SEDIMENTATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE IN THE SHARPLES-SUPER CENTRIFUGE[1]

By M. SCHLESINGER and I. A. GALLOWAY

From the National Institute for Medical Research, London, N.W. 3

The experiments recorded in this paper deal with the centrifugation of the virus of foot-and-mouth disease in the Sharples-Super Centrifuge by means of a new technique outlined already by one of us (Schlesinger, 1936). The questions discussed are, the application of the method to the estimation of the particle size and particle weight of the virus from the sedimentation rate and the sedimentation equilibrium, the information obtained about the specific gravity of the virus and the use of the method for preparative purposes. The experimental procedure and the results obtained are given in detail, the theoretical explanation of the principles underlying the method and its application to different problems are given briefly. The results of experiments recorded in the present paper were referred to already in general terms in communications made to the International Society for Microbiology at the Congress in London, July 1936. (See Congress *Proceedings*.)

Methods

The apparatus and method of use. The apparatus employed is the experimental model of the Sharples-Super Centrifuge with a "closed bowl". This "closed bowl" is a steel cylinder of about 20 cm. length and 4·5 cm. diameter with a removable base and rotating with its axis vertical. The speed used in the present experiments did not exceed 31,000 r.p.m., which corresponds to a centrifugal force of about 24,000 times gravity at the periphery of the cylinder.

The cylinder is warmed and 5 or 10 c.c. of liquid agar are introduced and spun for about 20 min. The centrifugal force distributes the agar solution as a thin layer on the wall of the cylinder which as it cools becomes coated with a uniform gel. Now the rotation is stopped and the virus suspension is introduced. In the spinning cylinder the liquid system spreads as a thin film on the surface of the agar layer. The virus particles have, under the influence of the centrifugal force, to travel only the very short distance represented by the thickness of the liquid layer before reaching and entering the agar-gel. When the centrifuge is stopped, the liquid freed to a great extent from the virus flows down and collects at the bottom while the virus particles remain in the agar-

[1] This work was done on behalf of the Foot-and-Mouth Disease Research Committee who have given their permission for the publication of this paper. One of us (M. S.) wishes to express his thanks to the Academic Assistance Council for a grant.

gel lining the wall. If the agar-gel is scraped off and minced in fresh medium or in the "supernatant" the virus particles diffuse out again and the virus can be recovered. As long as this recovery is possible without loss, any role of adsorption phenomena in the effects obtained can be excluded with certainty. The extraction of the agar in fresh medium may be referred to as "washing" of the sedimented virus particles. The *sedimentation rate* of the particles can be estimated from the time which is necessary to obtain a given drop in the concentration of a given volume of virus suspension. That concentration of the supernatant which cannot be further lowered by continued spinning under suitably chosen conditions gives information about the *sedimentation equilibrium.*

The agar-coated surface of the cylinder has an area of 273 sq. cm., 5 c.c. of liquid when spread on this surface as a film has a thickness of 0·18 mm. After travelling this distance which is about a hundredfold shorter than the column formed by the same volume in the usual type of centrifuge tube, the virus particles have left the liquid system and are caught in the agar. Thus using a centrifugal force of 24,000 times gravity only 3 min. are required to free 5 c.c. of diluted virulent vesicle lymph of about 90–99 per cent of its virus content. Employing a force of only 2000 times gravity (i.e. a centrifugal force equivalent to that developed by the ordinary bucket type of centrifuge running at 3000 r.p.m.) the same result can be effected in 2 hours.

Technical details. The closed bowl has an internal radius of 2·23 cm. and a height of 19·5 cm. (i.e. the cylindrical part coated with agar). When a volume of 5 c.c. of agar is employed the surface of the cylindrical part of the centrifuge bowl appears to be coated uniformly with the gel.

The thickness of the agar lining and of the liquid layer is calculated from the relationship of the measured volume of agar or liquid to the surface area of the cylindrical part of the centrifuge bowl (the fact that the layer is cylindrical, i.e. the inner surface area is smaller than the outer one, is neglected since the thickness is small if compared with the radius, i.e. an error of <1 per cent). The surface area of the cylinder is calculated from a direct measurement of the length of that part of the cylinder coated with agar and measurements of the radius made directly or calculated from a determination of the volume of liquid required to fill the cylindrical agar-coated part of the bowl. Any errors in estimations of the thickness of the gel or liquid layer are determined by errors in the measurements of their volume, i.e. <5 per cent, and these would have practically no effect on the experimental results.

Errors in the estimation of the thickness of the agar layer might arise if swelling of the gel occurred or if the centrifugal force applied expressed fluid from it. However, if such changes took place they would be detected by changes in the volume of the supernatant, but such have not been observed when employing 1 per cent agar at 9000 r.p.m. or 2 per cent agar at 31,000 r.p.m.[1]

[1] *It has been found that under other experimental conditions e.g. when employing (a) 1 per cent agar at 31,000 r.p.m. or (b) 2 per cent agar at 9000 r.p.m. some liquid (about 10 per cent) is expressed from (a) or sucked into (b) the gel.*

The cylinder is warmed in a Bunsen flame to 50–60° C., the agar solution is introduced and the bottom cap screwed in and luted with paraffin wax. In the centre of the screw in the bottom cap there is a small opening which can be used for introducing the virus suspension and removing samples of supernatant from the cylinder with capillary pipettes. Into this small opening fits a screw which when dipped in oil and sufficiently tightened ensures efficient sealing without additional luting. The cylinder is inverted to be fitted in the centrifuge. On the completion of each experiment the bottom cap was removed, after softening of the paraffin wax by careful warming, the gel examined to make sure that there was no solution of continuity in its structure and then the agar layer was scraped off for extraction. After use the cylinder was sterilized by boiling.

