THE DETERMINATION OF SEDIMENTATION CONSTANTS OF PROTEINS AND VIRUSES WITH THE HELP OF THE SPINCO PREPARATIVE ULTRACENTRIFUGE

by

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The high speed angle centrifuge has become a valuable tool for the purification of viruses, and other high molecular biological materials. Little quantitative experimental work on the rate of sedimentation of giant protein molecules and viruses in the angle centrifuge has however been done. Usually the amount of centrifugation to remove a certain molecular species from solution has been determined empirically. The exact determination of sedimentation rate allows not only the calculation of particle size but also the more complete separation from contaminating substances than is possible by cruder methods of centrifugation.

PICKLES[1] has already used the high speed angle centrifuge for the determination of approximate sedimentation constants. He has pointed out the importance of avoiding convection currents and the need to determine an exact sedimentation boundary.

We have noticed that, when solutions of proteins of high molecular weights are centrifuged in the Spinco preparative ultracentrifuge, the sedimenting boundaries are not very sharp but that their positions in the centrifuge tubes can nevertheless be determined with sufficient accuracy by direct measurement. We have utilized the instrument successfully for analytical studies not only of protein solutions but also viruses.

THEORETICAL

SVEDBERG AND PEDERSEN[2] have shown that the sedimentation constant S can be calculated from the distance that a boundary migrates $(x_2 - x_1)$ during the time interval $(t_2 - t_1)$ in a centrifuge rotor which has an angular velocity of ω by means of the equation:

$$S = \frac{2\,(x_2 - x_1)}{(x_2 + x_1)\ \omega^2\ (t_2 - t_1)} \qquad (1)$$

If x_1 is the horizontal distance of the initial boundary from the axis of rotation in the angle centrifuge and x_2 that of the boundary after centrifugation, then $x_2 = x_1 + H \sin \alpha$, where H is the difference in the heights of the boundaries in the vertical centrifuge tube before and after centrifugation (uncorrected sedimentation distance) and α is the angle of inclination of the centrifuge tube to the vertical. When substituting $H \sin \alpha$ for

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$(x_2 - x_1)$ SVEDBERG AND PEDERSEN'S equation becomes applicable also to the calculation of sedimentation constants from data obtained with the angle centrifuge.

EXPERIMENTAL

To test equation 1 solutions of whelk (Caminella sincta) haemocyanine were centrifuged at different speeds for a constant period and the resultant change in level of the boundary in the centrifuge tube measured directly. Such measurements of the boundary displacements during centrifugation of the haemocynanine are possible on account of the distinct blue colour of the solutions. The average speed of centrifugation was determined by dividing the total number of revolutions of the rotor by the time of centrifugation in minutes. This takes into account the periods of acceleration, constant speed and deceleration. To minimize the acceleration period the centrifuge dial was set to a higher speed than was required until the speed of the rotor approached the required velocity when it was turned back to that speed.

Sources of error

1. *Temperature.* It soon became apparent that if reliable sedimentation constants are to be computed the increase of temperature of the rotor during centrifugation had to be taken into consideration. When a pre-cooled rotor is spun in the centrifuge the temperature of the rotor increases rapidly until an approximate equilibrium value is reached. This equilibrium value is dependent on the degree of vacuum in the centrifuge chamber as well as on the velocity of the rotor. In Table I and Fig. 1 are given equilibrium temperatures obtained for different speeds of centrifugation. From this table the approximate rotor temperature for any velocity between 10,000 and 34,000 can be calculated. The rotor can therefore be cooled to the appropriate temperature before the start of centrifugation.

TABLE I

EQUILIBRIUM TEMPERATURES FOR DIFFERENT ROTOR VELOCITIES

r.p.m.	*Time in minutes*	*Rotor temperatures °C*
10,000	95	7.0
14,000	95	7.5
20,000	95	8.0
20,000	95	9.0
25,000	100	10.0
25,000	100	11.0
31,000	100	12.5
34,000	102	12.5
34,000	102	12.5

The effect of heat convection currents on the sedimentation boundary has been discussed in detail by PICKLES[1] who has pointed out that unusually sharp sedimenting boundaries are formed when convection currents are set up during or after centrifugation. PICKLES has further pointed out that with convection currents the material below the boundary does not show the usual gradation in concentration but is homogeneously distributed throughout the tube. With the precautions taken in the present experiments of pre-cooling the rotor to the centrifugation equilibrium temperature neither of these effects has been noticed when solutions of haemocyanine were centrifuged.

