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# CHARACTERIZATION OF T4D VIRUS BY SEDIMENTATION FIELD-FLOW FRACTIONATION

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## SUMMARY

Sedimentation field-flow fractionation (a non-destructive separation method) has been used to measure the molecular weight and density of T4D virus. The molecular weight was obtained under experimental conditions in which the major parameters governing retention in field-flow fractionation were varied to examine their effect on the calculated result. The parameters varied are (1) carrier density, (2) carrier velocity, (3) field strength, (4) injected sample volume and (5) column volume. Retention was not affected by flow-rate or sample volume injected. Effective molecular weights, calculated from a large number of experiments run under different conditions, had a mean and standard deviation of $(100 \pm 1) \cdot 10^6$. The actual molecular weight, subject to greater uncertainty, was determined to be $317 \cdot 10^6$. The density calculated for the virus was 1.47 g/ml.

A study of whether the sedimentation field-flow fractionation technique is destructive or not showed that the infectivity of the virus is affected very little by passage through the channel and detector system.

## INTRODUCTION

Field-flow fractionation (FFF) is a method for separating and characterizing small particles and macromolecules, usually in the size range under 1 μm, but which can be extended to larger particles. The method is based on a coupling between the parabolic velocity profile of a carrier fluid flowing laminarly through a ribbon-like channel and a perpendicular field which compresses suspended particles into layers against one wall of the channel. The mean thickness of each particle layer is determined by some field-coupled property (such as mass) or a combination of such properties of the particles. Those particles concentrated in slower flow regions closer to the wall migrate through the channel more slowly (*i.e.*, they are more retained) than those with greater layer thicknesses which extend into faster flow regions nearer the channel center. Normally, for particles less than 1 μm in diameter, smaller particles travel faster (are less retained) than their larger counterparts. Discussions of the theory and instrumentation of the method were presented by Giddings *et al.*[1].

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Sedimentation field-flow fractionation is a subtechnique using a gravitational or centrifugal field to achieve particle separation and mass characterization[1–4]. Sedimentation FFF has the highest intrinsic resolving power of any of the developed FFF subtechniques. This paper reports the determination of virus density and molecular weight.

Besides characterizing the above physical properties of T4D virus, we have carefully examined the effects of different experimental conditions on the calculated values for the physical properties, with good results. The influence of the sedimentation FFF process on the infectivity of the virus was tested.

Compared with more conventional sedimentation techniques used for analytical purposes, sedimentation FFF has the following advantages: (1) simpler sample introduction and operation, (2) more rapid analysis, (3) more experimental versatility with more properties subject to characterization and (4) advantages in sample detection and collection as a consequence of FFF being an elution technique. The most serious disadvantage of sedimentation FFF is that it is limited to small samples, although this is often not a problem in analytical studies.

In previous work sedimentation FFF has been applied to the separation and characterization of polystyrene latex beads[2–4] and to the determination of molecular weights of two different viruses: T2 virus and gypsy moth nuclear polyhedrosis virus (NPV)[5,6]. The molecular weight of T2 virus determined as a function of the field strength was $(227 \pm 11) \cdot 10^6$ when the amount of virus injected was 0.2 mg and $(236 \pm 7) \cdot 10^6$ when the amount injected was 0.1 mg. In the second study, the effective molecular weight (the actual molecular weight less the equivalent buoyancy molecular weight) of NPV under varying field and flow conditions was found to be $(160.6 \pm 16.0) \cdot 10^6$. In both instances the density of the virus had to be obtained from outside sources in order to calculate actual molecular weights. In the present study the density of the T4 virus is one of the parameters obtained from the experiments. For this purpose we must use carriers with different densities: sodium citrate buffer with pH 7.6 buffer as well as buffer solutions containing sucrose or cesium chloride. The T4 virus densities obtained from outside sources have been used only for comparison purposes. A more detailed study of the influence of carrier density on retention and the methodology for the simultaneous determination of density and particle diameter of spherical particles has been developed using polystyrene latex beads. This work is reported elsewhere[7].