The agar-gel. The agar solution was made up freshly for each experiment by dissolving the agar powder (British Drug Houses) in a medium corresponding as far as possible to that of the virus suspension. It was found however that the medium most commonly used for the suspension of the virus of foot-and-mouth disease, buffered phosphate solution, could not be used for the agar-gel on account of a precipitate which sometimes formed. The agar was therefore dissolved in distilled water when the virus was suspended in buffered phosphate solution or in broth and distilled water when broth was present in the virus mixture. The agar solution made in this way proved to be neutral, and since the buffering salts from the virus suspension would enter the agar-gel very quickly during centrifugation no disadvantage was to be expected from the omission of the phosphate salts from the gel. The procedure adopted was justified by the results of the experiments. The volume of agar used was either 5 or 10 c.c. as indicated in the tables, giving a thickness of gel of 0·18–0·36 mm. In the experiments using lower speeds, 18,000 to 9000 r.p.m., and especially in those concerning the sedimentation equilibrium, 1 per cent agar was used to facilitate the entrance and free motion of the virus particles in the gel. In the majority of the experiments done at the speed of 31,000 r.p.m. however 2 per cent agar was employed and the results proved to be as satisfactory as when 1 per cent agar was used. An experiment was made to determine whether the virus actually penetrates even the 2 per cent agar-gel. After 30 min. spinning of a 5 c.c. amount of virus suspension the whole supernatant was removed and the surface of the agar rinsed thoroughly with 5 c.c. of fresh buffered phosphate solution while shaking and rotating the cylinder. This rinsing liquid was removed to test for virus potency. The agar lining was then scraped off, minced and then left in contact with another 5 c.c. of fresh buffer solution for 2 hours, this fluid after removal of the agar by centrifugation and filtration constituting the agar extract. The rinsing liquid had the same virus content or titre as the supernatant, i.e. 1/1000th that of the stock virus suspension, while the agar extract had a virus potency equivalent to that of the original stock virus. Thus the assumption that the virus particles actually enter the gel is correct and 2 per cent agar is just as suitable for the agar lining when working

at high speeds as 1 per cent agar and has the advantage that the agar layer lining the cylinder does not break or crack during spinning as sometimes happens with 1 per cent gels, particularly when larger volumes, 10–20 c.c., of virus suspension are centrifuged.

Testing of different systems. These were titrated by the inoculation of serial tenfold dilutions, intradermally, into the metatarsal pads of each of two guinea-pigs. The highest dilution of the system tested producing a generalized foot-and-mouth disease infection in one or both guinea-pigs inoculated indicated the limiting infective dilution or titre. The systems titrated as a rule were (*a*) the stock virus suspensions, (*b*) the supernatants (i.e. samples of liquid removed from the cylinder after different times of spinning; in certain experiments the same liquid was centrifuged for a further period after removal of a small sample of supernatant, in others the whole supernatant[1] was removed and replaced by fresh stock virus, thus the same agar-gel was used many times consecutively), (*c*) the deposit, i.e. the gel extract. In preparing this extract the agar lining was scraped off the walls of the cylinder by means of a curved glass rod, suspended in a quantum of liquid (chiefly either fresh buffered phosphate solution or the whole supernatant from which only a small sample had been removed) corresponding to the volume of stock virus used, and finally the agar was removed by centrifuging and/or filtration after 2 hours' extraction.

The speed of the centrifuge. With the type of Sharples-Super Centrifuge employed, the cylinder is rotated by means of a driving belt from an electric motor. The speed of the centrifuge has been estimated roughly in each experiment by counting the revolutions of the axis immediately connected with the driving belt and multiplying this figure by 400, as indicated by the makers, to give the speed of the cylinder. The figures obtained by this method have been checked by means of the speed-measuring apparatus described in 1936 by Elford, to whom our thanks are due for assistance. As a result of this control, it was found necessary to reduce the speed measurements estimated by the former method by 4 per cent. Variation of the speed was effected by the use of interchangeable pulleys of different diameter. The three speeds employed in the present experiments were approximately 31,000, 18,000 and 9000 r.p.m.

Precautions during centrifugation. It is advisable when working with viruses or other infective agents to place a vessel containing disinfectant below the spinning cylinder in case untoward circumstances may cause leakage of virus. An additional precaution is to tie a cloth soaked in the disinfectant round the metal fitting at the lower end of the centrifuge into which the cylinder fits.

[1] Actually even when exercising the greatest care in removing the supernatant with capillary pipettes 0·6–0·8 c.c. of liquid always remained in the cylinder and this was taken into account in calculations.

EXPERIMENTS WITH UNPURIFIED VIRUS

In all the experiments the results of which are recorded in Table I unpurified virus was employed, i.e. collodion membrane filtrates, A.P.D. 0·5–0·7 μ, of a 2 per cent suspension of virulent vesicle lymph in buffered phosphate solution at *p*H 7·6. The results show that whereas 2–3 min. centrifugation at 31,000 r.p.m. of 5 c.c. amounts of virus was sufficient to effect about a 10–100-fold drop in titre of the supernatant liquid (i.e. to spin out 90–99 per cent of the virus), and 15–30 min. produced 1000–10,000-fold drops in titre, the whole amount of virus could be recovered by extraction of the agar-gel. The recovery of the virus was just as complete when the extraction was made in the liquid layer or supernatant as when it was effected in fresh medium. In the former case not only the relation of the volume of gel to the liquid system but also the physico-chemical conditions were the same during extraction as during centrifugation. The recovery of the virus without loss under these conditions was conclusive evidence that adsorption played no role whatever in the results recorded. On the other hand, the possibility of recovering the virus from the gel when using fresh medium for the extraction showed that this centrifugation method could be used for purification of the virus. It has been found, however, (*vide infra*) in experiments with purified and partially purified virus that the non-adsorbability of the virus by the agar-gel is dependent on the presence of impurities, probably the proteins of the vesicle lymph, and this places certain limits on the use of the method for washing purposes.