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2. *Distortion of the "lusteroid" tubes during centrifugation.* During centrifugation the lusteroid tubes used in the centrifuge collapse on the side nearest the axis of rotation. This is especially noticeable when the tubes are half empty. The consequence of this distortion is that the meniscus level is pushed up higher in the tube. On slowing down of the centrifuge the tubes regain their original shape almost completely and the clear fluid above the sedimenting boundary is now spread over a larger area than during centrifugation. The result of this distortion is that the distance of sedimentation appears smaller and in consequence sedimentation constants computed from short distances of sedimentation will be considerably smaller than the true values.

In an experiment to test out the magnitude of the error due to tube distortion, a solution of dye was subjected to centrifugation at 10,000 to 30,000 r.p.m. for 20 minutes.

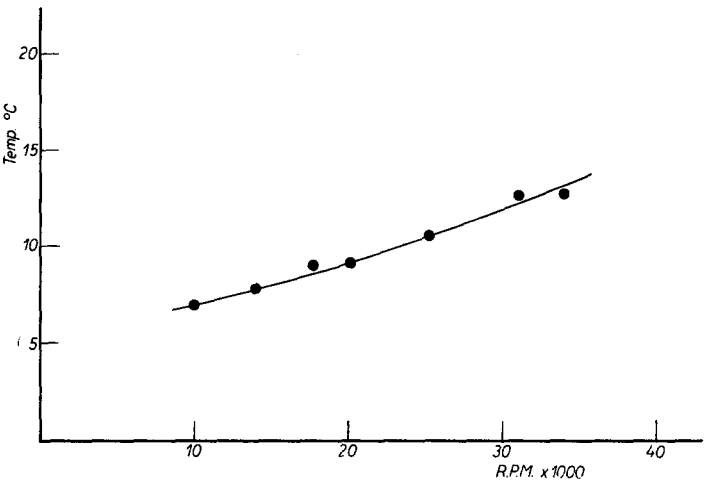

Fig. 1. Variation of rotor equilibrium temperature with rotor velocity.

From the level of the stain adhering to the walls of the centrifuge tube after centrifugation it could be calculated that during centrifugation the horizontal distance of the meniscus from the axis of rotation was 5.35 cms instead of 5.2 cms, as would be the case with a rigid centrifuge tube.

There are two methods whereby the error from tube distortion can be overcome:

(i) By computing sedimentation constants from results of experiments in which the uncorrected sedimentation distance was at least 2 cms. This is borne out by the results of experiments with C. sincta haemocyanine recorded in Table II. The results show that sedimentation constants calculated from boundary displacements of less than 1.9 cm are too low. The values calculated from 1.9 cm or more agree very well with those obtained by SVEDBERG AND PEDERSEN[2] with haemocyanine of this type.

(ii) By computing sedimentation constants not by direct calculation but by comparison of the sedimentation of the substance under investigation with that of a substance with known sedimentation constant.

It can readily be shown from SVEDBERG AND PEDERSEN's formula as applied to sedimentation in the angle centrifuge that

$$S_1 \omega_1^2 t_1 \eta_1/\eta_{20} = S_2 \omega_2^2 t_2 \eta_2/\eta_{20}$$

References p. 208.

