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The Diffusion Constant and Molecular Weight and Shape of Neurotropic Horsesickness Virus.

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THE methods usually employed for determining the particle size of viruses and bacteriophages are based upon the results of ultrafiltration and ultracentifugation experiments, the final calculations being dependent upon the assumption that the particles are spherical (Elford 1938). That this assumption is not always correct was shown by Lauffer (1938) and Robinson (1939) who demonstrated that the virus of tobacco mosaic is rod-shaped. Similarly Pedersen and Goard (1941) showed that the virus of mouse encephalomyelitis consists of elongated particles. It is apparent, therefore, that unless the shape and also the molecular weight of a particle is known the calculated dimensions of a virus particle as determined by these methods may not be accurate.

Svedberg (1925) [see also Svedberg and Pedersen (1940)] developed a technique for determining the exact size and weight of colloidal particles based upon a combination of sedimentation, diffusion and partial specific volume data. This principle has been adapted to an evaluation of the molecular weight and shape of horsesickness virus. Since sedimentation and specific volume data for this virus had previously been collected from the results of ultracentrifugation experiments (Polson 1941) it merely remained to determine the diffusion constant to enable a calculation of the molecular weight and shape to be made.

When the substance under investigation is obtainable in the pure state the diffusion constant may be determined by the optical method of Lamm (1937) but when there is an association with other components as in the case of horsesickness virus, then it becomes necessary to resort to an analytical method. The method generally used in such cases is that developed by Northrop and Anson (1928-29)—a method based upon diffusion through a porous plate. This technique has been of great value in the study of enzymes but when applied to a virus it has limitations. Bourdillon (1941) proposed an additional method which is sound theoretically but in practice is applied only with great difficulty. A critical appreciation of the two methods has been published by Markham, Smith and Lea (1942). In this report a description is given of an additional method which has been applied with success to the study of horsesickness virus; it is based upon the diffusion of a substance in solution through the interface between that solution and the solvent.

THEORETICAL.

According to Fick's law the amount of a substance ds that diffuses through a layer A cm² in dt seconds is given by the equation:—

$$ds = -\mathrm{AD}\frac{dc}{dx} \cdot dt \qquad (1)$$

in which $\frac{dc}{dx}$ is the concentration gradient at that level and D is the diffusion constant.

A solution of Fick's equation is the following:—

$$\frac{dc}{dx} = \frac{\mathrm{Co}}{2\sqrt{\pi \mathrm{Dt}}} e^{-x^2/4\mathrm{Dt}} \qquad (2)$$

Where C_o is the original concentration and x is the distance from the original boundary at which the concentration gradient $\frac{dc}{dx}$ exists.

Consider the case where a substance diffuses through an interface formed between a solution of the substance and its solvent and calculate the amount that passes through this interface, where $x=0$, in a given length of time.

By combining equations 1 and 2 we get—

$$\frac{ds}{dt} = \frac{-\mathrm{AD\,Co}}{2\sqrt{\pi \mathrm{Dt}}} e^{-x^2/4\mathrm{Dt}}$$

The value of $e^{-x^2/4\mathrm{Dt}} = 1$ as $x = 0$.

By putting $\frac{\mathrm{A\,Co}\sqrt{\mathrm{D}}}{2\sqrt{\pi}} = \mathrm{K} = \text{constant}$

we get $-ds = \frac{K\,dt}{\sqrt{t}}$

The negative sign indicates that the substance diffuses out of the more concentrated portion of the fluid. The total amount of substance that diffuses out in any period of t seconds is given by—

$$-s = -\Sigma ds = K\int_{t=o}^{t=t} \frac{dt}{\sqrt{t}}$$

$$= 2\,K\,t^{\frac{1}{2}}$$

$$\therefore -s = \frac{Co\,A}{\sqrt{\pi}} \cdot \sqrt{\mathrm{Dt}}$$

$$\therefore D = \frac{s^2}{Co^2\,A^2} \cdot \frac{\pi}{t} \qquad (3)$$

From this equation the diffusion constant can be calculated. To convert the diffusion constant at one temperature into that at another temperature the following equation was employed.

$D_1 = D_2 \frac{T_1}{T_2} \cdot \frac{\eta_2}{\eta_1}$ where D_1 and η_1 are the diffusion constant and viscosity at the absolute temperature T_1 and D_2 and η_2 those at the absolute temperature T_2 respectively.