EXPERIMENTS WITH PURIFIED VIRUS

In centrifugation experiments made with samples of virus previously purified by washing on graded collodion membranes of suitable porosity, A.P.D. 25 mμ, and in contrast with the results of experiments with unpurified virus recorded in Table I, it was found that the virus could not be recovered from the agar-gel by extraction after centrifugation. It was considered that one or other or both of two possible factors might account for this result, viz. (1) an increased sensitiveness of purified virus to the toxic effects of the metal of which the cylinder is made, or (2) the adsorbability of purified virus by the agar-gel. Experiments were made to test the first of these possibilities and it was shown that purified virus left in contact for a relatively short time with the unlined wall of the metal cylinder lost some of its potency, while a control sample of purified virus kept in a glass tube for the same period maintained its titre. Very striking also was the difference in the behaviour of purified as compared with that of unpurified virus left in contact with an agar-gel. Experiments were made in agar-lined Petri dishes in which the relation of the volume of the virus to the surface area of the gel, the time of contact and other conditions were the same as those obtaining in centrifugation experiments. A hundredfold drop in titre was observed in the case of purified virus left in contact with the gel whereas unpurified virus remained unaltered under these

Table I. *Data on the sedimentation rate of the virus of foot-and-mouth disease*

No. and date of experiment		Strain of virus	Time of centrifuging in min.	Vol. of agar-gel in c.c.	Vol. of virus in c.c.	Agar extracted with	Limiting infective dilution			C_t/C_0
							Stock (C_0)	Supernatant (C_t)	Sediment	
14. x. 36	1	Waldmann C (Riems)	(A) 1½	5	5	—	1 : 1000 (++)	1 : 1000 (+0)	—	1–0·1
	2	,,	(A) 3	5	5	5 c.c. *M*/45 phosphate solution *p*H 7·6	1 : 1000 (++)	*1 : 100 (+0)	1 : 1000 (++)	0·1–0·01
	3	,,	(B) 3	5	5	—	*1 : 100 (+0)	<1 : 10	—	≦0·01
	4	,,	(B) 5	5	5	5 c.c. *M*/45 phosphate solution *p*H 7·6	1 : 1000 (++)	1 : 10 (+0)	1 : 1000 (++)	0·01–0·001
9. x. 36	5	Vallée O (G. F.)	(A) 2½	5	5	,,	1 : 10,000 (++)	1 : 1000 (++)	1 : 10,000 (++)	0·1
	6	,,	(A) 5	5	5	,,	1 : 10,000 (++)	*1 : 1000 (+0)	1 : 10,000 (++)	0·1–0·01
	7	,,	(B) 5	5	5	,,	*1 : 1000 (+0)	1 : 10 (+0)	1 : 1000 (+0)	0·01
24. vii. 36	8	Vallée A (L)	5	5	5	—	1 : 10,000 (++)	≦1 : 100	—	≦0·01
	9	,,	15	5	5	Supernatant	1 : 10,000 (++)	≦1 : 10	1 : 10,000 (++)	≦0·001
27. xi. 36	10	Vallée O (G. F.)	15	5	2½	—	1 : 10,000 (+0)	1 : 10 (++)	—	0·01–0·001
19. iii. 36	11	,,	15	10	5	—	1 : 1000 (+0)	Undiluted (++)	—	0·01–0·001
	12	,,	30	10	5	Supernatant	1 : 1000 (+0)	Undiluted negative (00)	1 : 1000 (+0)	0·001–0·0001
24. iii. 36	13	,,	30	10	5	5 c.c. *M*/45 phosphate solution *p*H 7·6	1 : 1000 (++)	Undiluted (++)	1 : 1000 (+0)	0·001
2. iii. 36	14	,,	60	10	5	Supernatant	1 : 10,000 (++)	1 : 100 (++)	1 : 10,000 (+0)	0·01
17. iii. 36	15	,,	60	10	10	Supernatant	1 : 1000 (+0)	Undiluted (++)	1 : 1000 (++)	0·01–0·001
2. iv. 36	16	,,	60	5	20	20 c.c. *M*/45 phosphate solution *p*H 7·6	1 : 10,000 (+0)	1 : 100 (+0)	1 : 10,000 (+0)	0·01

In third column, experiments on 14. x. 36 and 9. x. 36: A = 1st agar-gel; B = 2nd agar-gel.

C_0 = original concentration of virus.

C_t = concentration of virus after centrifuging for time = t.

In experiments 2 and 3 and 6 and 7 C_t marked * was used as C_0 marked * for centrifuging experiment with 2nd agar-gel.

(++), (+0), (00). These signs after the titres of the stock, supernatant or sediment indicate that two, one or neither of two guinea-pigs inoculated showed lesions.

Speed = 31,000 r.p.m. = centrifugal force 24,000 times gravity.

conditions. The drops in titre of the purified virus in these experiments, made in Petri dishes, were sufficiently high to explain the non-recovery of the purified virus from the agar-gel by extraction in the centrifuging experiments without consideration of other possible factors. It was thought that broth might help to protect purified virus against adsorption by the agar-gel. A series of experiments were made in which broth was mixed 1 : 10 both with the agar-gel and the suspension of purified virus. (Higher concentrations of broth than 1 : 10 were not used since it has previously been shown (Galloway & Elford, 1936) that digest broth is itself harmful to purified virus.) The results of these experiments were inconclusive.