TABLE II

ULTRACENTRIFUGATION EXPERIMENTS AND COMPUTED SEDIMENTATION CONSTANTS OF CAMINELLA SINCTA HAEMOCYANINE

Protein concentration 2.75%. $x_1 = 5.35$ during centrifugation.

r.p.m.	*Rotor equilibrium temperature °C*	*H in cms.*	η/η_{20}	*Time in minutes*	S_{20}
10,000	7	0.5	1.42	100	86.6
14,200	7.5	1.0	1.4	100	85.0
17,400	7.5	1.9	1.4	100	101.5
20,000	9.0	2.7	1.35	103	99.2
22,400	10.0	3.6	1.31	104	98.1
24,490	11.0	4.6	1.27	104	98.2
		Average of values in range 17,400 to 24,490 =			99.2

η/η_{20} is the ratio of viscosity of the medium to that of water at 20° C.

where S_1, ω_1, t_1 and η_1/η_{20} are the sedimentation constant, rotational velocity, time of centrifugation and viscosity ratio, respectively, for one substance and S_2, ω_2, t_2 and η_2/η_{20} corresponding values of another. Thus for a given value of H, *i.e.*, the uncorrected sedimentation distance as measured on the centrifuge tube in a vertical position $S\,\omega^2\,t\,\eta/\eta_{20}$ is constant. If therefore two particulate substances, one of known sedimentation constant and the other unknown, are sedimented by centrifugation through a distance (H), the unknown sedimentation constant can be calculated from the relative speeds and time of centrifugation required.

The advantage of this comparative method of calculation is that many experimental errors including that due to distortion of the tube are cancelled out. The usefulness of the method is illustrated by the results obtained with Jasus lalandi haemocyanine recorded in Table III. The haemocyanine of this species has not been investigated by SVEDBERG but the sedimentation constant of a related species the European lobster, Palinurus vulgaris, has been found to be 16.3 Svedberg units[2]. Assuming this value also to be correct for the haemocyanine of the closely related species, Jasus lalandi, then the observed values for the sedimentation constant at 20° C (S_{20}) are too low. The explanation for this is that calculations were made from total uncorrected sedimentation distances (H) of less than 2.0 cms, when as already shown with C. sincta haemocyanine, tube distortion effects play an important part.

TABLE III

ULTRACENTRIFUGATION OF JASUS LALANDI HAEMOCYANINE

Protein concentration 3.1%. $x_1 = 5.35$ cms. $a = 26°$.

r.p.m.	*Rotor equilibrium temperature °C*	η/η_{20}	*H im cms.*	*Time in minutes*	S	S_{20}
20,000	8	1.38	0.35	103	11.05	14.7
25,000	10.5	1.28	0.5	104	10.07	12.3
30,000	12.0	1.23	0.75	105	9.86	12.1
35,000	12.5	1.21	1.0	106	9.56	11.4
					Average =	12.6

References p. 208.

In Figs. 2 and 3 are given the sedimentation distances (H) of the two haemocyanines, C. sincta and J. lalandi, during centrifugation at different speeds for approximately constant time. From a comparison of the speeds required to give approximately equal sedimentation of the two haemocyanines and a knowledge of the S_{20} of C. sincta haemocyanine the S_{20} of J. lalandi haemocyanine could be calculated. The values obtained (Table IV) are in closer agreement with SVEDBERG's estimate for P. vulgaris haemocyanine than those recorded in Table III.

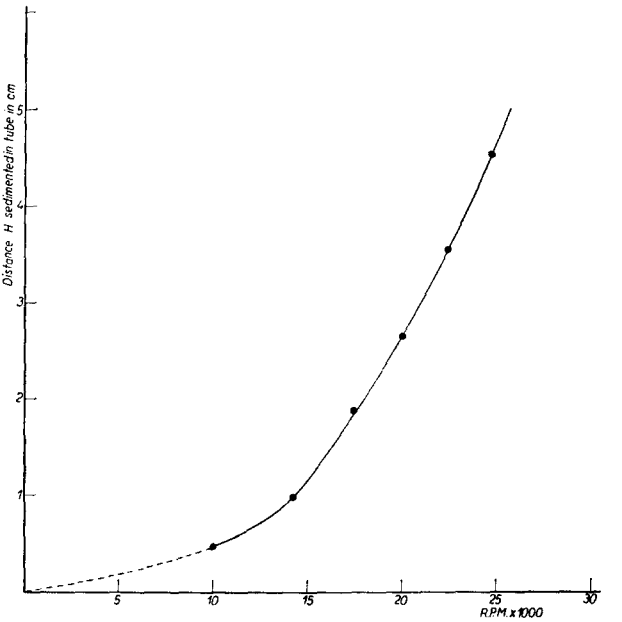

Fig. 2. Distance (H) which the haemocyanine of Caminella sincta sedimented in the centrifuge tube at different rotor velocities and approximately constant time 105 minutes.