Technique, Materials and Apparatus.

To determine the amount of substance which diffuses through the interface between a solution and the solvent the simple apparatus shown in Fig. (1) was designed.

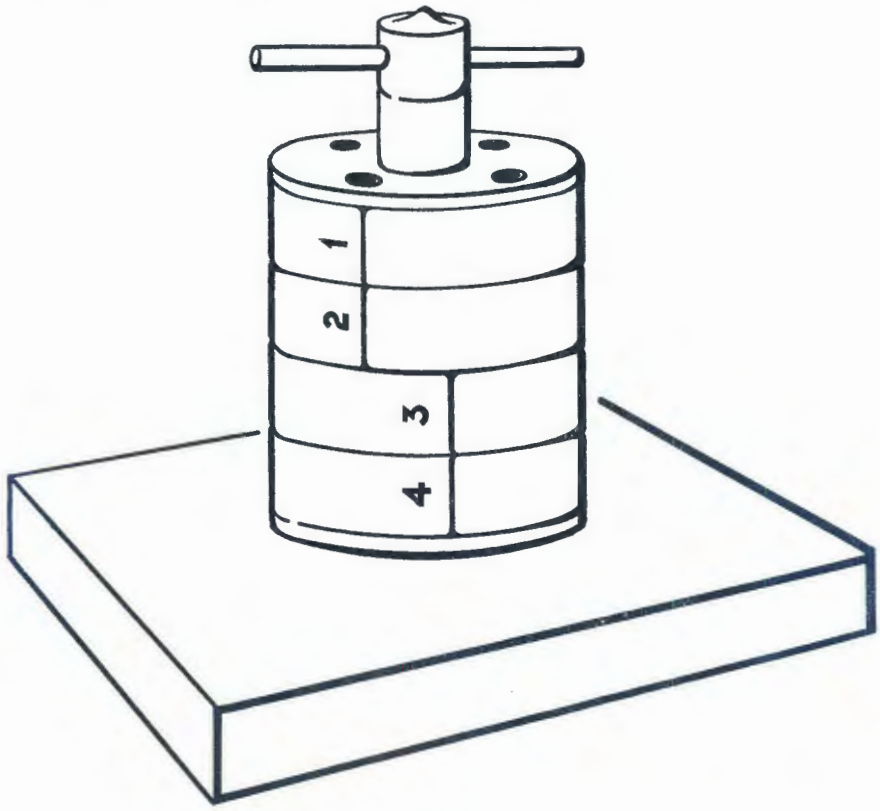

Fig. 1.

A 5 mm. hole was drilled through the centre of a cylindrical piece of metal (1) approximately 4 cm. in diameter and 5 cm. long. A closely fitting pin was turned to be inserted in the hole in such a manner that the head will clamp the complete apparatus vertically to a wooden base, when the thumb screw at the threaded end was tightened, to squeeze the completed segments together. The cylinder was then cut into the following sections:—

(*a*) a thin section about 3 mm. thick to serve as a cover;

(*b*) 3 sections (1, 2 and 3) to have a final thickness of exactly 1 cm. each;

(*c*) a basal section 1·5 cm. thick.

The surfaces of the sections were carefully ground and polished so that when in apposition they fitted perfectly. With the segments threaded on to the central pin, 4 holes 5 mm. in diameter were drilled vertically about midway between the periphery and the centre of the metal cylinder so that

each punctuated to a depth of 1 cm. into the basal metal section. In this way there were formed 4 cylindrical holes 5 mm. in diameter and 4 cm. long which could be broken into covered lengths of 1 cm. with a minimum of disturbance to the contents, simply by rotating the individual segments. Similarly the continuity of these holes could be re-established, without removing the top cover, by rotating the segments until suitable marks on the outside were in position.

This diffusion cell resembles the cells described by Schuhmeister (1879) and by Dummer (1919) somehow. The diffusion column was built up of two cylinders one inverted over the other and the diffusing solution was placed in a lower cylinder. The solvent was contained in a similar cylinder which was inverted and placed on a sliding frame. The inverted cylinder was then slid over the first one and the diffusion allowed to start, and on completion of the diffusion experiment the frame was slid back and the contents of the cylinders analysed. A very detailed review of the above method and other methods for the experimental study of diffusion has been published by Williams and Cady (1934).

Wool grease was used as a lubricant for the surfaces.