Experiments of particular interest were those made with virus which had been washed on a gradocol membrane of suitable porosity, A.P.D. 25 mμ, only to a point where the sulphosalicylic acid test was just negative and guinea-pig protein was still detectable by serological precipitin tests. In a centrifuging experiment made with this preparation it was possible to obtain almost complete recovery of the virus from the agar-gel by extraction on completion of the centrifugation, whereas in direct tests for adsorption of the virus by an agar-gel spread in Petri dishes no change in potency could be detected. Thus it appears that small residues of protein which may be undetectable by the sulphosalicylic acid test are sufficient to protect the virus against adsorption by an agar-gel. These results are important with regard to the use of this special method of centrifugation for the purification of the virus. They show, and it has been demonstrated further by experiments in which repeated centrifugation was carried out and the serial extracts examined by parallel tests for protein by the sulphosalicylic acid test and virus potency by inoculation of guinea-pigs, that a purification of the virus up to a certain limit is possible. However, beyond that point indicated by the limit of the sensitiveness of the sulphosalicylic acid test for protein, purification by this centrifugation method cannot be continued since the virus can then no longer be recovered by extraction of the agar.

THE ESTIMATION OF THE SEDIMENTATION RATE

The estimation of the rate of sedimentation of the virus particles is based upon titrations of the supernatant after different times of centrifugation. Since the sedimentation, in the very thin layer of liquid existing when quanta of 5 c.c. of virus are employed,[1] may be regarded as being undisturbed by convection effects, and the centrifugal force is constant throughout the layer (since the thickness of the layer is very small compared with the radius of rotation), the calculation is very simple. The relative drop in concentration in the supernatant indicates immediately the distance travelled by the particles in

[1] Sedimentation cannot be regarded as being uninfluenced by convection effects when quanta of virus greater than 5 c.c., e.g. 10–20 c.c., are centrifuged.

proportion to the whole thickness of the layer; the quotient of the distance travelled and the time of spinning is the velocity of sedimentation, from which the sedimentation constant and the particle radius can be calculated (*vide infra*), e.g. with a layer of thickness 0·20 mm. the drop in virus content of the supernatant would be 1 : 2 if the particles travelled 0·10 mm., 1 : 4 if the particles travelled 0·15 mm. and 1 : 10 corresponding to a sedimentation of 0·18 mm. Further sedimentation through a distance of less than 0·02 mm. should now bring about sudden drops to 1 : 100, 1 : 1000, etc. There are, however, a number of disturbing factors which may interfere with a decrease in titre greater than 1 : 100 to 1 : 1000. With the present simple arrangement, before removal of the samples for testing, the cylinder must be inverted whereby the liquid part of the system flows once again over the agar lining and there may be time for some virus to diffuse out into the "supernatant". Again small traces of the stock virus when this is being introduced into the bowl may contaminate certain parts of the cylinder, e.g. the opening in the screw cap, where they would not come under the influence of centrifugation and might later become mixed with the "supernatant" in sampling.

It is probably to such circumstances that are to be attributed the apparently paradoxical results sometimes obtained. (E.g. cf. Exps. 14 and 12 in Table I. In the former, where the time of centrifugation was 60 min., the relationship C_t/C_0 was only 0·01, while in the latter, in which the centrifugation time was 30 min., the relationship C_t/C_0 was 0·001 to 0·0001.) It must be emphasized, however, that the influences of the factors discussed here have only to be considered in those experiments in which the concentration of the virus in the supernatant falls below 1/100th to 1/1000th that of the stock virus suspension and the result of Exp. 14 (*vide* Table I) represents the most exaggerated effect of their influence we have observed.

Taking these circumstances into consideration as well as the fact that the smallest difference in virus concentration which can be demonstrated satisfactorily by animal inoculations is 1 : 10, the estimation of the sedimentation rate will have to be based upon the following considerations: no detectable drop in the titre of the supernatant means that the distance travelled by the virus particles is shorter than the thickness of the liquid part of the system, a drop in titre of 1 : 10 in the supernatant indicates that the distance travelled by the virus particles corresponds approximately to the thickness of the liquid layer, while drops exceeding 1 : 10 to 1 : 100 show that the time of centrifugation was longer than the time necessary for the virus particles to travel through the whole thickness of the liquid layer.

The results recorded in Table I show that the time required for the particles to travel the distance of 0·18 mm. (5 c.c. of virus spread over the surface area of 273 sq. cm.) under the influence of a centrifugal force of 24,000 times gravity ($2{\cdot}3 \times 10^7$ dynes) is less than 5 min. and lies between $1\frac{1}{2}$ and 3 min. (the time necessary for starting and stopping the centrifuge is taken into account).

The sedimentation constant s (the distance travelled in a second under the

influence of a force of one dyne in a medium of the viscosity of water at 20° C.) was calculated from the above data using the formula

$$s = \frac{v}{F} \times \frac{\eta}{\eta_0}, \qquad \ldots\ldots(1)$$

where v = velocity of sedimentation, F = the centrifugal force, η = the viscosity of the medium employed, η_0 = the viscosity standard, and was found to be $4\text{–}9 \times 10^{-12}$.

In a number of experiments made at a speed of 18,000 r.p.m., i.e. with a force of 8000 times gravity ($7{\cdot}9 \times 10^6$ dynes), no drop in titre of the supernatant was detected after 3 min. centrifugation, while a drop in titre of 1 : 10 was observed after 5–7 min. From these figures the value of s was calculated to be $5\text{–}8 \times 10^{-12}$.

