ULTRACENTRIFUGATION STUDIES OF YELLOW FEVER VIRUS

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In recent years a number of reports have appeared giving the results of analytical studies on viruses by high speed centrifugation. In most of these studies two general procedures have been used. One of these is the so called inverted capillary method developed by Elford (1) from the original Bechhold and Schlesinger (2) technique. It employs an electrically driven centrifuge of commercial type and depends on correlating after each of a series of runs the activity of sampled material with the time of centrifugation. No preliminary concentration or purification of the virus is required.

The other procedure is an adaptation of the ultracentrifugation method developed by Svedberg (3) for the study of proteins. The original method utilizes the principle of recording photographically the sedimentation of the material while the centrifuge is actually in rotation. The light used is of a wave length which is absorbed considerably more by the solute than by the solvent. Serial photographs are taken while the material is being centrifuged in a transparent cell, the sedimentation being indicated by differences in the amount of light transmitted at various levels in the cell. The behavior of sedimenting material in the revolving cell has also been investigated by various refractive index methods which are based on the fact that light rays are deviated in passing through regions of graded refractive index such as exist at sedimenting boundaries. In general, the latter methods yield the more absolute and dependable measurements and are especially suitable for studying mixtures of proteins, the individual concentrations of which must be known. In any case, a certain minimum concentration is necessary for detection by an optical method, and even then it may be impractical to study a substance in the ultracentrifuge if its behavior is masked by that of other sedimenting components present in higher concentrations and having similar optical properties.

Most investigators using Svedberg's method for the study of viruses have considered it necessary first to purify and concentrate the infectious agent. To attain this, both the salting-out and the differential centrifugation procedures have been applied. For purification of yellow fever virus these methods are not practicable. When removed from associating animal protein by selective precipitation, this virus rapidly becomes inactivated, and there is no method available for identifying it in the inactive form. Another objection is suggested by the work of Smith and MacClement (4). These investigators found that some of the plant viruses, when removed from the plant juice, suffered an alteration in some

of their physical properties. Although a partial purification of yellow fever virus by differential centrifugation should be possible, here again inactivation occurs rapidly when the associating proteins are removed. Furthermore, since the difference in the sedimentation rate of the virus and some of the serum proteins is not very great, the necessary, repeated washings and centrifugations would involve a considerable length of time for such a labile material.

In comparison with other viruses, the causative agent of yellow fever appears in the circulating blood of infected monkeys in a relatively high concentration. It has been shown by Bauer (5) and by Davis, Frobisher, and Lloyd (6) that serum taken from monkeys at the height of fever is frequently infectious in dilutions of 10^{-9}. It seems certain that more than one virus particle is required to initiate infection, and therefore it may safely be assumed that the actual concentration of virus in the blood of infected animals is considerably higher than the titration values indicate. Since it was considered highly desirable first to investigate the virus without subjecting it to purification processes which might alter its physical properties, preliminary experiments were undertaken with a view to determining whether its sedimentation characteristics could be investigated directly in the natural environment of the virus, *i.e.*, the serum of infected monkeys. The results indicated that this was possible, and the studies reported below were carried out on the virus as it appears in the circulating blood of monkeys suffering from experimentally induced yellow fever.

Materials and Methods

Virus.—The Asibi strain of virus, being the most virulent for *rhesus* monkeys, was used throughout these studies. Monkeys were bled from the heart under ether anesthesia. In order to obtain clear serum with the least amount of fat, the animals were fasted for 12 hours before bleeding. New glassware was used throughout, as it was found that when blood was collected in centrifuge tubes that had become somewhat etched through prolonged use, there appeared in the serum an excessive amount of hemoglobin which interfered with the optical analysis of the material. As soon as the blood clotted, the serum was centrifuged first for 30 minutes at a speed of 2500 revolutions per minute, and then for 1 hour at 13,000 R.P.M. The clear supernatant obtained after the second centrifugation served as the virus suspension. This was distributed in 1 to 2 cc. amounts in small test tubes, frozen rapidly in a mixture of alcohol and solid CO_2, and then stored at −18°C. until used for ultracentrifugation studies. Just before use, the frozen serum was thawed rapidly by immersion in a water bath at 40°C. Although most of the studies were carried out with unaltered serum, some experiments were performed with specimens concentrated in the following manner: After the second centrifugation at 13,000 R.P.M. the clarified serum was centrifuged in the concentration vacuum centrifuge of Bauer and Pickels (7, 10) for 3 hours at a speed of 27,300 R.P.M. Then the supernatant fluid was drawn off carefully with a sampling device described by Hughes, Pickels, and Horsfall (8) until only the bottom 0.7 or 0.8 cc. of fluid remained in

each tube. The resuspension of the sediment in the residue of the supernatant fluid resulted in approximately a tenfold increase in the concentration of the virus as compared with the original material.

