VIRUS OF AVIAN ERYTHROMYELOBLASTIC LEUKOSIS

IV. SEDIMENTATION, DENSITY AND HYDRATION*

by

D. G. SHARP AND J. W. BEARD

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

A particulate component has been demonstrated[1,2] in the plasma of chicks diseased with avian erythromyeloblastosis[3], and a close correlation has been found[4] between the particles, counted by sedimentation and electron micrography, and the infectious properties of the plasmas. These plasmas possess, likewise, the capacity to dephosphorylate[5] adenosine triphosphate, an enzymic activity which, also, is related to the particles[6,7]. From these results it has been judged that the particles constitute the etiological agent of the disease and that enzymic activity is a property of the virus. Some information about the physical characters of the virus has been obtained from electron pictures[2] and in studies involving sedimentation[4,7] of the agent in the ultracentrifuge and by electrophoresis[4,7]. In the present work, further investigation has been made of other physical properties of the material. These have included an examination of the sedimenting behaviour of the virus in partly purified preparations and directly in plasma in which the agent reaches amounts sufficiently great for such studies. Determinations have been made, also, of the hydrated density of the virus by sedimentation from bovine serum albumin solution[8] and of dry density, through use of D_2O[9]. The results of these studies and their correlation with findings previously reported are described in the present report.

MATERIALS AND METHODS

Sedimentation studies were made on virus directly in plasma and on virus concentrated by ultracentrifugation of plasma. Each experiment of the present work was made on the plasma from a single bird. Blood was obtained from young White Leghorn chicks inoculated[10] with previous passage virus at 3 days of age. The donors for the experiments were chosen for high plasma activity to dephosphorylate adenosine triphosphate as determined by the micro screening test[11]. Bleeding was effected by section of the saggital sinus, and the blood was collected in chilled, pointed 15-ml tubes with heparin as anticoagulant. The cells were sedimented at 2,000 $\times$ g for 15 minutes, and the plasma was pipetted off and centrifuged again at 3,715 $\times$ g for 20 minutes.

To obtain concentrated virus, plasma, generally not more than 3 ml volume from the small birds, was diluted with 0.85 % NaCl solution to fill a 5-ml lusteroid tube, and the virus was sedimented

* This work was supported by a Research Grant to Duke University from the American Cancer Society, on recommendation of the Committee on Growth; by a grant from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service; by the Dorothy Beard Research Fund; and by a gift to Duke University from Lederle Laboratories Division, American Cyanamid Company.

References p. 17.

by spinning at about 17,000 × *g* for 30 minutes. The supernatant plasma was poured and drained off, after which the interior of the tube was wiped dry, except that part occupied by the pellet. The pellet was then taken up, by pipetting, with 0.85 % NaCl solution.

In those experiments designed to determine wet and dry density of the virus, by methods previously described[8,9], the plasma from one bird was divided equally between two 5-ml lusteroid tubes, and saline was added to fill the tubes. The virus was sedimented as described above, and 0.5 ml of 0.85 % NaCl in H_2O was placed on one pellet, and one ml of 0.85 % NaCl in D_2O was placed on the other. After resuspension of the virus in these volumes, a small amount of the H_2O sample was analyzed for determination of sedimentation velocity (after addition of an equal volume of 0.85 % NaCl solution). The remainder was transferred to a dry-weighed glass tube, weighed, and an approximately equal volume of bovine serum albumin solution was pipetted in and weighed. The concentration of the albumin solution had been previously adjusted to produce a density in the mixture of about 1.03. The actual density of each mixture was calculated from the volumes of virus suspension and albumin solution as weighed, not pipetted. Sedimentation studies of this mixture and of the D_2O sample completed the series of three measurements made on each single plasma.

Sedimentation velocity measurements were made in a rotor of mean cell radius 6.5 cm. The cell height was 12 mm in the radial direction. Fields of centrifugal acceleration ranging from 5,700 × *g* to 16,700 × *g* were used, depending upon the viscosity and density of the fluid through which the virus particles were sedimenting. The time of sedimentation and photography of the boundaries was about 20 minutes. All runs were made at room temperature with careful adjustment of previously cold samples to room (rotor) temperature before starting. Boundary positions were observed and photographed on panchromatic film by means of simple Schlieren bands, using a line-filament tungsten lamp without light filters.

The density of the bovine serum albumin solutions was measured with a 2 ml pyknometer, and viscosity measurements were made with an Ostwald-type viscometer of water time 129.2 seconds at 29.3° C. The plasma samples used for direct analysis of sedimenting boundaries were never of sufficient volume to fill the viscometer; consequently, viscosity and density data were calculated from measurements made on diluted samples.

