

Biochimica et Biophysica Acta, 437 (1976) 589–598

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BBA 27941

DIFFUSION COEFFICIENT AND MOLECULAR WEIGHT OF TYPE 5 ADENOVIRUS BY PHOTON-CORRELATION SPECTROSCOPY

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(Received October 21st, 1975)

(Revised manuscript received March 4th, 1976)

Summary

The technique of photon-correlation spectroscopy (intensity fluctuation spectroscopy) is applied to light scattered from type 5 adenovirus undergoing Brownian motion in solution and the translation diffusion coefficient ($D_{20,w}$) measured to be 0.367 ± 0.003 Fick units. Using Svedberg's equation with previously determined parameters, a molecular weight of $165 \cdot 10^6 \pm 5 \cdot 10^6$ is obtained.

Introduction

Study of the spectrum of intensity fluctuations of laser light scattered from a suspension of particles undergoing Brownian motion, either directly or mixed with a reference beam from the same laser, has provided a new tool for the investigation of diffusion coefficients. The first experiment of this type [1], in common with much of the earlier work, achieved poor signal utilisation as only one frequency component of the scattered light was used at any time. Recently, the technique of photon-correlation spectroscopy, using a signal-processing scheme first proposed by Jakeman and Pike [2] and applied by Foord et al. [3,4], has resulted in simultaneous measurements over a number of frequency components, thus giving maximum utilisation of the scattered light. This enables low concentrations of the material under study to be used or, alternatively, allows much more rapid and accurate measurements than previously achieved. The technique has been applied successfully over a range of molecular

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weights from lysozyme and bovine serum albumin to haemocyanin [3–5] and the Tamm-Horsfall glycoprotein [6].

In the present paper, diffusion coefficient measurements on type 5 adenovirus are described. Combination of this result with the measured sedimentation coefficient and calculated partial specific volume enables the molecular weight to be calculated using the Svedberg equation. It has been shown [5,6] that molecular weights can be obtained rapidly and with good accuracy in this manner.

Since the light scattered by a solution containing a solute of given concentration is proportional to the molecular weight of the solute, this optical technique is particularly suitable for substances with a large molecular weight. It is precisely these substances, with molecular weights of around $100 \cdot 10^6$, whose diffusion coefficients or molecular weights are more difficult to determine with existing techniques such as ultracentrifugation, angular dependent light scattering or osmotic pressure. The technique of photon-correlation spectroscopy which is described below has proved ideally suited to this regime.

Photon-correlation spectroscopy

The measurement of diffusion coefficients by photon-correlation spectroscopy of the intensity fluctuations of scattered laser light was described by Foord et al. [4]. Detailed theoretical and experimental investigation of the factors affecting the shape of the correlation function [7,8] and the accuracy with which a spectral linewidth can be determined [9,10] have shown that the theory of the process is well understood.

In the simple case of a monodisperse substance undergoing only translational diffusion the correlation function of the intensity fluctuations of the scattered laser light has the form

$$g^{(2)}(\tau) = 1 + A \exp(-2K^2 D_T \tau) \quad (1)$$

where A is a constant determined by the various temporal and spatial properties of the recording system, D_T is the translational diffusion coefficient and τ is the delay time. K^2 is the squared magnitude of the scattering vector given by

$$K^2 = \left[\frac{2\pi n}{\lambda} \sin \frac{\theta}{2} \right]^2 \quad (2)$$

where n is the solution refractive index, λ is the laser wavelength in vacuo and θ is the scattering angle. Measurement of the correlation function at a known scattering angle enables the diffusion coefficient to be determined by suitable computation using Eqn. 1 above. Alternatively, a logarithmic plot of the exponential part of the correlation function ($g^{(2)}(\tau) - 1$) will give an observed correlation time ($\tau_c = 2$ [time to $1/C$]) which is related to the diffusion coefficient by the equation

$$\frac{1}{\tau_c} = 1 D_T \cdot K^2 \quad (3)$$

If the solution, however, is not monodisperse, or if rotational diffusion is also present, then the interpretation of the data in terms of the simple model given

above would yield a distorted result. It is therefore important to be able to determine the extent to which an assumed model does apply in any given situation. As will be discussed later, measurement of the correlation function at different angles will reveal the presence of aggregates, nonspecific dust contaminants or rotational diffusion. Thus, it is possible to assign an unambiguous model to describe the process from which one can confidently determine a diffusion coefficient.

