteristics of a virus, it is obviously essential to utilize the most precise and most accurate techniques available for the determination of the presence and the concentration of the agent. In the case of PVM the *in vitro* hemagglutination technique appears to be more precise and much more accurate for such determinations than the *in vivo* technique (6). Consequently, throughout this study calculations based on the results of *in vitro* titrations have been used. From the measurements which have been carried out with PVM so far, it appears that a reduction in, or complete loss of, the property of infectiousness is not necessarily accompanied by a significant alteration in the physical state of the virus particles themselves.

SUMMARY

A technique has been devised for obtaining free or uncombined pneumonia virus of mice (PVM). Free PVM, liberated from infected mouse lungs by means of this technique, is infectious and causes hemagglutination directly. The results of quantitative studies carried out in the high speed angle centrifuge indicate that the free virus is relatively small, with dimensions of the order of 40 millimicrons. When it is in combination with lung tissue particles, PVM appears to be a relatively large virus with minimal dimensions of the order of 140 millimicrons. Non-infectious virus particles, released from combination with lung tissue particles by heating, are similar to the infectious free virus in size.

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