Ultracentrifuge.—The air-driven vacuum ultracentrifuge described by Bauer and Pickels (9) and by Pickels (10) was used in these studies. In addition to the optical absorption system of Svedberg and the refractive index scale method of Lamm (11), it was provided with an automatic refractive index system designed especially for the ultracentrifuge and utilizing a scanning arrangement similar to that described by Longsworth (12) for electrophoresis measurements. The speeds employed ranged from 30,600 to 46,800 R.P.M. In most of the experiments cells were used which accommodated a fluid column of 10 mm. thickness along the light path; a few runs were made with 3 mm. cells. Clean cells were always used excepting a few cases in which the same material was run a second time after standing.

Viscosities were measured with a small Ostwald viscometer requiring only 0.6 cc. of fluid. This had been checked against a larger and more accurate viscometer with water and with several typical sera. It was found that the temperature coefficient of viscosity for monkey serum at room temperature was approximately the same as that for water or salt solution. Therefore, it was considered justifiable, for the sake of comparison, to correct all sedimentation rates for the viscosity to a standard temperature of 25°C., especially since most of the runs were made near this value.

EXPERIMENTAL

The approximate size of the virus particles was known from the ultrafiltration studies of Bauer and Hughes (13). The behavior of the virus under varying conditions in the high speed concentration centrifuge had also been investigated (7, 8). From these diverse observations it was possible to predict its approximate sedimentation rate.

Since absorption studies with the ultraviolet light which is strongly absorbed by the serum proteins were out of the question, attempts were made with several unaltered infective sera to detect a rapidly sedimenting virus boundary by the use of Lamm's refractive index scale method.

The 365 mμ line of mercury, which is known to be transmitted fairly readily by serum proteins, was used for illumination. It was observed that in some of these runs a faint absorption boundary could be detected which sedimented at the approximate rate predicted for yellow fever virus. No trace of such a boundary was found with either normal or immune sera. Although there was in the infective sera this distinct differential absorption of the 365 mμ line by a group of rapidly settling particles, the refractive index gradients were apparently so small that the scale method did not prove adequate for the measurement of sedimentation rates. Therefore, the absorption method, using the 365 mμ line of the mercury arc, seemed to be by far the more sensitive of the two, and it was adopted instead of the scale method as a possible approach to our problem.

It was assumed that the rapidly settling boundaries were associated with virus, and, therefore, for the sake of brevity, they will be referred to hereafter as virus boundaries.

Experiments with Infected Monkey Serum.—A total of thirteen different specimens of unaltered infected monkey serum was studied. Two of these were pooled from several monkeys; the others were individual specimens, each from a different animal. Five individual specimens and one pool showed no visible trace of virus boundaries, probably because of insufficient concentration. Most of these sera giving negative results were taken at the onset of fever, and all were taken before the monkeys were moribund. The six individual specimens and one pool which did exhibit virus boundaries represented sera taken at the height of fever, while the animals were moribund, or at death.

Normal serum, as well as most of the specimens from infected monkeys which showed no virus boundary, absorbed less than 40 per cent of the 365 mμ light used, an exposure time of 2 seconds being sufficient for photography. On the other hand, the amount of light absorbed by the virus varied among the different experiments but was usually of the order of 25 per cent. In the specimens showing virus boundaries, varying amounts of some highly absorbing, slowly sedimenting particles, presumably serum proteins carrying bile pigment, made it necessary to increase the exposure time to as much as 1 minute in some instances. In addition to measurements made by the absorption method, photographs were taken with the automatic refractive index system, but in only one instance was the virus boundary sufficiently well defined by this method for an approximate measurement. The sedimentation rate coincided with that obtained by the absorption method. All refractive index measurements were made with the principal green and yellow lines of the mercury spectrum.