Materials and Methods

Cell culture and virus preparation

Adenovirus type 5 (strain Ad. 75) was propagated in monolayers of HeLa cells, grown at 37°C in 20 oz Roux bottles using 199 medium with 10% calf serum supplement and maintained with 199 medium only as previously described [11]. The cells were infected with about 10^4 TCD₅₀ of virus and after about three days, when complete cytopathic effect was established, were harvested, concentrated by low speed centrifugation and then taken up in a minimal volume of Tris/saline buffer (0.15 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane adjusted to pH 7.5 with 1 M HCl) and stored frozen at -4°C. This procedure was carried out on a weekly basis commencing three weeks prior to purification.

Virus purification

Frozen infected cells were thawed, disrupted with fluorocarbon (Arcton 113, ICI Chemicals, London), the debris removed by low speed centrifugation and the extract layered on to a discontinuous gradient consisting of 3 ml CaCl in Tris/saline buffer of bouyant density 1.48 g/ml, 3 ml of 1.36 g/ml and 3 ml of 1.28 g/ml. This was then centrifuged in polycarbonate tubes for 1 h at $94\,000 \times g$ (mean) in an MSR 23 ml swing-out rotor. The base of the tube was punctured with a needle and the appropriate fraction collected in a small glass vial. The CaCl was removed by dialysis against Tris/saline buffer and the virus suspension then centrifuged at $3\,000 \times g$ for 1 h to remove larger aggregates. The clarified preparation was then carefully drawn off, leaving a minimal volume at the bottom of the tube and transferred to a preformed CaCl density gradient in Tris/saline buffer (bouyant density 1.48–1.1 g/ml), and then centrifuged at $200\,000 \times g$ (mean) for 3 h. Only one band of opalescence was observed and this was collected from above using a bent needle and syringe. The virus concentrate was then divided into two portions and exhaustively dialyzed against phosphate-buffered saline (Dulbecco A buffer, i.e. phosphate-buffered saline pH 7.2) or borate-buffered saline (0.05 M borate buffer pH 7.6)

Sedimentation properties of purified virus particles

The sedimentation behaviour of the two samples were studied in an MSE analytical ultracentrifuge using Schlieren optics. Material for analysis was appropriately diluted in phosphate- or borate-buffered saline at relative concentrations of 1, 0.75, 0.5 and 0.25, placed in 20 mm cells and the *s* values obtained by extrapolation to zero concentration. In each case the virus sedimented as a single peak: Run 1, phosphate-buffered saline, $11\,960 \pm 5$ rev./

min, 750 ± 7 S; Run 2, borate-buffered saline, $11\,980 \pm 5$ rev/min, 748 ± 7 S. All runs were carried out at 20°C and the values obtained compare with a previously determined value in buffer of 745 S [11]. The sedimentation coefficient was corrected to zero concentration in water as described by Tanford [12] and calculated to be 773 ± 8 S.

Clarification of buffers and solutions

It was essential that all the virus preparations were free of dust contaminations. The three buffers, Tris/saline, phosphate-buffered saline and borate-buffered saline were initially centrifuged at $180\,000 \times g$ (mean) in 30 ml volumes for 30 min in a Spinco model L ultracentrifuge. The upper three-quarters of the buffer in each centrifuge tube was then carefully removed with a pipette avoiding agitation and stirring of the buffer. Each pipette used was rinsed out three times with its own volume of buffer, withdrawn from the top of the tube while the bottles used for storage were themselves rinsed three times with 0.1 vol. introduced from a washed pipette. The washings were discarded by inversion of the bottles. All other glassware and ancillary equipment was treated in a similar manner to minimise dust contamination. A stock solution of CaCl in Tris/saline buffer used for density gradient purification of the virus was passed through Millipore filters (Millipore Corp., Mass., U.S.A.) of mean pore sizes 1200 and 300 nm sequentially.