## THEORETICAL

### *Theory of retention*

The basic theoretical framework for FFF systems has been adequately covered in numerous papers (for example, ref. 1) and will not be repeated here in detail. In sedimentation field-flow fractionation the flow channel is accommodated inside a centrifuge basket and is spun with an angular velocity $\omega$. The particles within the channel experience a force $F(r)$ given by

$$F(r) = (\varrho_s - \varrho)\, V \omega^2 r \tag{1}$$

where $\varrho_s$ is the solute or particle density, $\varrho$ is the solvent density, $V$ is the particle

volume and $\omega^2 r$ is the centrifugal acceleration at radius $r$. If we express $V$ in terms of molecular weight $M$ and particle density $\varrho_s$, and use the symbol $G$ for the acceleration $\omega^2 r$ (which can be considered constant across the narrow channel), eqn. 1 gives

$$F = (MG/N)(1 - \varrho/\varrho_s) \tag{2}$$

where $N$ is Avogadro's number. The drift velocity, $U$, for a particle with friction coefficient $f$ is given by

$$U = \frac{F}{f} = (MG/Nf)(1 - \varrho/\varrho_s) \tag{3}$$

The friction coefficient $f$ is given by the Einstein equation:

$$f = \frac{kT}{D} \tag{4}$$

where $k$ is Boltzmann's constant, $T$ is the absolute temperature of the system and $D$ is the particle diffusion coefficient.

The key retention parameter in sedimentation FFF is the dimensionless ratio $\lambda = l/w$, where $w$ is the channel thickness and $l$ is the effective thickness of the solute layer, $l = D/|U|$. Thus

$$\lambda = D/|U|w \tag{5}$$

The reason why $\lambda$ plays a key role is that it links together the experimental measurement of retention volume ($\lambda \approx V^0/6V_r$, where $V^0$ is the channel volume and $V_r$ is the retention volume) and the physico-chemical parameters which give rise to $|U|$ and $D$ in eqn. 5.

*Molecular weight*

Substituting eqn. 4 into eqn. 3 and the latter into eqn. 5, we obtain

$$\lambda = \frac{RT}{|GMw(1 - \varrho/\varrho_s)|} \tag{6}$$

where $R$ is the gas constant, $Nk$. From eqn. 6 we can obtain the equation from which the true molecular weight $M$ can be obtained, provided that the particle density, $\varrho_s$, is known:

$$M = \frac{RT\varrho_s}{Gw\lambda|\varrho_s - \varrho|} = \frac{RT\varrho_s}{Gw\lambda|\Delta\varrho|} \tag{7}$$

*Effective molecular weight*

Rearranging eqn. 7, we obtain

$$M = \frac{|\Delta\varrho|}{\varrho_s} = M' = \frac{RT}{Gw\lambda} \tag{8}$$

where $M'$ is the effective molecular weight, that is, the absolute value of the mass of the particle in Dalton units minus the mass of displaced fluid. $M'$ can be obtained from sedimentation FFF experiments without the benefit of known density values.

*Molecular weight and density*

Using eqn. 7, we can acquire both the density and mass of solute particles by running experiments with different solvent densities. To do so, we convert eqn. 7 into the equation

$$\varrho = \varrho_s \pm \frac{\varrho_s}{M} \cdot M' \tag{9}$$

which shows that a plot of $\varrho$ *versus* $M'$ gives a straight line, the intercept being equal to solute density $\varrho_s$ and the slope being $\varrho_s/M$. The slope is positive when $\varrho_s < \varrho$ and negative when $\varrho_s > \varrho$. The present study concerns the case in which $\varrho_s > \varrho$, that is, the virus density is greater than the carrier density.

## EXPERIMENTAL

*Sedimentation FFF apparatus and operation*

Two centrifugal channels were used in this study, one with dimensions 83.3 × 0.0254 × 2 cm and the other with corresponding dimensions 47.5 × 0.0254 × 1 cm. Most experiments were performed in the former, the system with the greater channel volume. These experiments included studies of the influence on retention of (1) solvent density, (2) solvent velocity, (3) field strength and (4) volume injected. Experiments performed in the second system were used for (1) collection of fractions to test the mildness of the FFF technique and (2) independent calculation of the molecular weight of the T4D virus at a higher rpm (revolutions per minute) value (2200) than available for the first system. In both systems the carrier liquids were pumped into the channels by a Gilson Minipuls 2 peristaltic pump.

Detection in the system with the larger channel volume was accomplished by means of an Altex Model 153 UV detector, operating with a 254-nm light source, and in the second system by a Laboratory Data Control UV detector, Model 1204, also operating with a 254-nm light source. The detector output in both systems was monitored by a Houston Instrument Omniscribe recorder. Rotation rates of the centrifugal channels were determined using a digital interval counter built in this laboratory, which measured the time interval between successive passes of a magnet attached to the basket with respect to a fixed coil.