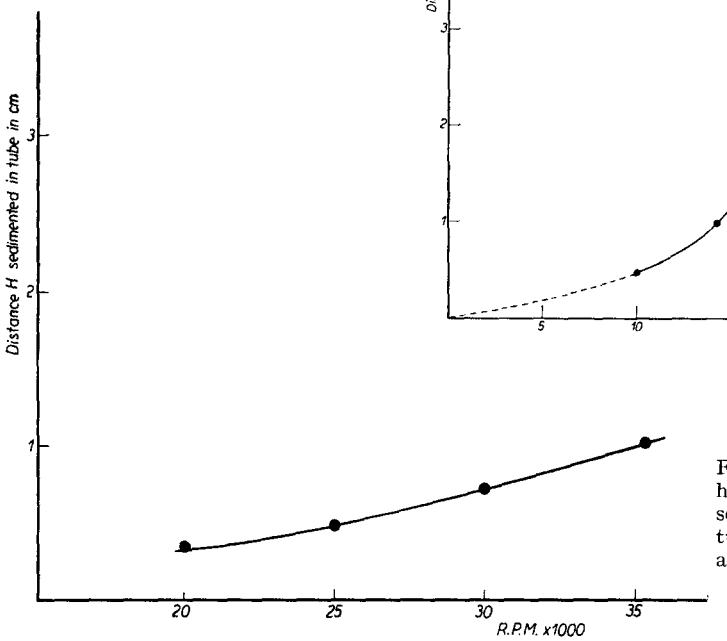

Fig. 3. Distance (H) which the haemocyanine of Jasus lalandi sedimented in the centrifuge tube at different rotor velocities and approximately constant time.

TABLE IV

SEDIMENTATION OF JASUS LALANDI AND CAMINELLA SINCTA HAEMOCYANINES

C. sinta S_{20} = 100 Svedberg units. Time of centrifugation t = 100 minutes.

Jasus lalandi					*Caminella sincta*			$\therefore S_{20}$
r.p.m.	*Time in minutes*	η/η_{20}	T °C	H	*r.p.m.*	η/η_{20}	T °C	*Jasus lalandi*
20,000	103	1.38	8.0	0.35	8,000	1.45	6.5	16.3
25,000	104	1.28	10.5	0.50	10,000	1.43	7.0	17.11
30,000	105	1.23	12.0	0.75	12,150	1.42	7.2	17.9
35,000	106	1.215	12.5	1.0	13,800	1.41	7.4	16.9
							Average =	17.05

References p. 208.

ULTRACENTRIFUGATION OF VIRUS SUSPENSIONS

Because of the relatively sharp sedimenting boundaries obtained on centrifugation of the haemocyanines and the consequent accurate determination of molecular size it was decided to apply the same method to the study of viruses. As the amount of virus protein in most suspensions of viruses is very low it is impossible to observe directly any virus boundary. A reasonably accurate determination of the boundary position can however be made by testing the virus content of the fluid at different heights in the tube after centrifugation. An abrupt change in virus content of samples from adjacent levels will indicate the zone in which the virus boundary lies. The position of the sedimenting boundary can conveniently be determined as the level at which the virus concentration is midway between the concentrations of the virus in the layers above and below the zone of abrupt change (see Fig. 6).