The experiments the results of which are recorded in Table I were made with three strains of virus of different type, viz. strain G.F. of the Vallée O type, strain Riems of the Waldmann C type, and a strain of the Vallée A type. This last strain, kindly sent by Prof. Levaditi, was the one used by Levaditi *et al.* (1936) in experiments to which reference has already been made by Elford & Galloway (1937). It will be seen that there is no difference in the sedimentation rates observed with the three strains.

In Exps. 3 and 7 (see Table I), the supernatants obtained after centrifugation of two different strains of virus in Exps. 2 and 6 were centrifuged further for the same period of time, 3 and 5 min. respectively, while employing a new agar lining. By the combined effects of the two consecutive spinnings about 99·99 per cent of the virus was removed from the liquid, and what is most significant the sedimentation rate in the second centrifugation was not lower than in the first as would have been the case if even a very small proportion of the virus were present in higher dispersion than is indicated by the sedimentation constants recorded above. These results therefore point to a homodispersity of the virus suspension.

If we assume the virus particles to be spheres having a density of 1·30 (see p. 456), their diameter can be calculated from the relationship (Stokes's law)

$$v = \frac{2r^2 (\sigma - \sigma_1) F}{9\eta} \quad \text{or} \quad \left[s = \frac{2r^2 (\sigma - \sigma_1)}{9\eta_0} \right], \qquad \ldots\ldots(2)$$

where v = the velocity of sedimentation of a small spherical particle, σ = the density of the particle, σ_1 = the density of the medium, η = the viscosity of the medium (or η_0 = the viscosity standard), F = the centrifugal force. The sedimentation constant of $4\text{–}9 \times 10^{-12}$ corresponds to a particle diameter of 16–23 mμ (average 20 mμ).

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Experiments with mixtures of the virus and the S 13 strain of bacteriophage

In all experiments, except three, made for the estimation of the sedimentation rate of the virus a quantum of a broth filtrate of S 13 bacteriophage was added to the stock virus suspension. It will be recalled that the virus of foot-and-mouth disease, Galloway & Elford (1931) and the S 13 strain of bacteriophage, Elford & Andrewes (1932) have been estimated to have the same size value, 8–12 $m\mu$ by ultrafiltration analysis. In the present experiments on the completion of centrifugation the virus and bacteriophage content of the stock mixtures, "supernatants" and "sediments" were estimated in parallel; in the case of the virus by inoculation of serial tenfold dilutions intradermally into the metatarsal pads of guinea-pigs and in the case of the "phage" by the method of plate counts. In agreement with the results of the previous filtration experiments, the sedimentation constants derived for the bacteriophage were similar or only slightly smaller than those obtained for the three strains of virus of different type.

The sedimentation equilibrium of the virus

As the suspended virus particles move under the influence of the centrifugal force towards the periphery of the cylinder, their concentration in the most distant layers increases more and more as compared with the layers nearer the axis of rotation. Owing to the difference in concentration of virus thus produced, diffusion of the particles begins to take place from the periphery towards the axis. The diffusion between two levels becomes more and more significant as the concentration gradient increases and finally the number of particles carried back by diffusion will be just the same as the number moved towards the periphery by the centrifugal force. At this stage there is a certain constant value of concentration of virus particles at each level which decreases in a simple logarithmic relation to the distance from the periphery, and this value remains unchanged however long centrifugation at the same speed may be continued. The sedimentation equilibrium of the virus is thus established and from the data of the conditions determining this equilibrium an estimation of the volume and weight of the virus particles can be derived. It should be emphasized that the figures for the particle weight obtained in this way are entirely independent of any assumption concerning the shape of the particles. The weight estimation of the virus from the sedimentation equilibrium data may be used in combination with sedimentation rate estimations for detecting deviations from the spherical shape of the particles which is assumed when applying Stokes's Law.

In the present experiments made to determine the sedimentation equilibrium of the virus of foot-and-mouth disease in the agar-lined cylinder of the Sharples centrifuge, it was necessary to arrange the speed so that it was low enough to ensure that the average concentration of virus in the liquid layer or

"supernatant" in the equilibrium was about 1/10th to 1/100th that of the original concentration. If the centrifugation conditions had been such as to determine lesser concentrations of virus in the supernatant then the results might have been vitiated by the factors discussed on p. 452.

It was also necessary to make certain by testing samples of the supernatant after different intervals that continued centrifugation did not lead to a further decrease in the virus content of the liquid layer.

When the sedimentation equilibrium was established the equilibrium constant k could be calculated from the relationship of the measured average concentration of virus C_s in the liquid layer and C_w the average concentration of virus in the whole system, liquid plus gel, from the relationship

$$\frac{c^{kx_1}-1}{c^{k\,(x_1+x_2)}-1}=\frac{x_1}{x_1+x_2}\cdot\frac{C_s}{C_w}, \qquad \ldots\ldots(3)$$

where x_1 = thickness of liquid layer or supernatant, x_2 = thickness of gel layer. (In order to simplify the calculation the thickness of the liquid layer used was always half that of the gel layer, i.e. $x_1=x_2/2$.)

The particle size value v can be estimated from the relationship

$$k=\frac{N}{RT}\cdot v\,(\sigma-\sigma_1)\,F, \qquad \ldots\ldots(4)$$

where R = the gas content, T = the absolute temperature, N = Avogadro's number, σ = density of virus particles, σ_1 = density of medium, F = the centrifugal force.