The sedimentation of yellow fever virus in unaltered serum from an infected monkey is illustrated by the serial photographs in Fig. 1. Near the meniscus of the fluid can be observed variations in the photographic density caused by the slow sedimentation of light-absorbing pigments and of the non-absorbing serum proteins, the high concentrations of which cause such extreme refractive index gradients at their boundaries that the light is deviated too much to pass through the photographic lens. Fig. 2 shows tracings of the microphotometer curves obtained from the alternate photographs of Fig. 1. As illustrated here, all the boundaries showed a considerable spread, only a small proportion of which could possibly be explained on the basis of diffusion alone. A lowering of concentration below the boundary, of about the magnitude indicated in the illustration, is to be expected in any normal sedimentation because of the non-uniform centrifugal field of force, as has been shown by Svedberg and Rinde (14). To a first order approximation, all of the eight observed boundaries were consistent with respect to the amount of spread and the rate of sedimentation. While it was impossible to differentiate completely that portion of the spread due to varying sedimentation rates from that due to diffusion and minor spurious effects, it was estimated that as an average representation of the results, practically all of the material associated with the rapid boundary in infected monkey serum having a viscosity of 14 millipoises

sedimented at rates lying between approximately 18 and 30 $\times$ 10^{-13} cm./sec./dyne, with an average rate of approximately 23 $\times$ 10^{-13} cm./sec./dyne.

The average rate of the virus boundary was well removed from that of any other serum protein, being a little more than twice that of the most rapid and least concentrated component observed in normal, infected, and immune sera. Minor distortions in some of the microphotometer curves, especially those corresponding to the first stages of sedimentation, complicated the measurements to a small degree. These may have been the result of refraction or absorption disturbances associated with the other components of the sera, or they may have been caused by an aggregation phenomenon akin to that observed by Pickels and Smadel with the virus of vaccinia (15).

There was only one instance in which the microphotometer curves indicated a distinct and pronounced departure in the behavior of the material from the average. This occurred with the single sample which was prepared by pooling several infective sera. The light absorption decreased very rapidly and unevenly through the cell during the centrifugation of this specimen. The concentration of material in the virus boundary was unusually low. The average rate was estimated to be approximately normal, but the determination of maximum and minimum rates was impossible. In consideration of the very high light absorption of the pigments incorporated in the medium, it appeared that the abnormal character of the microphotometer tracings was due mainly to an increased precipitation of the pigment-carrying proteins, although the virus or other associated substances (precipitinogen) may have been partly responsible.

Experiments with Virus Concentrated in Serum.—Seven ultracentrifugation experiments were made with virus concentrated in the native serum as already described. Two of these were with individual specimens from monkeys. The others were concentrated samples of the same pool mentioned above.

The viscosity values of the concentrated preparations were considerably higher than those of the unaltered material because of an increase in the protein content occasioned by the concentration process. The increased concentration of the virus as observed in the ultracentrifuge is readily apparent from the typical photographic record shown in Fig. 3.

The behavior of the boundaries in the concentrated preparations was fairly consistent with regard to the regularity of movement, the amount of spread, and the corrected rate of sedimentation. As an average representation of the results from these runs, the sedimentation rates of the particles constituting the single rapid boundary in concentrated serum having a viscosity of 26 millipoises ranged from approximately 10 to 16 $\times$ 10^{-13} cm./sec./dyne, with a mean value of about 13 $\times$ 10^{-13} cm./sec./dyne. The successive displacement of the boundary bore the same general rela-

tionship to that of the heavy globulin component as was observed with unaltered sera.

During five of these runs photographs were taken with the automatic refractive index system, and in every instance there was observed a measurable boundary which corresponded to that obtained by the absorption

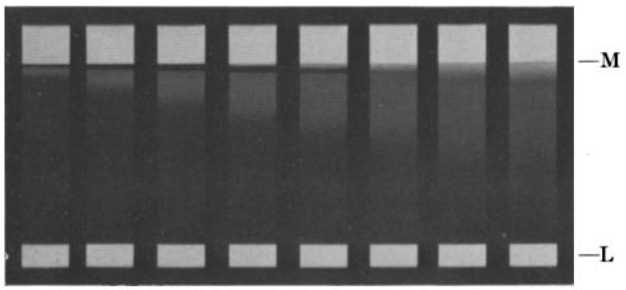

FIG. 1. Sedimentation of blurred boundary associated with yellow fever virus in unaltered serum from infected monkey; speed, 30,600 R.P.M.; mean centrifugal force, 68,000 gravity; interval between photographs, 10 minutes; exposure time, 10 seconds; illumination, 365 mμ line of mercury arc. *M* indicates the meniscus of the fluid and *L* the light intensity standard. Irregularities near the meniscus are caused by sedimentation of serum proteins.