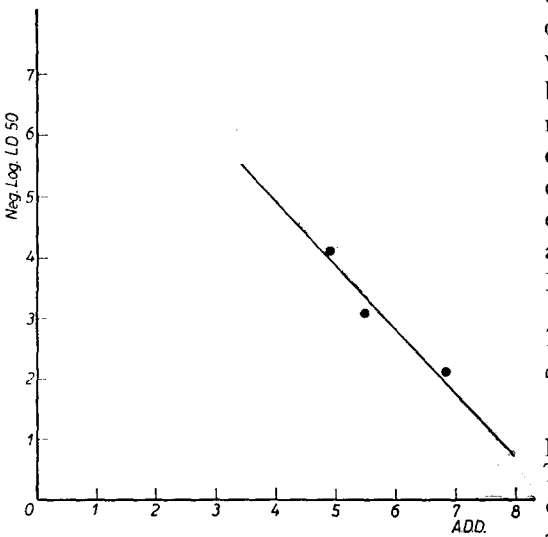

Fig. 4. Neg. log LD_{50} plotted against the average day of death in a titration of the virus of lumpy skin disease.

The sedimentation constant of lumpy skin disease virus

This method of centrifugation was applied to the virus of lumpy skin disease. This virus was adapted to the developing chicken embryo (VAN DEN ENDE *et al.*,[3,4]) and it has subsequently been passaged for many generations in eggs. The virus is very stable and sufficiently accurate titrations can be made by determining the average time taken to kill embryos after allantoic inoculation (GOLUB[5]). Table V and Fig. 4 show the results of a typical titration of the virus content of a 10% chick embryo emulsion.

TA

THE AVERAGE DAY OF DEATH (A.D.D.) OF EGGS /

Eggs inoculated after 9 days pre

Dilution	*Number dying*							
	1	*1.5*	*2*	*2.5*	*3*	*3.5*	*4*	*4.5*
Original Fluid $\times 10^{-2}$	—	—	—	—	1	2	—	3
$\times 10^{-3}$	—	—	—	—	—	1	1	1
$\times 10^{-4}$	—	—	—	—	—	—	—	—
$\times 10^{-5}$	1	—	—	—	—	—	—	—
$\times 10^{-6}$	—	—	—	—	—	—	1	—
$\times 10^{-7}$	—	—	—	—	—	—	—	—

References p. 208.

EXPERIMENTAL

Before centrifugation a centrifuge tube is marked off in intervals of 1 cm and filled with virus suspension to a level of 2 cms from the top of the tube. In the case of lumpy skin virus we invariably used a 10 % emulsion in broth of chick embryos which had been infected with the virus 3 days previously. The tube is then placed in the centrifuge rotor which has been pre-cooled to the equilibrium temperature for the selected speed of centrifugation. The rotor is rapidly accelerated to the required speed, and the number of revolutions carefully noted. The average velocity of centrifugation is taken as the number of revolutions divided by the total time of centrifugation in minutes.

After the centrifuge has stopped the tube which contains the virus suspension is removed carefully and immediately lowered into a glass beaker which contains water of the same temperature as that of the centrifuge rotor. Successive 1 cm layers of fluid are then removed with an instrument designed to lower a fine pipette with its tip bent upwards into the centrifuge tube (Fig. 5). The virus content (LD_{50}) of each layer is determined by the GOLUB method and the titres plotted against the average distance of each sample from the original meniscus in the vertically held centrifuge tube.

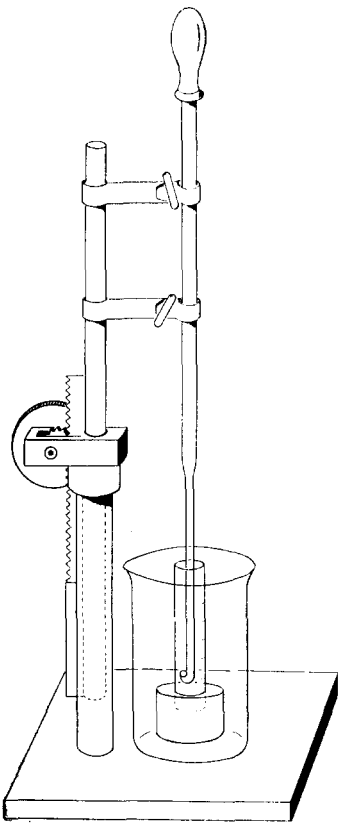

Fig. 5. Sampling device for removing fluid at different levels in centrifuge tube.