The collection of fractions for the test of mildness of the FFF technique was performed with a Gilson Model FC-80K micro-fractionator. The temperature was 25°C during the fractionation experiments.

*Preparation of T4D virus*

The host cells used to propagate the T4D virus were of *E. coli* type B growing exponentially at the time of infection in a medium containing Tris–tryptone. After 3 h the infected cells were artificially lysed through addition of chloroform with gentle shaking. After a few minutes of clearing, the phage-bearing supernatant was decanted

from the debris-coated chloroform phase. Remaining debris was removed by low-spin centrifugation for 10 min in a Beckman Spinco ultracentrifuge. The dilute suspension of virus particles was subsequently spun for 4.5 h at 10,000 rpm in a GSA-type rotor in order to pellet the virus. The now clear supernatant was discarded and the pellet left to disperse at a low temperature (4°C) overnight, covered with a small volume of 10 m*M* Tris buffer (pH 7.8) containing 5 m*M* magnesium chloride and 150 m*M* sodium chloride. When suitably dispersed, the sample was layered on to a discontinuous (densities: 1.7–1.5–1.3 g/ml) cesium chloride gradient and spun for 3.5 h at 20,000 rpm in an SW27 swinging-bucket rotor. The virus-containing band was harvested by puncturing the centrifuge tube at the location of the band. The collected sample was subsequently dialyzed overnight at 4°C in the above-mentioned Tris buffer to eliminate the gradient former.

*Test of infectivity*

Each sample to be tested was given a series of 10-fold dilutions. For each test a 0.1-ml portion of the virus sample was added to 2.5 ml of soft agar dissolved in growth medium. To this mixture were added a few drops of an *E. coli* B suspension and the mixture was then quickly poured over an agar plate and incubated overnight at 30°C. For each sample the dilution yielding between 50 and 350 plaques per plate was used as a basis for determining the total number of infectious units in that fraction.

*Materials*

In addition to the T4D virus, the materials used and their purpose were as follows:

(i) The carrier solution in all FFF experiments involving T4D phage was 0.1 *M* sodium citrate containing 0.005 *M* magnesium chloride and adjusted to pH 7.6 with nitric acid. All chemicals were of reagent grade and solutions were prepared with doubly distilled water.

(ii) Sucrose (reagent grade) from Amachem (Portland, OR, U.S.A.) and cesium chloride (optical grade, ultra pure) from NBC (Chicago, IL, U.S.A.) were used for the preparation of solutions with densities different from that of the sodium citrate buffer solution. Densities were determined pycnometrically to be 1.004 g/ml for sodium citrate buffer, 1.035 g/ml for sucrose solution in sodium citrate buffer and 1.038 g/ml for cesium chloride solution in sodium citrate buffer.

(iii) Polystyrene latex beads with a diameter of 0.357 $\mu$m from Dow Diagnostics (Indianapolis, IN, U.S.A.) were used for confirmation of the performance of both FFF systems prior to the virus runs.

## RESULTS AND DISCUSSION

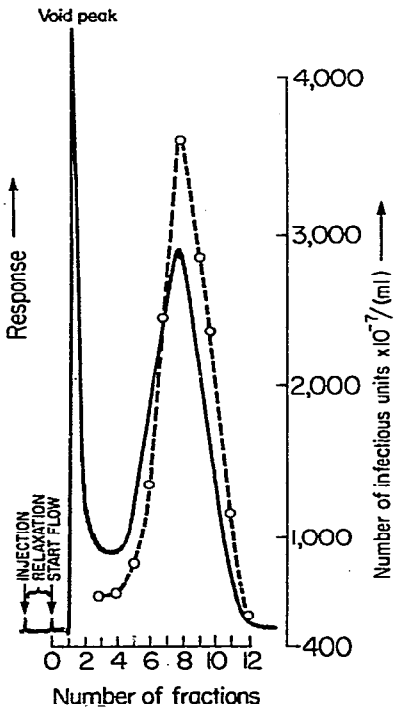
*Persistence of infectivity*

A sample analysed by sedimentation FFF must maintain its integrity through injection into the channel and passage through the length of the channel, both of which subject the sample to moderate but generally controllable shear stresses. Finally, the sample must exit the channel through a special (and possibly high shear) seal which leads the sample material into the stationary detector and/or fraction collector. For physico-chemical measurements (molecular weight, density, etc.), the sample

need not maintain its viability or even its identity in passage through the seal, as long as detectable fragments reach the detector to signal the nature of the particles' migration through the channel. However, for biological tests, biological viability must be maintained. A major object of this work was to test for viability by measuring the persistence of T4D infectivity upon passage through the sedimentation FFF apparatus, especially the seal.