It was found that with a speed of 9000 r.p.m. (2000 times gravity), 10 c.c. of 1 per cent agar (thickness of gel 0·36 mm.) and 5 c.c. of virus suspension a 1 : 100 drop in the concentration of the virus in the supernatant was obtained in 2 hours, and in different experiments where samples of the liquid layer were tested after 3, 4, 5 and 6 hours' centrifugation no further drop in titre was detected. Again using 5 c.c. of 1 per cent agar (thickness of gel 0·18 mm.) and 2·5 c.c. of virus, the drop in titre in the supernatant after 2 hours under similar centrifugation conditions was 1 : 10, and this concentration of virus in the liquid layer could not be reduced further by continued centrifugation in four different experiments. That the drop in titre of virus in the supernatant in the latter series of experiments was less than in the former is due to the fact that the distance from the periphery of the liquid layer examined (supernatant) in the latter case was half that of the liquid layer examined in the former case.

That the virus diffused out of the agar-gel just as readily as it was thrown into it by the centrifugal force and that we were dealing with a real equilibrium which could be reached from either direction was demonstrated by the following experiments:

(*a*) When employing 5 c.c. of 1 per cent agar (gel thickness 0·18 mm.), 2½ c.c. of virus and centrifuging at 31,000 r.p.m. for 15 min. the concentration of virus in the supernatant was reduced to 1/1000th of the original titre of the

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stock virus suspension. Further centrifugation was then carried out at the diminished speed of 9000 r.p.m. for 3 hours and the virus titre of the supernatant was found to have *increased* to 1/100th of the titre of the stock virus. After centrifugation at the lower speed for a further period of 3 hours the virus content of the supernatant again *increased* and was now 1/10th that of the original stock virus.

(*b*) When employing 10 c.c. of 1 per cent agar (gel thickness 0·36 mm.), 5 c.c. of virus and centrifuging at 18,000 r.p.m. for 30 min. the concentration of virus in the supernatant was reduced to 1/1000th that of the stock virus. After further centrifugation at 9000 r.p.m. for a period of 12 hours the virus titre of the supernatant had not changed, but centrifugation for a further period of 6 hours led to an *increase* in the virus concentration of the supernatant so that it was now 1/100th that of the stock virus.

Thus the same fixed distribution of virus was obtained when starting with a virus concentration below the equilibrium value as in the direct experiments described above.

If the weight of a virus particle taking the specific gravity as 1·30 is calculated either from the relationship $C_s : C_0 = 1 : 10$ with a thickness of gel layer 0·18 mm., or from the relationship $C_s : C_0 = 1 : 100$ with a thickness of 0·36 mm., the result is very much the same viz. circ. 1×10^{-17} g., where C_s = the virus concentration in the supernatant and C_0 = the virus concentration of the original stock suspension. (N.B. since the volume of the whole system liquid plus gel was three times that of the liquid alone the value C_w used in formula (3) is $C_0/3$.) If this figure is multiplied by $N = 6 \times 10^{23}$ (Avogadro's number = number of molecules in a gram molecule) we obtain 6×10^6 as the "molecular weight" of the virus of foot-and-mouth disease which is slightly higher than that recorded by Svedberg & Chirnoaga (1928) for haemocyanin (Helix). The diameter of a sphere of this weight and density would be 25 mμ.

The average size value of the virus estimated from the sedimentation rate data (*vide supra*) was 20 mμ. If this discrepancy between the figures obtained by the two methods of estimation is real and not due to the disturbing factors discussed on p. 452, tending to make the estimation of the size value from sedimentation rate data too low, then it would suggest that the shape of the virus particle is other than spherical. However, in view of the manifold difficulties encountered and especially those concerning the estimation of virus concentration by animal tests the two estimations might be regarded as being in very good agreement.

Estimation of the specific gravity of virus particles by centrifugation

An attempt to measure the relative density of the virus was made by examining its sedimentation in media of increased density. By dissolving sucrose or sucrose and sodium chloride in the virus suspension its specific gravity was raised as high as 1·28. The agar-gel employed in these experiments

was prepared in such a way that it contained exactly the same proportion of sugar, or salt and sugar, as the virus suspension. In order to avoid any possible error due to inactivation of the virus or its adsorption by the agar in the altered medium, a control virus mixture was kept, for the duration of the centrifugation experiment, spread upon the surface of Petri dishes coated with a similar agar-gel to that used in the actual centrifugation experiments. As will be seen from the results of the experiments recorded in Table II, even the most concentrated mixtures of salt and sugar had no deleterious effect on the virus and definite sedimentation was observed, even in those media of which the specific gravity was as high as 1·27 and 1·28. These results show that in the media used the density of the virus particles must be higher than 1·28. Now by comparison of the results of the experiments (see Table II) where the drop in virus titre

Table II. *Experiments to estimate the relative density of the virus of foot-and-mouth disease*

	Mixtures					Limiting infective dilution			
	Sucrose g.	NaCl g.	Virus c.c.	Time of spinning in min.	Specific gravity of mixture	Control mixture C_0	Supernatant C_t	C_t/C_0	Remarks
Exp. 1	2·3	1	3·5	30	1·28	10^{-3}	10^{-2}	0·1	Vol. of agar-sucrose-NaCl mixture used for gel = 10 c.c.
Exp. 2	2·0	1	3·5	30	1·27	10^{-4}	10^{-3}	0·1	
Exp. 3	2·3	0·7	3·5	30	1·25	10^{-4}	10^{-2}	0·01	
Exp. 4	2·0	0·5	3·5	30	1·22	10^{-4}	10^{-1}	0·001	Vol. of virus mixture = 2 c.c.
Exp. 5	52·5% sucrose			60	1·25	10^{-5}	10^{-4}	0·1	Vol. of agar-sucrose mixture used for gel = 10 c.c.
				120	1·25	10^{-5}	10^{-3}	0·01	Vol. of virus-sucrose mixture = 5 c.c.

after centrifugation was 1 : 10 with the measurements of the sedimentation rate (see Table I and p. 453) an attempt can be made to calculate the density value of the virus particles. Taking into consideration the effect which would be produced by the increased viscosity of the medium, 6·5 poises (i.e. about 6·5 times that of water) in Exp. 2 and 12 poises in Exp. 5, we obtain for the specific gravity of the virus particles the figure 1·35 from the former experiment and even 1·45 from the latter experiment. Again the results of Exp. 1 (viscosity 9 poises) lead to the density value 1·39.