RESULTS

The virus content of an occasional plasma is great enough to register a Schlieren band in the ultracentrifuge without concentration of the virus. Three of these infrequently occurring plasmas were found by the micro enzyme test previously described[11]. One of them, X 44, Table I, gave sedimenting boundaries which, although not prominent nor of optimum contrast for photography, were, nevertheless, quite adequate for measurement (Fig. 1a) even after dilution. The individual pictures were taken at 3 minute intervals while the plasma was held in a mean centrifugal field of 7,488 × *g* at 25.5° C. The calculated values of viscosity and those for density of the plasmas, obtained with diluted specimens, are given in Table I. In order to extrapolate from the viscosity data to the relative viscosity of undiluted plasma, it was assumed that the variation in viscosity with dilution of leukotic plasma would be the same as that of normal plasma. Calculations of the sedimentation rates of the virus in the various plasmas, which are shown, also, in Table I, make use of the data on the density of the virus determined in the next experiment.

TABLE I

SEDIMENTATION RATES OF THE VIRUS PARTICLES DETERMINED DIRECTLY IN DIFFERENT PLASMAS OF DISEASED CHICKS

Plasma	*Plasma dilution factor*	*Plasma density*	*Relative viscosity*	$\eta s \cdot 10^{15}$
× 77	None	1.018	1.67	504
× 117	None	1.015	1.50	496
× 44	None	1.018	1.70	532
× 44	1.642	1.012	1.43	603

References p. 17.

Density measurements were made by observing the rates of sedimentation of virus particles in 0.85% NaCl solution in H_2O; in 0.85% NaCl solution in D_2O; and in 0.85%

Fig. 1. (a) Schlieren diagram of virus particles sedimenting in dilute plasma (see text); (b) diagram of particles sedimenting in bovine serum albumin solution; and (c) diagram of particles sedimenting in D_2O solution.

NaCl solution in H_2O containing bovine serum albumin. The studies in the H_2O- and D_2O-saline were made on 6 preparations of concentrated virus from different individual plasmas, and those with bovine serum albumin employed the virus from 5 of these plasmas. The type of boundary observed and the relative rates of sedimentation in H_2O and D_2O are illustrated in the diagrams of Figs. 1b and 1c. The virus particles were well dispersed in both media as shown by the uniformity of light scattered in the portion of the photographs below the sedimenting boundaries. The observed sedimentation rates, multiplied by the absolute viscosity of the suspending media, are shown plotted against the density of the media in Fig. 2. The line drawn by inspection through the data obtained with H_2O-saline and with bovine albumin indicates a limiting hydrated density of 1.059 for the virus. Similar treatment of the data for D_2O-saline requires some estimate of the expected error in the density calculated for the virus because of the nature of the intersection of the extended line with the zero axis and the variability of the observed data. A straight line, calculated by the method of least squares, extends to an intercept at zero sedimentation rate at density 1.29 with a calculated

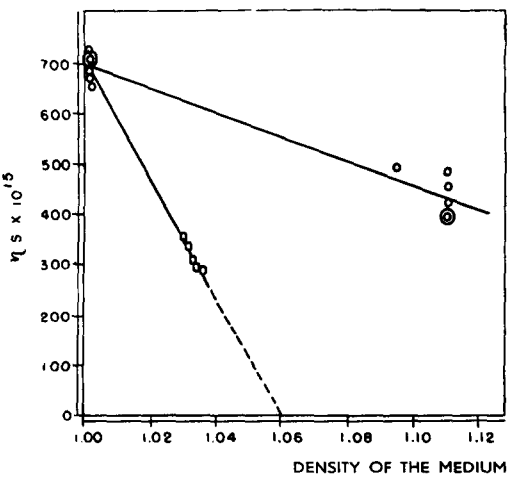

Fig. 2. Sedimentation rates of virus particles in bovine serum albumin solutions (squares) and in D_2O solution (circles). Each point represents the rate with a different virus preparation. The line through the circles was drawn by the method of least squares and that through the squares was drawn by inspection.

References p. 17.

standard deviation of the mean of + 0.030 or — 0.025. The corresponding values of the reciprocal are 0.772 + 0.014 or — 0.019.

Calculations of sedimentation constants have been made from the data obtained with the 6 specimens of virus studied in H_2O-saline solution with the usual viscosity and buoyancy corrections employing the value 0.772 for partial specific volume. The mean sedimentation constant so calculated is 693*s*. The latest electron micrographs give reason to believe that these particles are essentially spherical in plasma and in physiological salt solutions, and from this and the above values it is possible to calculate a diameter of 144 mμ for the hydrated particles.