Light scattering measurements

The experimental techniques used here have been outlined previously [3,4]. Light from a Spectra Physics 125 He-Ne laser was incident on a spectrofluorimetry cell (1 cm optical path) containing about 0.5 ml of the adenovirus solution. The light scattered from samples covering a range of concentrations between $16\text{ }\mu\text{g/ml}$ and 5 mg/ml was studied at angles between 35°C and 92°C . Photon-correlation analysis was performed using a 'Malvern' K7023 Digital Correlator System (Precision Devices and Systems Ltd., Spring Lane Malvern, Worcs.), which utilises the signal processing techniques described by Foord et al. [3,4]. From the observed correlation functions at each angle the diffusion coefficient was calculated. The equipment and test samples were at room temperature and each measured to $\pm 0.1^\circ\text{C}$ at the beginning and end of each run. When necessary, the correction for temperature employed the equation

$$D_{20} = D_{T_{\text{abs}}} \frac{293}{T_{\text{abs}}} \cdot \frac{T_{\text{abs}}}{20} \quad (4)$$

This correction works well over the range $T = 18\text{--}25^\circ\text{C}$ which is used widely [13] and has been successfully applied by one of us (C.J.O.) for determining D for a number of physically well characterized proteins [3–6]. Each scattering angle was calculated from the angle between the incident and scattered light measured in air. In addition, measurement of the correlation time at each angle, with a sample of haemocyanin (derived from *Murex trunculus*) allowed a scattering angle to be calculated by substitution in Eqns. 1 and 3 above; this calculated value could be expected to agree with the initially measured value [5], and constituted an additional check in the assumed values. This particular protein sample is known to be monodisperse and to have a diffusion coefficient

(at this concentration of 4.5 mg/ml) of 1.07 ± 0.01 Fick units [4,5]. In every case the agreement between the two angle determinations was within the 1% experimental error in the correlation time. Finally an appropriate value for the adenovirus molecular weight was obtained by comparison of the observed scattered intensities for the two substances since the concentrations of both, were known. A similar angular dependence for the scattering from each substance was assumed which for finite particle sizes is not strictly true. However, a useful estimate can be obtained over a wide range of molecular weights.

Results and analysis

The observed autocorrelation functions at angles of $35^\circ 22'$ and $92^\circ 54'$, respectively, for an adenovirus concentration of $23 \mu\text{g/ml}$ are shown in Figs. 1 and 2. These are presented as logarithmic plots of the exponential part of the correlation function obtained by normalising the data using numerical values stored by the correlator. A rotational component would be apparent through curvature of the plot for the $92^\circ 54'$ scattering angle; nonspecific dust con-

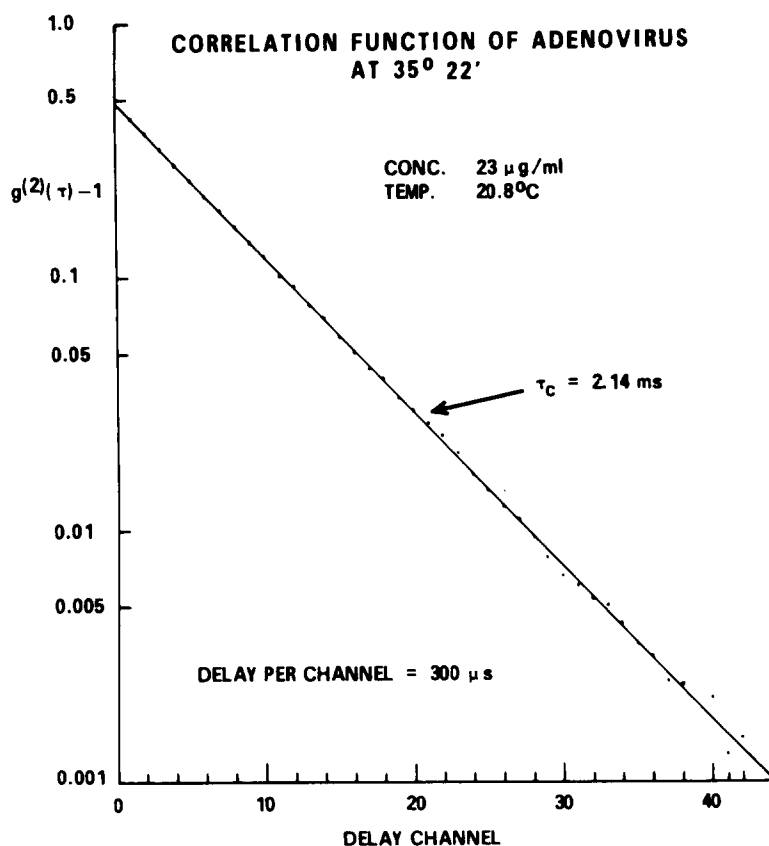


Fig. 1. The exponential part of the observed autocorrelation function ($g^{(2)}(\tau)-1$) for laser light scattered from a $23 \mu\text{g/ml}$ sample of adenovirus at an angle $35^\circ 22'$. The measurement took 150 s and determined D_T with an accuracy of $\pm 1\%$.