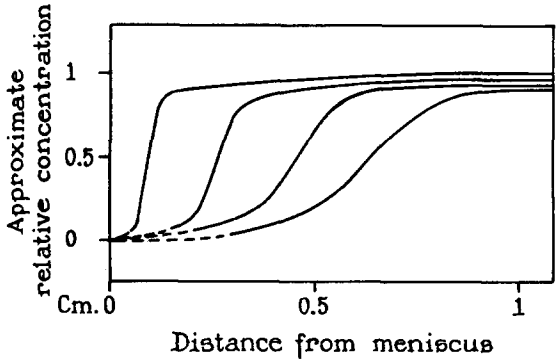

FIG. 2. Curves obtained from microphotometer tracings of the alternate photographs in Fig. 1. The decreasing of the concentration below the boundary is caused by the radial variation in the centrifugal force.

method. Although the virus material was present in obviously small amounts, it was possible, by measuring the area under its refractive index curve, to estimate the value of its refractive index increment as approximately 10^{-4}.

Fig. 4 illustrates one of three instances in which multiple boundaries

were observed in place of the usual single blurred boundary. Both the pooled preparation and an individual specimen exhibited the phenomenon. Four boundaries could be differentiated in two instances, three in the other. The combined spread of the several boundaries was approximately the same as that of the usual single boundary. The individual spread of each of the multiple boundaries was just about what would be expected for a single substance on the basis of diffusion alone. The rapidly sedimenting material was more or less equally distributed among the several boundaries. The boundaries in the concentrated material (viscosity of 26 millipoises) had sedimentation rates of approximately 9.9, 12.5, 14.2, and 16 $\times$ 10^{-13} cm./sec./dyne.

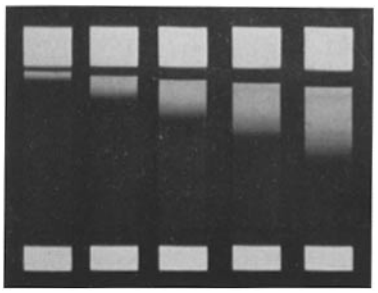

FIG. 3

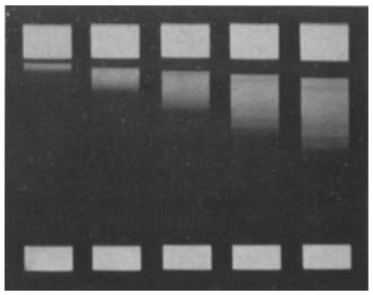

FIG. 4

FIG. 3. Sedimentation of boundary associated with yellow fever virus after a tenfold concentration of the particles in serum by preliminary centrifugation; speed, 46,800 R.P.M.; interval between photographs, 10 minutes; exposure time, 2 minutes.

FIG. 4. Multiple boundaries which sometimes replace the single blurred boundary associated with yellow fever virus. These apparently represent aggregations or associations which when less stable account for most of the boundary spread.

Relation of the Rapidly Moving Boundary to Virus Activity.—Although every consideration indicated that the rapidly moving boundaries were intimately associated with the virus activity, it was considered desirable to establish more stringent correlations if possible. Accordingly, samples of normal and immune sera were carried through the same concentration procedure as followed with the infective specimens. Ultracentrifugation of these concentrated specimens showed no boundary having a rate near that observed with infective serum.

Attempts were made by various methods to sample off material at several levels in the vicinity of the boundary after centrifugation and to demonstrate differences in their biological activities which would correlate with the observed differences in light absorption. Although infectivity experi-

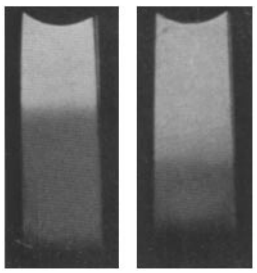

FIG. 5. Immobilized boundaries associated with yellow fever virus. The photographs were taken with stationary cells which had been carefully removed from the ultracentrifuge after a partial sedimentation of the virus.