In making the calculation of the particle size of the virus from sedimentation rate data and sedimentation equilibrium data we have used the density value 1·30, thus allowing for the possible dehydrating effects of the substances added to the virus suspension in the experiments made to determine the specific gravity. The fact that in the present experiments there was no indication of a higher density of the virus particles in the sugar-salt mixtures than in the medium containing sugar alone (i.e. though in the latter case the same specific gravity was obtained at a much lower molar concentration) would suggest that the role played by osmotic dehydration is not a big one. What the normal

water content of the virus particles actually is, however, is unknown and the actual density of the virus particles in a state when their water content is unaltered still remains an unsolved problem.

Discussion

Estimations of the size value of the virus of foot-and-mouth disease have been made with a new centrifugation technique from two different measurements, the rate of sedimentation of the virus particles and their distribution in the sedimentation equilibrium.

The figures obtained for the sedimentation constant vary from 4×10^{-12} to 9×10^{-12}, corresponding to particle diameters of 16–23 mμ or an average of 20 mμ. If the true density of the virus proved to be 1·40 instead of 1·30 (the figure actually used in making the calculation of the size value), then the particle diameters of the virus would be 14–20 mμ or an average of 17 mμ instead of 20 mμ.

It has been shown definitely that the sedimentation of the virus in the experiments recorded here was due entirely to the effect of the centrifugal force and that adsorption phenomena played no role. All other possible sources of error would tend to make the sedimentation rate values and thus the figures for the particle diameter derived therefrom too low. If the virus particles were not spherical they would sediment at a slower rate than spheres of the same weight. Such factors as currents due to mechanical or thermal effects during centrifugation, incomplete penetration of the virus into the structure of the gel, etc., would all tend to increase the time required to produce a given drop in virus concentration and thus lead also to low estimations of the particle diameter. It will be seen that the estimations of size value of the virus made from the data of these centrifugation experiments are definitely higher than those arrived at from ultrafiltration analysis (Galloway & Elford, 1931). Factors which may be responsible for the difference have been considered in the paper by Elford & Galloway (1937 *loc. cit.*).

It might be argued that the infective particles of which the sedimentation rate has been measured may not be single virus elements. Bechhold & Schlesinger (1931) and Schlesinger (1932) have pointed out that the most conclusive answer to such an objection would be provided by a demonstration of the monodispersity of the virus. Now taking the special case of foot-and-mouth disease the first indication of the monodispersity of the virus was the form of the filtration curves obtained in experiments by Galloway & Elford (1931). In concurrent experiments made by Elford & Galloway (1937) combining centrifugation and ultrafiltration methods, further evidence of the monodispersity of the virus has been obtained. The possibility of there being virus-units having a size larger than the average has been definitely excluded. The results of experiments recorded in the present paper have shown that 99·99 per cent of the virus of a stock virus filtrate (collodion membrane

A.P.D. 0·5–0·7 μ) could be removed by two consecutive centrifugations using different agar-gels and that the sedimentation rate of the virus from the second fraction was not less than from the first as would have been the case if even a small proportion of the infectivity were due to particles of a smaller size than the average.

Three strains of virus have been employed in the present series of experiments, the G.F. strain (Vallée O type), Riems strain (Waldmann C type) and a strain received from Professor Levaditi (Vallée A type). Levaditi *et al.* (1936) gave a size value for this last strain of virus of 3–5 mμ. The sedimentation rate for all three strains of virus was found to be the same in our experiments. That the three standard types of virus do not differ in their particle size as was suggested by Modrow (1929) was indicated already in the filtration experiments made by Galloway & Elford (1931). Elford & Galloway (1937) further report that they have found that the strain examined by Levaditi and his co-workers behaves in ultrafiltration experiments in exactly the same way as other strains of virus of different type previously studied. According to sedimentation rate measurements recorded in the present paper all three strains examined have the same size value, about 20 mμ.

The estimation of the sedimentation equilibrium of a virus had not been attempted prior to the experiments recorded in this paper. Measurements of this kind have been up to the present the prerogative of Svedberg's special centrifuges in which observations are confined to the direct optical registration of changes in concentration of the substance examined. From the sedimentation equilibrium data the calculation of the weight of a single virus particle is possible, independently of any assumption about its shape. A value for the "molecular weight" of the virus is obtained by multiplying the weight of a single particle by Avogadro's number $N = 6 \times 10^{23}$. The weight of a single foot-and-mouth disease virus particle has been estimated from the sedimentation equilibrium data in the present experiments to be circ. 1×10^{-17} g., and this figure multiplied by 6×10^{23} gives a "molecular weight" of 6,000,000. This corresponds roughly to the "molecular weight" of haemocyanin (Helix) 5,000,000, as estimated by Svedberg and Chirnoaga (1928). The diameter of a sphere with a weight of 1×10^{-17} g. and a specific gravity of 1·30 would be 25 mμ.