The results of several experiments recorded in Fig. 6 show that the distance (H) of sedimentation of lumpy skin virus during centrifugation at 30,000 r.p.m. for 105 minutes lies between 4.6 and 5.4 cms, with an average of 5.01 cms.

The sedimentation constants (S_{20}) calculated from these values is 66.4 (Table VI). The sedimentation con-

ANTOIC INOCULATION OF LUMPY SKIN VIRUS

ion. LD_{50} of Original Material 6.08.

on (day)									Number surviving on 10th day	A.D.D.
5·5	6	6·5	7	7·5	8	8·5	9	9·5		
3	1	—	—	—	—	—	—	—	0	4·9
2	3	2	—	—	—	—	—	—	0	5·5
1	—	5	—	4	—	—	—	—	0	6.8
1	—	3	—	1	2	—	—	—	2	—
—	1	—	1	—	1	—	—	1	5	—
—	—	1	—	2	—	—	—	—	7	—

References p. 208.

stant calculated by comparison with the rate of sedimentation of C. sincta haemocyanine is 72.4 (Table VII). The reason for this discrepancy is at present obscure. The value of 66.4 Svedberg units is probably nearer to the correct value as it was obtained by the method of calculation which gave an exact value for C. sincta haemocyanine. With a sedimentation constant for lumpy skin virus of 66.4 and assuming a particle density of 1.33, the particle size can be calculated from the modified Stokes equation $r^2 = \frac{9}{2} \frac{S \eta_{20}}{(d - \varrho)}$ to be 18.9 mμ

where r = radius of the particle,
S = sedimentation constant in SVEDBERG units,
η_{20} = viscosity of water at 20°C,
d = 1.33 the particle density, and
ϱ = density of the dispersion medium.

The particle diameter calculated from S_{20} of 72.4 is 19.9 mμ.

TABLE VI

ULTRACENTRIFUGATION OF THE VIRUS OF LUMPY SKIN

Sin α = 0.4384. x_1 = 5.35 cms.

r.p.m.	*H*	*T* °C	η/η_{20}	*Time in minutes*	S_{20}
30,000	4.6	12.5	1.22	105	62.6
30,100	5.1	12.5	1.22	105	67.5
30,000	4.95	12.5	1.22	106	65.7
30,200	5.4	12.5	1.22	105	69.9

Average = 66.4

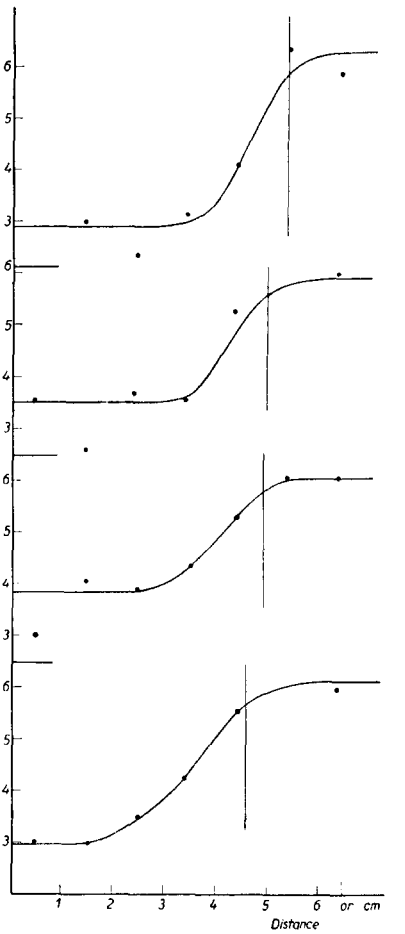

Fig. 6. Neg. log LD_{50} of samples taken at various levels in the centrifuge tubes in different experiments conducted at approximately constant rotor velocity and time of centrifugation.