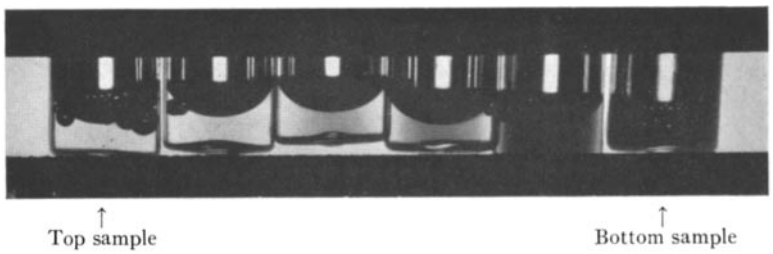

FIG. 6. Samples taken at successive levels from stationary ultracentrifuge cell containing immobilized boundary associated with yellow fever virus. Differences in concentration are indicated by the differential absorption of the 365 mμ wave length used for illumination.

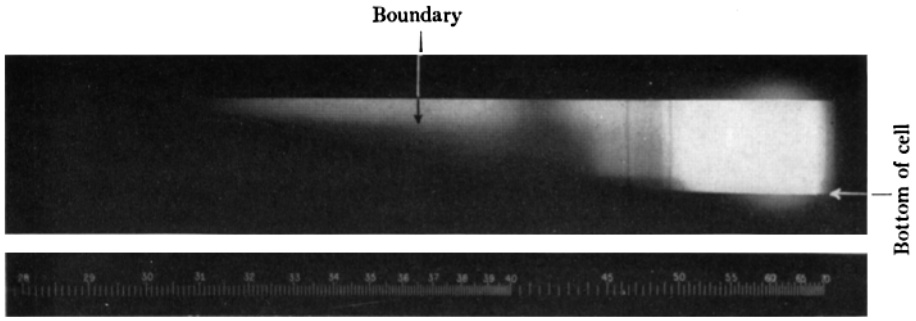

FIG. 7. Spectrum obtained, using hydrogen arc as distant light source, with ultracentrifuge cell placed directly in front of spectrograph slit. Differential absorption between the immobilized boundary associated with yellow fever virus and the other serum components above the boundary can be seen in the 320–400 mμ region.

ments were carried out with material centrifuged in the concentration centrifuge, as well as in the analytical apparatus, the latter proved the more satisfactory.

It was found that by carefully removing the rotor of the ultracentrifuge after a run and then slowly tilting it until the fluid column in the cell was vertical, the boundary could be restored with very little remixing to the position it had just before centrifugation ceased. In Fig. 5 the two photographs of stationary cells, taken with the 365 mμ line of mercury and showing boundaries of concentrated virus migrated to different depths, illustrate the possibilities of this technique. There remained the problem of removing samples of fluid from the different levels of the cell with the least possible remixing of the particles believed to be virus. Difficulty was experienced with various methods tried, mainly because of the small size and consequent capillary attraction of both the cell reservoir and the smaller hole through which it was filled. However, success was finally gained with a rather simple apparatus. A blunted needle attached to a ¼ cc. syringe filled with mercury was carefully lowered through the centrifuged serum until its end almost touched the bottom of the cell reservoir. By means of a mechanical movement obtained with the aid of a slow clock motor, the mercury was gradually pushed from the syringe, and as the displaced fluid collected in the form of a globule about the needle outside the cell hole, it was drawn off in equal amounts with another syringe.

A specimen of concentrated virus suspension which had given sedimentation pictures similar to those of Fig. 3 was sampled as just described. The six 0.1 cc. samples were placed in small glass tubes and then photographed with the 365 mμ line of mercury. The pictures are shown in Fig. 6. The great difference in the concentration of light-absorbing material in the two bottom samples and the two top samples is readily seen. The biological activity of each sample was tested, and an approximate correlation was obtained between the virus activity and the absorption of the 365 mμ line of mercury.