The estimation of the particle diameter from the sedimentation equilibrium is not affected greatly by errors in the assumed density of the virus and/or errors in the estimated virus concentrations in the different fractions. The particle diameter calculated with the density value of 1·40 instead of 1·30 would be 23 mμ. Again if the equilibrium concentration of the virus was actually 1 : 10 or on the other hand 1 : 1000 instead of the estimated 1 : 100 (in experiments employing an agar-gel of thickness 0·36 mm.) then the particle diameters would be 20 or 28 mμ respectively instead of 25 mμ. However in the present experiments the existence of errors of this magnitude in the estimation of the equilibrium concentration is improbable. The equilibrium concentration

value was arrived at from the results of several experiments made under similar conditions and in which more than one titration was made.

It is quite certain also that, under the conditions of the present experiments with foot-and-mouth disease virus, a real equilibrium was being estimated, for it was shown that the diffusion of the virus particles out of the gel (1 per cent agar) was just as free as their entrance into the structure of the gel. An error in the estimation of the thickness of the gel or liquid layer (see p. 447) even as high as 10 per cent would lead to an error of not more than 3 per cent in the particle diameter value derived from the data of the sedimentation equilibrium. Other deviations from the conditions assumed to obtain in these experiments (if, for example, the cylinder were not perfectly concentric and therefore the thickness of the agar layer not quite uniform) would tend to lead to too low an estimation of the particle size for the virus. The good agreement between the equilibrium concentration values obtained with agar layers of different thickness and the results of similar experiments made with S 13 bacteriophage and haemocyanin (Helix) suggest that errors of a great magnitude in either direction in the estimation of the particle diameter are improbable. In view of the facts mentioned when discussing the sedimentation rate estimations, one would be inclined to regard the particle size estimation derived from the sedimentation equilibrium as probably nearer the true size value. If on the other hand the discrepancy between the size values arrived at by the two different methods of estimation is real, then this would suggest that the individual virus particles of foot-and-mouth disease are not spherical in shape. However, the placing of such interpretations on the results must at the present stage be considered as speculative. It might well be argued, in view of all the possible sources of error in such experiments, that the two estimations of particle size value, 20 mμ from the sedimentation rate and 25 mμ from the sedimentation equilibrium, can be regarded as being in very good agreement. It is interesting to note that concurrent centrifugation experiments employing the Ecco centrifuge and another special technique, have led to an estimation of a particle size value of 20 mμ for the virus of foot-and-mouth disease from sedimentation rate data (Elford & Galloway, 1937).

Definite sedimentation of the virus by centrifugation was obtained in media containing sucrose or sucrose and sodium chloride in proportions which raised the specific gravity up to 1·28. The estimations of the density of the virus made from the data obtained in these experiments gave a figure 1·40 on the average. This value is slightly higher than that found by Svedberg and his co-workers as the specific gravity of protein molecules. The uncertain factor in the method used in the present experiments for the determination of the density of the virus is the degree of dehydration effected by the high concentrations of sugar and salt which have to be used in attaining the requisite density values of the suspending media. In calculating the particle size of the virus, a density value of 1·30 has been used. Much lower values than this for the virus particles "in their native state", i.e. in a medium with unaltered osmotic equilibria, are

unlikely since they would lead to improbably high values for the particle size. The conclusion that appears to be justified is that the true specific gravity of the virus is similar to that of protein molecules, or is at least definitely higher than the figures obtained for bacteria suspended in a medium with physiological osmotic properties (e.g. the normal density of *B. coli* has been found to be 1·094 (Ruffilli, 1933)).

SUMMARY

1. By means of a new technique, using the "closed bowl" of the Sharples-Super Centrifuge, in which the distance which the virus particles have to travel under the influence of a given centrifugal force until they are caught mechanically in an agar-gel lining the wall of the bowl amounts to only a few tenths of a millimetre, the potency of 5 c.c. of a foot-and-mouth disease virus suspension can be reduced by 90–99 per cent in 3 min. using a centrifugal force of 24,000 times gravity. The virus can be recovered without loss by extraction of the agar-gel either in fresh medium or even in the liquid layer or "supernatant", thus showing that adsorption phenomena play no role whatever in the effects obtained by centrifugation.

2. The non-adsorbability of the virus by the agar, however, is determined by the presence of a certain though very small amount of impurities presumably protein which seem to have a "protective" effect. The limit of non-adsorption of the virus by the gel appears to be in that region of "purity" where the sulphosalicylic acid test for protein is negative whereas serological precipitation reactions for host protein are still positive. Virus purified beyond this limit is strongly adsorbed by an agar-gel.

3. Measurements of the sedimentation rate were made on three strains of virus of different type Vallée A, Vallée O and Waldmann C and no differences between the three strains were observed. The average diameter of the virus particles determined from the sedimentation rate data was 20 mμ. All the evidence now available for the monodispersity of the virus is discussed. The fact that the infective particles are uniform in size indicates that the measurements recorded here concern actually the single virus elements.

4. The sedimentation equilibrium of the virus has been estimated. The value calculated on this basis for the weight of a single virus particle is circ. 1×10^{-17} g. The diameter of a sphere of this weight and density 1·30 would be 25 mμ. The size values for the virus, 25 mμ estimated from sedimentation equilibrium data and 20 mμ estimated from sedimentation rate data, are discussed in relation to the possible shape of the virus particles.

5. The virus can be sedimented in sucrose and sucrose-salt solutions of densities up to 1·28. On the basis of the sedimentation rates in these media the specific gravity of the virus particles is estimated to be about 1·40. This value however may be abnormally high due to the dehydrating effect of the high concentration of salt or sugar. The application of the centrifuge technique

described here to the concentration and purification of the virus of foot-and-mouth disease in conjunction with ultrafiltration methods will be the subject of a second paper by Galloway & Schlesinger (1937).

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