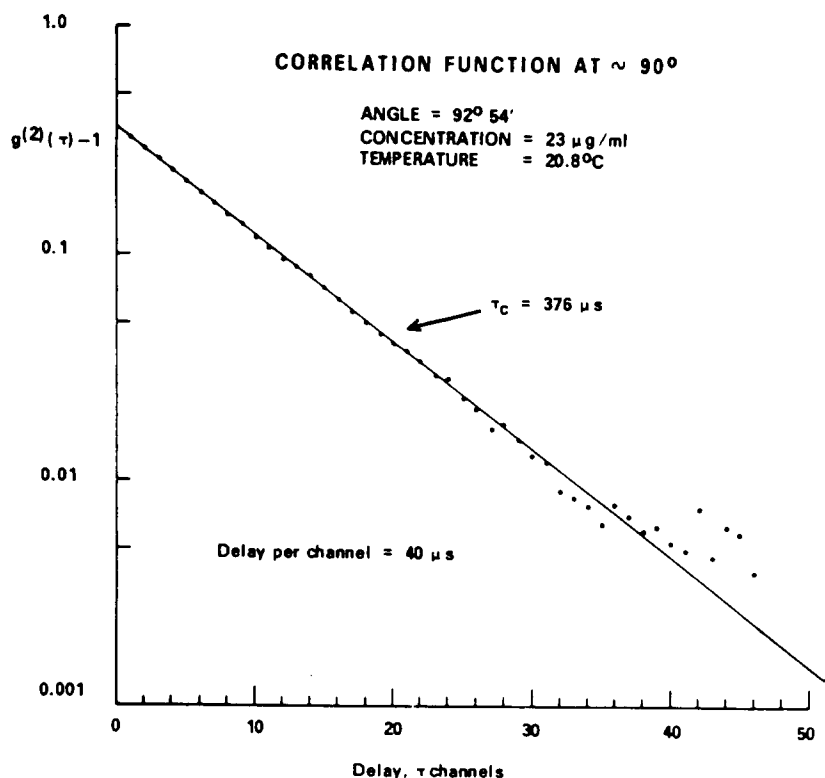


Fig. 2. The exponential part of the observed autocorrelation function ($g^{(2)}(\tau)-1$) for laser light scattered from a 23 $\mu\text{g/ml}$ sample of adenovirus at an angle of $92^\circ 54'$. The measurement took 40 s and determined D_T with an accuracy of $\pm 1\%$.

taminants or large aggregates on the other hand would show as curvature of the $35^\circ 22'$ plot. In this case the plot at both angles is straight, showing that the scattering process is adequately described in terms of a single diffusion process. The absence of contaminants confirmed the efficacy of the preparative techniques used while the absence of rotational effects would be expected since adenovirus is known, from electronmicroscopy, to have a spherically symmetrical icosahedral structure [14].

The angular dependence of the reciprocal of the coherence time (τ_c) of the intensity of fluctuations was measured with a sample concentration of 53 $\mu\text{g/ml}$ (Fig. 3). From Eqn. 3 it can be seen that the dependence of $1/\tau_c$ on K^2 should be linear with a gradient equal to the diffusion coefficient. This can be seen to be the case for the adenovirus sample, confirming the deduction from the data summarised in Figs. 1 and 2 that the scattering corresponded to a single diffusion process. From the angular dependence data the calculated value of the translational diffusion coefficient is found to be $D_{20,w} = 0.367 \pm 0.004$ Fick units. Similar angular distributions at concentrations of 0.4 mg/ml and 23 $\mu\text{g/ml}$ gave results of 0.371 and 0.358 ± 0.004 Fick units, respectively. The latter result probably indicates a systematic error due to slight dust contaminants in the buffer solution, since the result is concentration-dependent.