Virus.

Neurotropic horsesickness virus was used, the particular strain being that known arbitrarily as 1180 [Alexander (1935)] after approximately 210 serial passages through mice. To prepare the final emulsion 4 brains obtained from mice killed under ether anaesthesia when moribund was emulsified in 40 c.c. of 20 per cent. normal horse serum saline. After preliminary clarification by centrifugation at 3,000 r.p.m. for 15 minutes in Clay Adams angle centrifuge the supernatant was transferred to lusteroid tubes and placed in an air driven angle super-centrifuge, the speed slowly increased to 10,000 r.p.m. after which it was slowly brought to a standstill. It is believed that the resultant supernatant contains the virus in a mono disperse phase without gross aggregation of particles. " Merthiolate " in a concentration of 1 in 10,000 was used as a preservative.

As solvent into which the virus was allowed to diffuse, 10 per cent. normal horse serum saline was used. Estimations of the virus contents of emulsions were made by intracerebral injection of 0·05 c.c. amounts of serial two fold dilutions in 10 per cent. serum saline. The 50 per cent. endpoints (L.D. 50) were calculated by the method of Reed and Münch.

Experimental.

The four cylindrical cavities in the basal segments of the apparatus were carefully filled with the clarified virus emulsion. The other three segments were placed in position with their cylinders in apposition to one another but not to those containing virus emulsion. These cylinders were then filled with 10 per cent. serum saline and the whole apparatus placed in a controlled constant temperature room until temperature equilibrium had been attained. The three top sections were then carefully rotated until the cylindrical cavities were in apposition to form four continuous columns of fluid with a sharp interface between the virus-containing and non-virus-containing portions.

The apparatus was left in this state for definite periods of time, after which the segments 1, 2 and 3 were rotated so as to cut off the virus that diffused past the original interface into 1, 2 and 3. The volumes of the fluids

isolated in this way were measured accurately and their virus contents, as well as that of the original stock solution kept under the same conditions as the diffusion cell, were determined by mouse inoculation as described above. Two separate titrations of the original stock virus emulsion were made.

In the following tables the results of such titrations are given.

TABLE 1.

Diffusion of strain 1180. Diffusion time = 42 hours.
Volume into which the virus diffused = 0·6 c.c.
Cross sectional area of a cylinder A = 0·196 cm.
Temperature of diffusion = 20° C.

Dilutions.	Original.		Dilution.	Cell Contents.			
	1.	2.		1.	2.	3.	4.
1/1000.....	3445	444	1/100.....	455	440	440	440
1/2000.....	3457	447	1/200.....	440	500	500	400
1/4000.....	5570	680	1/400.....	000	450	600	500
1/8000.....	4600	460	1/800.....	000	000	700	000
1/16000.....	6000	600	1/1600.....	500	000	000	000
1/32000.....	0000	000	1/3200.....	000	300	000	000

In this and all the subsequent tables the numeral indicates the number of days after intracerebral injection the mouse died.

0 = Survived.

X = Death from some other cause than horsesickness usually traumatic injury.

— = Injection not done.

From the above tables the following were calculated:—

L.D. 50 Original 3·9031.
L.D. 50 cell contents 2·2695.

TABLE 2.

Strain 1180; Diffusion time 42 hours; Volume = 0·6 c.c. A = 0·196.
Temperature of diffusion = 20° C.

Dilutions.	Original.		Dilution.	Cell Contents.			
	1.	2.		1.	2.	3.	4.
1/1000.....	3444	4447	1/100.....	5540	5446	5554	4456
1/2000.....	4444	4444	1/200.....	5444	4555	5566	4556
1/4000.....	4444	4444	1/400.....	5556	6670	5566	4446
1/8000.....	4556	4560	1/800.....	4000	0000	7000	5000
1/16000.....	4550	4440	1/1600.....	7000	5000	0000	5000
1/32000.....	5000	4400	1/3200.....	0000	0000	0000	0000
1/64000.....	7000	5000	1/6400.....	0000	0000	0000	0000
1/128000.....	0000	5000	1/12800.....	0000	0000	0000	0000

L.D. 50 Original = 4·3424. L.D. 50 Cell Contents = 2·7324.

TABLE 3.

Strain 1180; Diffusion time = 66 hours; Volume = 0·6 c.c. A = 0·196.
Temperature of diffusion 20° C.