There is a narrow zone of high shear in the seal between its moving and stationary parts. Within the seal there is a frictional sliding between a spinning rubber O-ring and a stationary chrome-plated shaft, and it could be argued that passage through the seal would involve exposure to a narrow zone of elevated temperature. Any damage to the eluting particles would, of course, not be detected by the UV monitor in line with the channel, and it is therefore important to use a functional criterion as evidence for the structural integrity of the virus particles after passage through the FFF system.

Using a standard plaque test, the number of infectious units contained in a 50-$\mu$l injection was estimated to be $24 \cdot 10^{10}$. The effluent from the spinning column was collected in twelve fractions, each of 2-ml volume. The infectivity distribution following fractionation was determined by the method discussed earlier and can be seen in Fig. 1. About 75% of the injected infectivity is accounted for in the collected fractions. In view of the large uncertainties involved in this test, this number should not



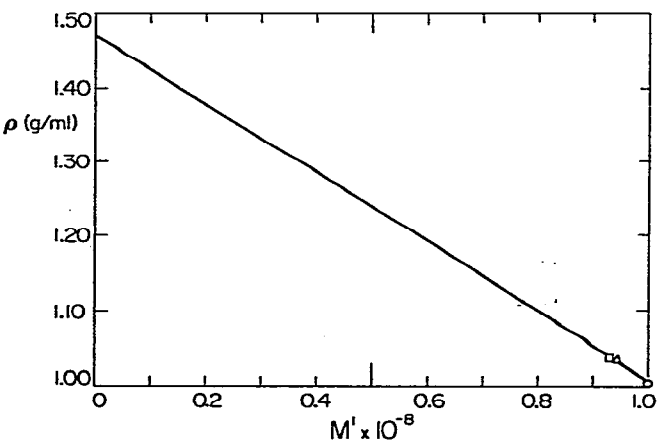




Fig. 1. Absorbance (254 nm) (solid line) and infectivity distributions of T4D virus (broken curve) *versus* number of collected fractions.

Fig. 2. Plot of solvent density, $\varrho$, *versus* $M'$. ○, Sodium citrate buffer; △, sucrose solution; □, cesium chloride solution. Solid line, least-squares fit.

be taken literally but rather to indicate that no significant destruction of the T4D virus takes place during an FFF run.

*Molecular weight parameters*

The main object of the molecular weight work was to ascertain the self-consistency of molecular weight parameters measured under different conditions. However, we attempted a few experiments with our newly developed technique for determining density and molecular weight simultaneously. This technique has been shown to work very effectively with colloidal polystyrene latex beads[7]. The full exploitation of the technique with T4D was hindered by the limited amount of sample remaining after the extensive tests described below, and by the fact that several T4D samples were unexpectedly disintegrated by exposure to sucrose solution of density $> 1.04$ g/ml. (The disintegration was readily apparent because the viral material all eluted in the void peak, in which only particles and molecules of sub-viral mass appear.) Nonetheless, we give our limited results here because we feel the methodology has considerable promise in future virus characterization work.

*Determination of virus density and molecular weight.* Fig. 2 shows a plot of $\varrho$ *versus* $M'$, which, according to eqn. 9, should be a straight line. From the intercept we find a virus density of $1.47 \pm 0.08$ g/ml. The true molecular weight determined from the slope is $317 \cdot 10^6$. The effective molecular weight (in the sodium citrate buffer) calculated from this plot is $100.2 \cdot 10^6$. There is considerable scatter in published density values for the T-even bacteriophages, with data ranging from 1.51 mg/ml[8,9] to 1.67 mg/ml[10]. Our density value for T4D of 1.47 mg/ml falls reasonably close to the lower end of this range, which is gratifying in view of the fact that our experimental value was obtained from the plot in Fig. 2 with only three experimental points by extrapolating the least-squares fitted line from the low density values of the carriers to the higher virus density.

We believe that much more accurate determinations of virus density could be made by this FFF technique if carrier solutions could be found spanning a considerably larger density range than the *ca.* 0.04 g/ml range employed here. With polystyrene latex beads, for example, we determined the particle density to be $1.047 \pm 0.0016$, in which the precision is 50 times better than the 0.08 g/ml reported here.