TABLE VII

SEDIMENTATION CONSTANTS OF LUMPY SKIN VIRUS CALCULATED FROM CORRESPONDING SEDIMENTATION DISTANCES OF CAMINELLA HAEMOCYANINE

Caminella haemocyanine					*Lumpy Skin Virus*					
r.p.m.	*H*	*T* °C	η/η_{20}	*Time in minutes*	*r.p.m.*	*H*	*T* °C	η/η_{20}	*Time in minutes*	S_{20}
24,500	4.6	11	1.27	104	30,000	4.6	12.5	1.22	105	68.2
25,500	5.1	11	1.27	104	30,100	5.1	12.5	1.22	105	73.3
25,250	4.95	11	1.27	104	30,000	4.95	12.5	1.22	106	72.5
26,000	5.4	11	1.27	104	30,200	5.4	12.5	1.22	105	75.8

Average = 72.4

References p. 208.

CENTRIFUGATION IN A PROTEIN GRADIENT

During experiments with the haemocyanines it was noticed that a protein gradient formed in the centrifuge tube during centrifugation. This gradient extended from the protein boundary downwards. This effect was treated theoretically by PICKLES[1]. PICKLES has also shown that convection-free sedimentation can be obtained when a protein or virus is centrifuged in a sugar gradient. As a density gradient can also be formed in a haemocyanine solution it was decided to ultracentrifuge lumpy skin virus in a solution of J. lalandi haemocyanine in this way to eliminate heat convection currents that might occur. The sedimentation constant of lumpy skin virus centrifuged in a solution of haemocyanine was 65.7S. We concluded therefore that a density gradient did not significantly modify any effect which covection currents may have had in our previous experiments.

ULTRAFILTRATION RESULTS WITH LUMPY SKIN VIRUS

In their original work on lumpy skin virus VAN DEN ENDE *et al.*[4] found that the virus passed a 53 mμ gradocol filter, but was retained by a 25 mμ membrane. In work by one of the authors (A.P. unpublished) it was established that the virus can be filtered through a 38 mμ membrane but not through a 32 mμ. Calculated by ELFORD's method this would indicate a particle size for the virus of lumpy skin of 12.6–19 mμ which is in agreement with our results obtained by ultracentrifugation.

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SUMMARY

Contrary to the views expressed by other workers that the angle centrifuge can only be used to determine approximate sedimentation constants of proteins and viruses (PICKLES[1]) we have found that with the electrically driven Spinco angle centrifuge accurate values can be obtained. Thus the sedimentation constant of whelk haemocyanine as determined by this method approximates closely to the value found by SVEDBERG. Furthermore the particle size of lumpy skin virus as determined by ultracentrifugation agrees with the value obtained by ultrafiltration. Such accurate measurement is possible only if factors such as temperature and time of centrifugation are carefully controlled and if the effect of distortion of the centrifuge tubes are taken into consideration. With the older types of angle centrifuges this was not possible as the rates of acceleration and deceleration were low. During these periods changes of temperature could take place and furthermore the total time of centrifugation could not be determined accurately.

More accurate values for the sedimentation constants of viruses than those recorded in this paper can undoubtedly be obtained with the aid of the sampling technique described if a larger number of smaller samples are taken. This will necessitate the use of larger numbers of eggs than were available to us. This method of particle size determination has definite advantages over ELFORD's capillary method (ELFORD AND GALLOWAY[6]). It can also be of great value to the electron-microscopist for whom an accurate knowledge of particle size is invaluable and who could use the same instrument for virus purification as well as particle size determination.

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RÉSUMÉ

Suivant certains auteurs (PICKLES[1]) la centrifuge à angle ne permet d'obtenir que des valeurs approximatives pour les constantes de sédimentation de protéines et de virus. Cependant, nous avons constaté que l'on peut obtenir des valeurs exactes à l'aide de la centrifuge à angle Spinco à commande électrique. En effet, la valeur de la constante de sédimentation de l'hémocyanine de buccin déterminée par cette méthode approche de près celle trouvée par SVEDBERG. De plus, la valeur trouvée pour la grosseur des particules de virus "lumpy skin" déterminée par ultracentrifugation est en accord avec la valeur obtenue par ultrafiltration. De telles mesures exactes ne peuvent se faire que si l'on règle soigneusement des facteurs tels que la température et le durée de centrifugation et si l'on tient compte de l'effet de distorsion des tubes à centrifuger. Ceci n'était pas possible avec les types de centrifuge plus anciens, les périodes d'accélération et de décélération étant trop longues. Pendant ces périodes, des changements de température pouvaient avoir lieu et de plus, la durée totale de centrifugation ne pouvait tre déterminée exactement.