Spectral Characteristics of Yellow Fever Virus.—Since the light absorption characteristics of the virus were obviously not the same as those of the associated proteins in the spectral region used for ultracentrifugation, it was considered of interest to compare their properties in other portions of the spectrum.

With a sedimentation boundary of concentrated virus immobilized, the centrifuge cell was placed directly before and against the slit of a quartz spectrograph. Arrangements were such that the light rays reaching the slit of the spectrograph were almost parallel and had passed through only that section of the cell below the top of the fluid column. A hydrogen discharge tube giving a fairly continuous spectrum was used as light source.

As illustrated by the photograph in Fig. 7, the immobilized boundary can be seen in some regions of the spectrum but not in others. The virus shows a strong absorption throughout the upper ultraviolet region and up to at least 440 mμ in the visible. Its absorption characteristics below

320 mμ could not be studied because of the extreme masking effect of the considerably more concentrated serum proteins which began to exhibit absorption throughout the depth of the cell. The unsedimented pigments, whose behavior is differentiated above the virus boundary, are seen to have an absorption band apparently not shown by the virus at about 410 mμ. The maximum optical contrast, for a short exposure, shown by the virus boundary occurs very close to the 365 mμ region already selected for photographing the sedimentation boundaries of the virus in the ultracentrifuge.

Ultracentrifugation Experiments with Virus Mixed with Normal and Immune Serum.— Two of the individual specimens of unaltered serum which had been taken from infected monkeys and which had shown virus boundaries in the ultracentrifuge were subjected to the concentration procedure described above. A 1 cc. sample of each of these concentrated specimens was mixed with an equal amount of an untreated pool of immune serum. A portion of each mixture was studied in the ultracentrifuge immediately after mixing, and another portion was centrifuged after the mixture had been allowed to stand at room temperature for 4 hours. Omitting the repeat runs, the same procedure was followed using mixtures of the same concentrated virus material with a pool of normal serum.

In none of these runs did the observed average sedimentation rate show any deviation from the others which was marked enough to be considered of significance. The corrected rates were close to 19×10^{-13} cm./sec./dyne, which is just about what would be expected, unless the size of the particles had been radically altered, in view of the intermediate viscosities of the mixtures. These were not measured but must have been near 16 millipoises. As a whole, the investigation of the mixtures was complicated by the same kind of irregularities in the light absorption which characterized the single unaltered infective pool already studied. These were apparently again the result of a pronounced and sometimes most erratic precipitation of either some of the virus particles, or some of the pigment-carrying proteins, or both. A disturbance in the equilibrium of the complex system seemed to be introduced by the centrifugation itself in some cases. Nevertheless, the identity of the rapid boundary was never rendered uncertain and its rate could be approximated.

DISCUSSION

The results of these studies allow certain conclusions in spite of the inherent limitations in investigating a small quantity of material suspended in such a complex, highly concentrated, highly viscous, and optically variable medium as undiluted serum, which itself suffers changes of state

during centrifugation. There can be little doubt but that the principal particles constituting the sedimentation boundary described above represented a specific, discrete, biological entity. Even after being concentrated as a sediment at the bottom of a centrifuge tube, these particles could readily be resuspended in the supernatant fluid and were found to possess the same original physical characteristics as revealed by their behavior in the ultracentrifuge. It has been shown that most of the yellow fever virus activity sedimented along with the rapidly moving boundary observed in these studies. Whether the bulk of this material consisted of yellow fever virus particles we were not able to determine because of inherent technical difficulties. However, it would have been a strange coincidence if there had occurred in the serum of monkeys only during the course of infection a new component having approximately the same sedimentation rate as the virus. Every consideration indicated that the material could not have been the precipitinogen described by Hughes (16). A number of the concentrated virus specimens were tested against precipitating antiserum with entirely negative results. Furthermore, this precipitinogen is known to be an albumin, whereas the particles under consideration sedimented more than twice as fast as the heaviest globulin molecules in normal or infected serum.