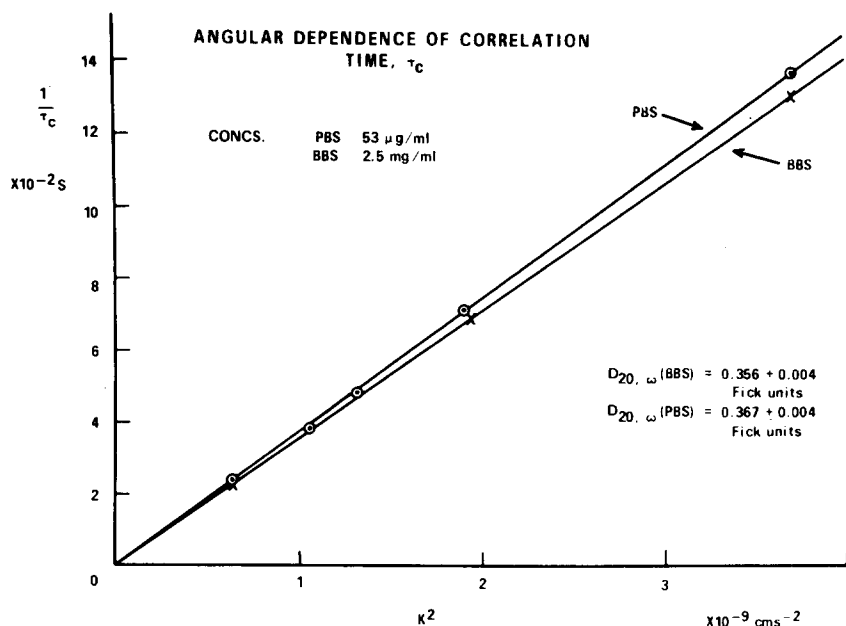


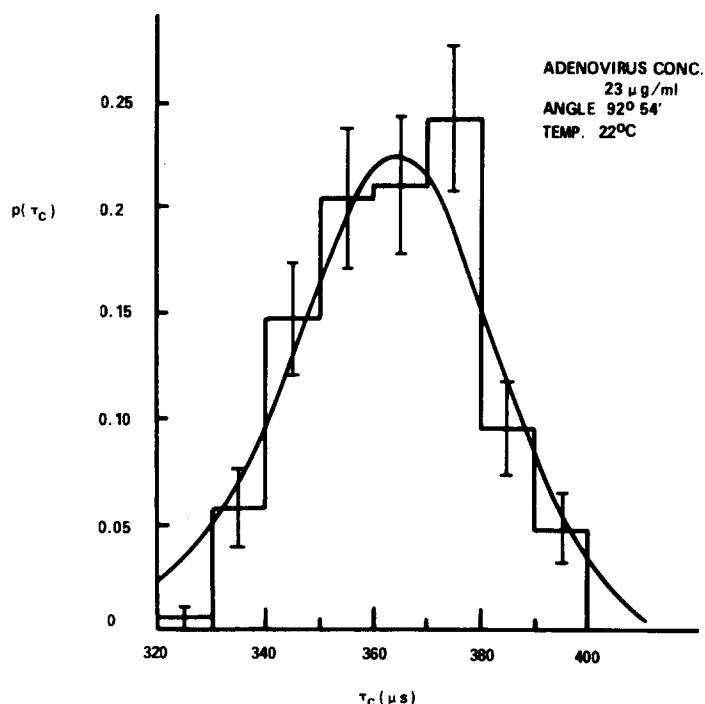
Fig. 3. The dependence of the reciprocal of the fitted correlation time (τ_c) against the scattering vector (K^2) for laser light scattered from a 53 $\mu\text{g/ml}$ sample in phosphate-buffered saline and from a 2.5 mg/ml sample in borate-buffered saline. Each point has an experimental error of $\pm 1\%$.

All the above results were obtained using phosphate-buffered saline. In order to check that the buffer had no untoward effects on the virus structure, the angular dependence in borate-buffered saline was also measured at the concentration of 2.5 mg/ml . This result, included in Fig. 3, was consistent with a single diffusion process with $D_{20, \omega}$ equal to 0.356 ± 0.004 Fick units.

A further indication that the samples are free from contaminants or any other cause of nonstatistical fluctuations can be obtained from the accuracy with which the coherence time can be measured. For this work a coherence area fraction of 0.19 was used which, with an incident laser power of 25 mW, gave a count rate of 15 counts per coherence time at 90° . A series of 192 runs, of 10^4 coherence times each was taken and the results fitted to Eqn. 1. The frequency distribution of the resulting coherence times was plotted as a histogram (Fig. 4). Also included is a theoretical curve showing the expected distribution of these results taken from Jakeman et al. [9,10] and also Hughes et al. [15]. The mean and standard deviation of the 192 runs were $364 \pm 20 \mu\text{s}$ while the predicted standard deviation was $\pm 18.5 \mu\text{s}$, which shows very good agreement between theory and experiment.

Every result quoted in the text is the mean of several runs, each usually ten times longer than those considered above, giving an overall accuracy of $\pm 1\%$ in each value quoted.