If the density values derived from the data of Fig. 2 are taken to be the true hydrated and dry densities of the virus particle, it is possible to make calculations of the water content of the particle. This procedure gives values of 80% water by volume or 75% by weight.

In previous work, mentioned above[4], studies were made to determine the length of time necessary for the removal by centrifugation at 169.1 R.P.S. of the particles, of enzymic activity and of the infectivity from plasma in the upper portion of a lucite rotor cell. Similar experiments have shown that the particles of Dow latex 580 G, which have a sedimentation constant of 1940*s*, sediment from this same cell region in 68.8 minutes at 73.7 R.P.S. Calculations utilizing the data obtained with the latex particles and with those observed earlier in the centrifugation of plasma and the present density value, 1.059, gave sedimentation constants of 645*s*, 597*s* and 700*s* for the particles, enzymic activity and infectivity, respectively.

Electron micrographs made from virus pseudoreplicas from an agar surface[2] have shown the particles to be round, and shadowcast pictures show them to be somewhat flattened, even after osmic acid fixation[2]. The diameters of these images vary from preparation to preparation of the same virus sample, ranging from 100 to 120 mμ. Virus particles similarly prepared for micrographs, except without osmic acid fixation, appear excessively flattened but not substantially different in diameter.

DISCUSSION

The data obtained in the present work serve to confirm and to amplify the knowledge of the physical properties of the virus particles occurring in the plasma of birds with erythromyeloblastic leukosis. Occurrence of the particles in high concentration in some of the plasmas has afforded an unusual opportunity for observation of boundaries of virus particles sedimenting directly in the natural suspending medium without prior concentration. The sedimentation rates of the particles in different plasmas were fairly uniform, considering the lack of complete control of conditions, but they were somewhat less than those, average 693*s*, observed with concentrated and purified virus. The reason for this is not apparent, though it may be surmised that unknown factors in undiluted plasma related to viscosity and osmotic effects may influence the outcome under the considerably different sets of experimental conditions. There seems no reason to doubt that the boundaries observed under the various conditions are those of the same material.

Of particular interest is the exceedingly low value, 1.059, of the hydrated density of the leukosis particles. It has been evident from the beginning that these particles are vastly different in physical properties from other viruses studied, except the Newcastle

References p. 17.

disease agent. The early electron micrographs of the particles[2] revealed a confusing pleomorphism varing from spherical bodies to filamentous and sperm-like shapes. Assuming that the apparent variation in particle size and shape was real, it was not possible to understand the relatively high degree of uniformity of the sedimentation boundaries of the concentrated and partly purified material seen in the analytical ultracentrifuge. It was soon suspected and later verified[2] that the strong effects of high salt concentration at the time of drying of the particles on the collodion film for electron microscopy was responsible for the irregular shapes. The very low density provides one explanation of the phenomenon. A particle containing 80% water is presumably capable of appreciable changes in size and shape during these conditions of desiccation. It might be predicted, also, that the virus of Newcastle disease in chickens, which has a similar appearance in the electron microscope[2,12] may be another agent in this exceedingly low density range. Data on the Newcastle disease virus from this[2] and other laboratories[13] would suggest this conclusion.

A vital problem in the study of the leukosis virus is the interrelationship of the particles, enzymatic activity and infectivity occurring simultaneously and proportionally in the plasmas of the diseased birds[4]. It is of interest that the sedimentation constant, 693*s*, of the particles calculated from the sedimenting boundaries of the concentrated materials is close to the values, 645*s*, 597*s* and 700*s* which were calculated with the present data together with those determined in other experiments[4] by particle counts and chemical and biological estimates for the sedimentation constants of the particles, the enzymatic activity and the infectious unit. The present experiments serve to correlate the particles demonstrated by visible sedimenting boundaries with the particles and activities previously identified by entirely different procedures.

SUMMARY

Studies have been made on the sedimentation properties and density of the virus of avian erythromyeloblastic leukosis. The virus occurs in some plasmas from diseased birds in concentrations permitting sedimentation studies of the particles directly in the plasma without prior concentration. Schlieren diagrams obtained by this means revealed well defined though somewhat diffuse sedimenting boundaries. The sedimentation rates observed under these not entirely controlled conditions varied from 496 to 603 *s*. Measurements on concentrated virus made under standard conditions give the average value of 693 *s*. The hydrated density of the virus determined by sedimentation in bovine serum albumin was 1.059, and that of the dry density found in analogous experiments with D_2O was 1.29. Calculation of water content with these data give the values of 80% by volume or 75% by weight. Particle diameter based on the sedimentation data was 144 mμ compared with 100 to 120 mμ found by electron micrography.