Dilution.	Original.	Dilution.	Cell Contents.			
			1.	2.	3.	4.
1/1000	4444	1/100	4560	4440	4440	4566
1/2000	4444	1/200	4555	4500	4500	4460
1/4000	4460	1/400	0000	5000	6000	4000
1/8000	4556	1/800	5550	0000	xx00	5000
1/16000	4000	1/1600	0000	0000	0000	0000
1/32000	4000	1/3200	0000	0000	x000	0000
1/64000	5000	1/6400	0000	0000	0000	0000
1/12800	0000	1/12800	0000	0000	0000	0000

L.D. 50 Original = 4·0531. L.D. 50 Cell Contents = 2·4472.

TABLE 4.

Strain 1180; Diffusion time = 92 hours; Volume 0·6 c.c. A = 0·196.
Temperature = 20° C.

Dilution.	Original.		Dilution.	Cell Contents.			
	1.	2.		1.	2.	3.	4.
1/1000	3344	4444	1/100	4400	4570	440	660
1/2000	3457	3444	1/200	4700	4450	670	360
1/4000	4450	3666	1/400	5000	0000	0000	370
1/8000	5700	4000	1/800	0000	0000	600	000
1/16000	4500	6000	1/1600	4000	0000	000	000
1/32000	4000	6000	1/3200	0000	0000	000	000
1/64000	0000	0000	1/6400	0000	0000	000	000
1/12800	0000	0000	1/12800	0000	0000	000	000

L.D. 50 Original = 4·0. L.D. 50 Cell Contents = 2·3674.

TABLE 5.

Strain 1180; Time 70 hours; Volume = 0·6 c.c. A = 0·196.
Temperature = 26° C.

Dilution.	Original.		Dilution.	Cell Contents.			
	1.	2.		1.	2.	3.	4.
1/1000	4444	4446	1/100	4440	4440	5444	5440
1/2000	4444	5566	1/200	4460	4600	5540	6448
1/4000	4555	4455	1/400	6700	4446	4000	7000
1/8000	6500	4500	1/800	4440	0000	5000	0000
1/16000	4000	4600	1/1600	0000	5000	6600	0000
1/32000	6000	0000	1/3200	0000	7000	0000	0000
1/64000	0000	0000	1/6400	0000	0000	0000	0000

L.D. 50 Original = 4·0934. L.D. 50 Cell Contents = 2·6170.

TABLE 6.

Strain 1180; Time 92 hours; Temperature 26° C.; A = 0·196.

The original level between the virus solution and the solvent was formed between sections 2 and 3. The volume of diffusate was 0·4 c.c.

Dilution.	Original.		Dilution.	Cell Contents.			
	1.	2.		1.	2.	3.	4.
1/1000.....	3300	3500	1/100.....	6550	5560	550	5600
1/2000.....	4555	5550	1/200.....	6655	5600	5600	5550
1/4000.....	6000	5500	1/400.....	6500	6000	5600	5000
1/8000.....	5550	5500	1/800.....	0000	0000	5000	0000
1/16000.....	5000	5500	1/1600.....	0000	0000	0000	0000
1/32000.....	6000	5000	1/3200.....	0000	0000	0000	0000
1/64000.....	0000	0000	1/6400.....	0000	0000	0000	0000
1/12800.....	—	—	1/12800.....	—	—	—	—

L.D. 50 Original = 3·7522. L.D. 50 = 2·4133.

Knowing the infective titre and the volumes of the different fluid fractions it is possible to calculate the number of M.L.D.'s of virus that diffused across the original boundary in each case. The data for the 6 detailed diffusion experiments are shown in columns 3 and 4 of Table 7 and in the 4th column is shown the diffusion constant calculated on the basis of diffusion in distilled water at 20° C.

TABLE 7.

Calculated diffusion constant.

Exp. No.	t in secs.	Co in M.L.D.	S in M.L.D.	$D \times 10^7$ cm²/sec.
1	151200	160,000	2232	1·05
2	151200	440,000	6480	1·05
3	237600	226,000	3360	0·76
4	252000	248.000	4968	1·15
5	331200	200.000	2796	0·48
6	331200	113,040	2072	0·74

Result.—The average diffusion constant is $0{\cdot}87 \times 10^{-7}$ cm.²/sec.