The concentration dependence of the diffusion coefficient from 16 $\mu\text{g/ml}$ to 5 mg/ml in phosphate-buffered saline was calculated from the data taken at the $92^\circ 54'$ scattering angle and the asymptotic value of $D_{20, \omega}$ at low concentrations was found to be 0.367 ± 0.003 Fick units. At the lowest concentra-



DISTRIBUTION OF 192 MEASUREMENTS

Fig. 4. The probability distribution, $p(\tau_c)$, of the observed correlation time τ_c for a set of 192 measurements at $92^\circ 54'$ on the sample of concentration $23 \mu\text{g/ml}$. Each run took 4 s and determined the coherence time with an observed standard deviation of 5.5%. The predicted probability distribution, shown as a continuous line, has a standard deviation of 5.1%

tion studied, viz. $16 \mu\text{g/ml}$, a low value of 0.355 ± 0.004 Fick units was obtained, probably as a result of contaminants in the buffer. A single measurement in borate-buffered saline was made and found to be in close agreement with the remainder of the results obtained with phosphate-buffered saline. As the concentration was measured a fall in the diffusion coefficient became apparent at about 0.5 mg/ml , reaching a decrease of about 5% at 5 mg/ml .

Finally, the scattered-intensity measurements over the different angles and concentrations were compared with those for the haemocyanin source. Making the assumption that the effect of the angular dependence differences will be averaged out over the range of angles chosen, the molecular weight was estimated to be $167 \pm 15 \cdot 10^6$.

Discussion

No previous measurements of the diffusion coefficient of adenovirus have been reported. If the value for $D_{20,w}$ determined here is substituted in the Stokes-Einstein equation

$$D_T = \frac{kT}{6\pi\eta r} \quad (5)$$

a Stokes' radius of 58.0 ± 0.6 nm is obtained. The photon-correlation technique is an absolute method of high accuracy yielding the average $D_{20,w}$. An interesting check on the validity of the diffusion coefficient value determined by the photon-correlation method is to use it for the calculation of a 'hard sphere' radius for the adenovirus particle, by determining the anhydrous molecular weight of the particle. The resulting radius can be compared with the radial values determined by electron microscopy. The molecular weight is calculable by substitution of the D_T value in the classical Svedberg equation

$$M = \frac{sRT}{D(1 - \bar{v}p)}$$

where R is the gas constant ($8.31 \cdot 10^7$ ergs), T is the absolute temperature, and $\bar{v}$ is the partial specific volume.

The sedimentation coefficient in water was calculated as 773 ± 8 S and $\bar{v}$ of 0.69 was calculated from the published chemical composition of the virus using a $\bar{v}$ for the virus protein [16] and 0.51 for DNA [17]. On this basis the Svedberg equation yields a molecular weight of $165 \pm 5 \cdot 10^6$ and the corresponding 'hard sphere' radius (treating virus density as $1/\bar{v}$ comes out at 30.5 ± 0.4 nm. This compares surprisingly well with some early estimations of radii of 30–32.5 nm [18] and 35 nm [14,19,20] as determined by electron microscopy. With regard to the molecular weight value, as obtained by velocity sedimentation, it is interesting to compare this value with that of $167 \pm 15 \cdot 10^6$ estimated by direct light scattering measurements, using haemocyanin as a reference standard (Results and Analysis).

If all the data are taken together, it is notable that there is a major discrepancy between the 'hard sphere' radii determined from (a) the anhydrous molecular weight or by electron microscopy, and (b) the Stokes' radii obtained by direct hydrodynamic methods. Similar observations have recently been made for reovirus type 3 and Rous Sarcoma virus using laser light scatter technique [21]. In so far as one set of observations must relate to the hydrated particle, it seems reasonable to assume that the greater part of the discrepancy must lie in the association of water with the virion. If it is assumed that the whole discrepancy is due to the presence of associated water, calculation indicates that this must amount to some 2.3 g of water per g of virus. This is far in excess of the values usually quoted for the probable concentration of protein bound water (based on serum proteins) and one can only assume therefore that it must represent water contained superficially and within the internal spaces of the particle. A similar conclusion was made in the case of the R17 bacteriophage by Pusey et al. [22] who observed a degree of solvation of 1.1 g of water per g of virus by a similar technique, a finding in harmony with early physical studies on the hydrated particles of the icosahedral insect virus, tipula iridescent virus [23].

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