It is well known that yellow fever virus in a susceptible host such as the monkey multiplies at a very rapid rate and that soon after the infection is established, antibodies are produced with even greater rapidity. As the disease progresses the virus is eventually overwhelmed by antibodies to such an extent that if the course of the infection is considerably prolonged, the recovery of unneutralized virus from the circulating blood becomes impossible, even though the disease itself may terminate fatally (Bauer, 5; Berry and Kitchen, 17). In human cases it is practically impossible to recover virus at death following even a very short type of infection. Just how long the neutralized virus particles remain in the circulating blood is not known. If their elimination is less rapid than the multiplication of the virus itself, then it may not be entirely illogical to assume that at least some, if not the majority, of the rapidly sedimenting particles in the infected monkey serum consist of neutralized or inactive virus. This material was present in greater concentration in the serum taken from monkeys just before death following a short and fulminating type of infection than in the specimens obtained at the onset of the disease. The addition of immune serum to the infectious material altered neither the sedimentation rate nor the optical properties of these particles. These facts seem to lend further support to the hypothesis that this material consisted of a

mixture of neutralized and active virus particles. The extremely high light absorption shown by this material in the 320 to 440 mμ spectral range is evidently an intrinsic property of the virus and probably is not affected appreciably by the neutralization process.

The spread of the sedimentation boundary was considerably greater than could be accounted for on the basis of diffusion alone. This spread in all probability resulted from varying degrees of association or aggregation of the virus particles with either themselves or some other substances such as antibodies. The latter may have acted as bonding agents between two or more virus particles, or they themselves may have been adsorbed in varying numbers on individual virus particles. The progression of such combinations into more stable forms would explain the observation that with the concentrated virus preparations there sometimes appeared in place of the usual widely spread boundary several well defined lesser boundaries, each being characteristic of a homogeneous system, and together covering about the same range of sedimentation rates as the single blurred boundary more generally obtained. Similar multiple boundaries obtained with the elementary bodies of vaccinia have been described by Pickels and Smadel (15). In the present instance, any logical explanation of the varied experimental observations would require that the rate of the primary virus particle and that of the slowest of the multiple boundaries be not greatly different. It may be concluded with reasonable assurance that the individual virus particles had a sedimentation rate in unaltered infected monkey serum of approximately 18×10^{-13} cm./sec./dyne, which was that of the least rapidly moving particles constituting the widely spread boundary.

To compute the sedimentation constant by correcting the observed rate to the standard conditions of water at 20°C. and to estimate the particle size, it is necessary to know the density of the particles and the effective viscosity and density of the medium. The unaltered sera used had specific gravities near 1.03. It is probable that the effective viscosity of the serum, *i.e.*, the viscosity as related to the resistance offered to the movement of the virus, was slightly less than the measured viscosity because of the simultaneous sedimentation of the serum proteins. However, the difference could not have been much since the average sedimentation rate of the virus and that of most of the protein were far apart. Furthermore, this conclusion also follows from a comparison of the rates in media of different protein concentration, *i.e.*, in unaltered sera, concentrated sera, and mixtures of the two.

Assuming a density value equal to that of most proteins, *i.e.*, 1.33 gm.

per cc., the particles constituting the rapid boundary had sedimentation constants ranging from approximately 27 to 45 $\times$ 10^{-13} cm./sec./dyne, the smaller value in all probability being very near that of the individual virus particle of yellow fever. Considering the infective unit to be spherical in shape, the particle diameter is computed from Stokes' law to be about 12 mμ. An assumed density value of 1.15 gm. per cc. would yield a minimum constant near 31 $\times$ 10^{-13} cm./sec./dyne and a particle diameter of approximately 19 mμ; a density of 1.50 gm. per cc. would give the minimum constant as approximately 26 $\times$ 10^{-13} cm./sec./dyne and the diameter as about 10 mμ. If the particles are not spherical, as is probably the case, but more nearly rod-shaped or perhaps plate-like, their minimum thickness or the diameter of their minor axis must be less than the values just given, and their length must be greater.

Ultrafiltration measurements of yellow fever virus by Bauer and Hughes (13) showed that the virus passed a membrane having a pore diameter of 50 mμ, but was held back by a 45 mμ membrane. According to Elford's formula (18) as applied to ultrafiltration measurements, the diameter of the virus particles is to be considered equal to one-third to one-half of the largest retaining pores, which would place the diameter of the yellow fever virus particle between 15 and 22 mμ. The agreement of the ultracentrifugation results are, therefore, considered satisfactory, since they indicate a probable diameter between 12 and 19 mμ on the assumption that the particles are spherical. The assumption of a less symmetrical shape would yield an even better agreement.