RÉSUMÉ

Les auteurs ont étudié les propriétés de sédimentation et la densité du virus de la leucosis érythromyéloblastique aviaire. La concentration du virus dans le plasma de certains oiseaux atteints est suffisante pour permettre l'étude directe sur le plasma de la sédimentation des particules sans concentration préliminaire. Des diagrammes de dispersion obtenus par ce moyen montrent des frontières de sédimentation bien définies quoique légèrement diffuses. Les vitesses de sédimentation dans ces conditions, qui ne sont pas toutes contrôlées, varient entre 496 et 603 *s*. Les mesures effectuées dans des conditions standard après concentration donnent une valeur moyenne de 693 *s*. La densité du virus hydraté, déterminée par sédimentation dans la sérumalbumine de boeuf, est de 1.059 et la densité à l'état sec, déterminée par des essais analogues en présence de D_2O est de 1.29. Ces données correspondent à une teneur en eau de 80% en volume ou de 75% en poids. Le diamètre des particules, déduit des résultats de la sédimentation, est de 144 mμ. La microscopie électronique indique un diamètre de 100 à 120 mμ.

References p. 17.

ZUSAMMENFASSUNG

Es wurden Untersuchungen der Sedimentationseigenschaften und der Dichte des Virus der Geflügel-Erythromyeloblastenleukämie durchgeführt. Das Virus kommt im Plasma gestorbener Vögel in Konzentrationen vor, die die Sedimentationsuntersuchungen der Teilchen im Plasma ohne vorherige Konzentration erlaubt. Unter diesen Umständen erhaltene Schlierendiagramme ergaben wohl definierte, jedoch etwas diffuse Sedimentationgrenzen. Die unter diesen nicht völlig kontrollierbaren Bedingungen erhaltenen Sedimentationsgeschwindigkeiten variieren von 496 bis 603 *s*. Messungen des konzentrierten Virus, die unter Standardbedingungen durchgeführt wurden, ergaben einen durchschnittlichen Wert von 693 *s*. Die durch Sedimentation in Rinderserumalbumin bestimmte, hydratisierte Dichte des Virus betrug 1.059 und die in analogen Experimenten mit D_2O erhaltene "trockene" Dichte 1.29. Die Berechnung des Wassergehaltes mit diesen Daten ergab Werte von 80 Volumprozent oder 75 Gewichtsprozent. Der aus den Sedimentationsdaten abgeleitete Teilchendurchmesser betrug 144 mμ, während durch Elektronenphotographie 100–120 mμ gefunden wurde.

REFERENCES

1 D. BEARD, E. A. ECKERT, T. Z. CSAKY, D. G. SHARP AND J. W. BEARD, *Proc. Soc. Exptl. Biol. Med.*, 75 (1950) 533.
2 D. G. SHARP, E. A. ECKERT, D. BEARD AND J. W. BEARD, *J. Bact.*, 63 (1952) 151.
3 E. JUNGHERR, L. P. DOYLE AND E. P. JOHNSON, *Am. J. Vet. Research*, 2 (1941) 116.
4 E. A. ECKERT, D. G. SHARP, E. B. MOMMAERTS, R. H. REEVE, D. BEARD AND J. W. BEARD, *J. Nat. Cancer Inst.* In Press.
5 E. B. MOMMAERTS, E. A. ECKERT, D. G. SHARP AND J. W. BEARD, *Proc. Soc. Exptl. Biol. Med.*, 79 (1952) 450.
6 E. B. MOMMAERTS, D. G. SHARP, E. A. ECKERT, D. BEARD AND J. W. BEARD, *J. Nat. Cancer Inst.* In Press.
7 D. G. SHARP, E. B. MOMMAERTS, E. A. ECKERT, D. BEARD AND J. W. BEARD, *J. Nat. Cancer Inst.* In Press.
8 D. G. SHARP, A. R. TAYLOR, I. W. MCLEAN, Jr., D. BEARD AND J. W. BEARD, *J. Biol. Chem.*, 159 (1945) 29.
9 D. G. SHARP, D. BEARD AND J. W. BEARD, *J. Biol. Chem.*, 182 (1950) 279.
10 E. A. ECKERT, D. BEARD AND J. W. BEARD, *J. Nat. Cancer Inst.*, in press.
11 E. B. MOMMAERTS, D. BEARD AND J. W. BEARD, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 479.
12 R. CUNHA, M. L. WEIL, D. BEARD, A. R. TAYLOR, D. G. SHARP AND J. W. BEARD, *J. Immunol.*, 55 (1947) 69.
13 W. J. ELFORD, C. M. CHU, I. M. DAWSON, J. A. DUDGEON, F. FULTON AND J. SMILES, *Brit. J. Exptl. Path.*, 29 (1948) 590.

Received November 26th, 1953