L'on pourrait sans doute obtenir, pour les constantes de sédimentation des virus, des valeurs plus exactes que celles rapportées dans ce mémoire, à l'aide de la technique d'échantillonnage décrite en prenant un nombre plus considérable d'échantillons plus petits. Il faudra pour cela un nombre d'œufs plus grand que celui dont nous disposions. Cette méthode de déterminer la grosseur des particules présente des avantages certains par rapport à la méthode capillaire d'ELFORD (ELFORD ET GALLOWAY[6]). Elle pourrait être d'une grande valeur en microscopie électronique où la connaissance exacte de la grosseur des particules est inestimable et où le même instrument pourrait être employé par purifier le virus et pour en déterminer la grosseur.

ZUSAMMENFASSUNG

Im Gegensatz zu der von anderen Autoren (PICKLES[1]) geäusserten Ansicht, dass die Winkelzentrifuge nur zur ungefähren Bestimmung der Sedimentationskonstante von Proteinen und Viren benutzt werden kann, fanden wir, dass mit der elektrisch angetriebenen Spinco-Winkelzentrifuge genaue Werte erhalten werden können. So nähert sich die mit dieser Methode bestimmte Sedimentationskonstante des Kinkhornhämocyanins weitgehend dem von SVEDBERG gefundenen Wert. Ferner stimmt die mit der Ultrazentrifuge bestimmte Teilchengrösse des "lumpy skin" Virus mit dem durch Ultrafiltration erhaltenen Wert überein. Derartig genaue Messungen sind nur möglich, wenn die Faktoren wie Temperatur und Dauer des Zentrifugierens sorgfältig kontrolliert werden und die Tatsache der Verformung der Zentrifugenröhrchen in Betracht gezogen wird. Bei den älteren Typen der Winkelzentrifugen war dies nicht möglich, da die Anlauf- und Auslaufzeiten zu gross waren. Während dieser Zeit konnten Temperaturänderungen stattfinden und ausserdem konnte die Gesamtzeit des Zentrifugierens nicht genau bestimmt werden.

Genauere als die in dieser Arbeit berichteten Werte für Sedimentationskonstanten von Viren können zweifellos mit Hilfe der beschriebenen Probenmethode erhalten werden, wenn eine grössere Anzahl kleinerer Proben gegeben ist. Dies wird die Benützung einer grösseren als uns zur Verfügung stehenden Anzahl Eier erfordern. Diese Methode der Bestimmung der Teilchengrösse hat bestimmte Vorteile gegenüber ELFORDS Kapillarmethode (ELFORD UND GALLOWAY[6]). Ebenso kann sie für den mit dem Elektronenmikroskop Arbeitenden von grossem Wert sein, da für ihn eine genaue Kenntnis der Teilchengrösse von unschätzbarem Wert ist und er das gleiche Instrument ebenso gut zur Virusreinigung wie zur Bestimmung der Teilchengrösse benutzen könnte.

REFERENCES

[1] E. G. PICKLES, *J. Gen. Physiol.*, 26 (1942–'43) 341.
[2] T. SVEDBERG AND K. O. PEDERSEN, *The Ultracentrifuge*, Oxford, Clarendon Press, 1940.
[3] M. VAN DEN ENDE AND G. S. TURNER, *J. Gen. Microbiol.*, 4 (1950) 225.
[4] M. VAN DEN ENDE, P. A. DON AND A. KIPPS, *J. Gen. Microbiol.*, 3 (1949) 174.
[5] O. J. GOLUB, *J. Immunol.*, 59 (1948) 71.
[6] W. J. ELFORD AND I. A. GALLOWAY, *Brit. J. Exptl. Path.*, 18 (1937) 155.

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