Comment.—There is a variation in the calculated diffusion constant in each experiment. This is almost certainly due to the difficulty of accurately determining the infective titre of the fluids by a biological test, and in the calculation the square of the factor S/Co amplifies any experimental error considerably. Nevertheless the variation about the mean of 6 experiments is sufficiently small to warrant the conclusion that a correct estimation of the figure has been obtained.

Molecular Weight of Horsesickness Virus.

From the well-known Stokes-Einstein equation for the diffusion constant of a particle $D = \frac{RT}{N} \cdot \frac{1}{6 \pi r \eta}$

in which R is the gas constant,
T the absolute temperature.
N Avogadro's number,
r the particle radius, and
η the viscosity of the medium,

a diameter of 48·8 m.μ is calculated for horsesickness virus. This value agrees very well with values previously calculated from ultracentrifugation experiments (45·4 m.μ) and ultrafiltration (40-60 m.μ) (Polson etc.), This result indicates that horsesickness virus has no means of movement in solution other than by Brownian motion, in which respect the virus particles behave like giant protein molecules. It was thus of interest to calculate its molecular weight and shape.

The formula derived by Svedberg for calculating the molecular weight of a protein from sedimentation and diffusion data is the following:—

$M = \frac{R\,T\,S}{D\,(1 - V\sigma)}$ where

S is the sedimentation constant,
D the diffusion constant
V the partial specific volume of the substance, and
σ the density of the medium.

From the sedimentation constant $S = 286 \times 10^{-13}$cm./sec./dyne the specific volume V = 0·8 c.c./gm., $D = 0{\cdot}87 \times 10^{-7}$ cm^{2}sec. and $\sigma = 1{\cdot}006$ gm/c.c. a molecular weight of 41,000,000 is calculated. The values of S and V were calculated from the particle size and density previously reported. This value for the molecular weight is slightly lower than the value previously reported, i.e., 44.500,000 (forthcoming article in Nature).

Shape of Horsesickness Virus Particles.

According to Svedberg the molar frictional constant $f = \frac{M(1 - V\sigma)}{S}$

When the molecular weight is known it is possible to calculate what the molar frictional constant, *fo* would be for a compact spherical and unhydrated molecule of the same mass, viz.,

$$fo = 6\,\pi\,\eta\,N\left(\frac{3\,M\,V}{4\,\pi\,N}\right)^{1/3}$$

If the ratio $f/fo = 1{\cdot}0$ the molecules must be spherical in shape, if f/fo, however, is greater than 1·0 the molecules are hydrated or their shape deviate

from the spherical. The influence of shape on the diffusion constant of a substance has been calculated by Herzog, Illig and Kudar (1933) and by Perrin (1936). The following equations have been obtained: —

$$f/fo = \frac{\sqrt{(1-\rho^2)}}{\rho^{\frac{2}{3}} \log \frac{1+\sqrt{(1-\rho^2)}}{\rho}} \qquad (\rho < 1)$$

for oblong ellipsoids, and

$$f/fo = \frac{\sqrt{(\rho^2-1)}}{\rho^{2}/_{3} \; arc \; tan \; \sqrt{(\rho^2-1)}} \qquad (\rho > 1)$$

for oblate ellipsoids.

In these formulae $\rho = b/a$ where b equals the equatorial radius and a half the length of the axis of rotation. When these equations are applied to horsesickness a molar frictional ratio is calculated which when substituted in the above equations yields for $b/a - 0{\cdot}71$ for oblong ellipsoids of rotation and $b/a - 1{\cdot}4$ for oblate ellipsoids of rotation. These values are calculated for unhydrated molecules; they might not be exact but they nevertheless indicate that the virus particles are essentially spherical in shape.

Summary.

(1) A new method for the analytical determination of the diffusion constant of a virus is described.

(2) This method is based upon the amount of virus which diffuses across the interface formed between an infective emulsion and the dispersion medium and the method of calculation is detailed.

(3) From the experimental data collected the diffusion constant is calculated as $0{\cdot}87 \times 10{-}7$ cm^2/sec.

(4) From the diffusion constant the size is calculated as 48·8 m.μ, a figure which corresponds closely to that obtained from ultrafiltration and ultracentrifugation experiments.

(5) By combining the diffusion constant with the sedimentation and partial specific volume data the molecular weight is calculated to be of the order or 41,000,000.

(6) From the above data it is shown that horsesickness virus particles are essentially globular in shape and not threadlike or elongated.

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