From the total refractive index increment of the particles constituting the rapid boundary in concentrated serum, their concentration was computed to be about 0.05 per cent, on the assumption that their specific refractive index increment was about the same as that of the serum proteins, *i.e.*, 0.002 at the higher wave lengths. It must be of this order of magnitude. In 1 cc. of the unaltered serum there was, therefore, approximately 0.00005 gm. of these large particles. If, for the purposes of approximation, the diameter of the individual virus particle is taken as 19 $\times$ 10^{-7} cm. and its density as 1.15 gm. per cc., its mass is computed to be 4.1 $\times$ 10^{-18} gm. Therefore, in the unaltered infective serum there were of the order of 10^{13} virus particles per cc. The concentration of virus activity was not determined in the specimens used in this study, but if for purposes of computation an activity titer of 10^{-9} is taken as the limiting value of the end point, each minimal infective dose would have contained approximately 10,000 particles. As concluded before from other considerations, the majority of these virus particles were probably already in the inactive state.

In a 1 cm. thickness of fluid, the small amount of virus present in unaltered infective serum absorbed about as much light (approximately 25 per cent) in the 365 mμ region of the spectrum as did all the normal serum proteins present in a combined concentration some 1000 times as great.

SUMMARY

1. It was possible to study in the ultracentrifuge by optical methods the behavior of yellow fever virus particles directly in the unaltered serum from infected monkeys.

2. The virus showed an extremely high light absorption in the spectral range of 320 to 440 mμ, which seemed to be its intrinsic property. In a 1 cm. thickness of fluid, the small amount of virus present in unaltered infective serum absorbed about as much light (approximately 25 per cent) in the middle of this range as did all the normal serum proteins present in a combined concentration some 1000 times as great.

3. The concentration of virus in the unaltered serum was found to be of the order of 0.00005 gm. per cc. 1 cc. of a 10^{-9} dilution, which, as has been shown, may constitute a minimal infective dose for monkeys, would contain approximately 10,000 virus particles. The probability that most of the virus particles were in the inactive form is discussed.

4. In infective serum having a viscosity of 14 millipoises, the particles sediment with a blurred boundary at rates lying between approximately 18 and 30 $\times$ 10^{-13} cm./sec./dyne. Evidence indicates that this spread is the result of an aggregation or association phenomenon.

5. Computations of size are in approximate agreement with those made from ultrafiltration studies. On the assumption that the density of the virus particle is near that of protein, its volume is computed to be at least that of a spherical particle having a diameter of 12 mμ. An assumed density of 1.15 gm. per cc. yields a diameter of 19 mμ, considering the shape as spherical.

BIBLIOGRAPHY

1. Elford, W. J., *Brit. J. Exp. Path.*, 1936, **17**, 399.
2. Bechhold, H., and Schlesinger, M., *Biochem. Z.*, 1931, **236**, 387.
3. Svedberg, T., *Naturwissenschaften*, 1934, **22**, 225.
4. Smith, K. M., and MacClement, W. D., *Proc. Roy. Soc. London, Series B*, 1938, **125**, 295.
5. Bauer, J. H., *Am. J. Trop. Med.*, 1931, **11**, 259.
6. Davis, N. C., Frobisher, M., and Lloyd, W., *J. Exp. Med.*, 1933, **58**, 211.
7. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, **64**, 503.
8. Hughes, T. P., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1938, **67**, 941.

9. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1937, **65,** 565.
10. Pickels, E. G., *Rev. Scient. Instr.*, 1938, **9,** 358.
11. Lamm, O., *Nature*, 1933, **132,** 820.
12. Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61,** 529.
13. Bauer, J. H., and Hughes, T. P., *Am. J. Hyg.*, 1935, **21,** 101.
14. Svedberg, T., and Rinde, H., *J. Am. Chem. Soc.*, 1924, **46,** 2677.
15. Pickels, E. G., and Smadel, J. E., *J. Exp. Med.*, 1938, **68,** 583.
16. Hughes, T. P., *J. Imunol.*, 1933, **25,** 275.
17. Berry, G. P., and Kitchen, S. F., *Am. J. Trop. Med.*, 1931, **11,** 365.
18. Elford, W. J., *Tr. Faraday Soc.*, 1937, **33,** 1094.