Once the viral density is known, the determination of molecular weight can be made with considerable precision. The results are consistent with one another over a wide range of experimental conditions, as illustrated below.

*Effect of solvent velocity on measured molecular weight.* Calculated values of the effective and true molecular weights of T4D virus at different flow-rates of carrier, $\langle v \rangle$, are given in Table I. The true molecular weight is calculated on the basis of several different densities, as shown in the table. Also given are the average molecular weights with their standard deviations. The calculated molecular weights are nearly constant over the 2-fold velocity range for any one assumed density value, standard deviations being only about 1% of the mean.

In all runs the carrier used was a sodium citrate buffer of pH 7.6. The rpm was constant at 1800.

*Effect of field strength on molecular weight.* Values determined for effective and true molecular weights of the T4D virus at different field strengths are given in Table II. The corresponding flow-rates of carrier, $\langle v \rangle$, are also given. The calculated mo-

TABLE I

MOLECULAR WEIGHTS OF T4D VIRUS CALCULATED AT DIFFERENT CARRIER VELOCITIES

The effective molecular weight, $M'$, is calculated from eqn. 8. The true molecular weight, $M$, is calculated from eqn. 7 based on the three different values of assumed density shown. Spin rate = 1800 rpm.

| $\langle v \rangle$ (ml/h) | $M' \cdot 10^{-6}$ | $M \cdot 10^{-6}$ | | |
|---|---|---|---|---|
| | | $\varrho_s = 1.51$ g/ml | $\varrho_s = 1.67$ g/ml | $\varrho_s = 1.47$ g/ml |
| 21.8 | 102.13 | 302.78 | 256.08 | 322.16 |
| 28.1 | 99.28 | 294.33 | 248.94 | 313.17 |
| 29.7 | 101.18 | 299.96 | 253.70 | 319.16 |
| 34.2 | 101.18 | 299.96 | 253.70 | 319.16 |
| 38.4 | 99.28 | 294.33 | 248.93 | 313.17 |
| 43.2 | 101.65 | 301.37 | 254.89 | 320.66 |
| Averages | $\bar{M}' = 101 \pm 1$ | $\bar{M} = 299 \pm 4$ | $\bar{M} = 253 \pm 3$ | $\bar{M} = 318 \pm 4$ |

TABLE II

MOLECULAR WEIGHTS OF T4D VIRUS CALCULATED AT DIFFERENT FIELD STRENGTHS

| Rpm | $\langle v \rangle$ (ml/h) | $M' \cdot 10^{-6}$ | $M \cdot 10^{-6}$ | | |
|---|---|---|---|---|---|
| | | | $\varrho_s = 1.51$ g/cm$^3$ | $\varrho_s = 1.67$ g/cm$^3$ | $\varrho_s = 1.47$ g/cm$^3$ |
| 2200 | 39.8 | 99.37 | 294.52 | 249.17 | 313.46 |
| 1800 | 38.4 | 100.78 | 298.70 | 252.71 | 317.91 |
| 1700 | 39.1 | 100.65 | 298.32 | 252.38 | 317.50 |
| 1600 | 39.8 | 99.80 | 295.78 | 250.24 | 314.80 |
| 1500 | 39.2 | 99.18 | 293.97 | 248.70 | 312.87 |
| Averages | | $\bar{M}' = 100 \pm 1$ | $\bar{M} = 296 \pm 2$ | $\bar{M} = 251 \pm 2$ | $\bar{M} = 315 \pm 2$ |

lecular weights show standard deviations of less than 1 % of the mean. Sodium citrate buffer was again used as the carrier.

It is worth noting that although the run at 2200 rpm was performed in the channel with a smaller column volume, the molecular weight calculated from the data of this run is in excellent agreement with values calculated from runs in the other system with greater volume and a lower rpm range.

Fig. 3 is a series of fractograms showing how the retention of T4D virus changes with spin rate. As the field strength increases, the resolution also increases, as predicted from theory[1]. Despite the considerable change in retention, the molecular weight parameters calculated from retention remain constant, as just shown.

*Effect of sample size.* In all of the previously described runs used for density and molecular weight calculations, the amount of virus sample injected was 50 $\mu$l. In order to determine if there is any dependence of retention on sample size, a run was performed with 25 $\mu$l of injected sample at 1800 rpm. The effective molecular weight was calculated to be $100.6 \cdot 10^6$, which is consistent with the previously obtained values.

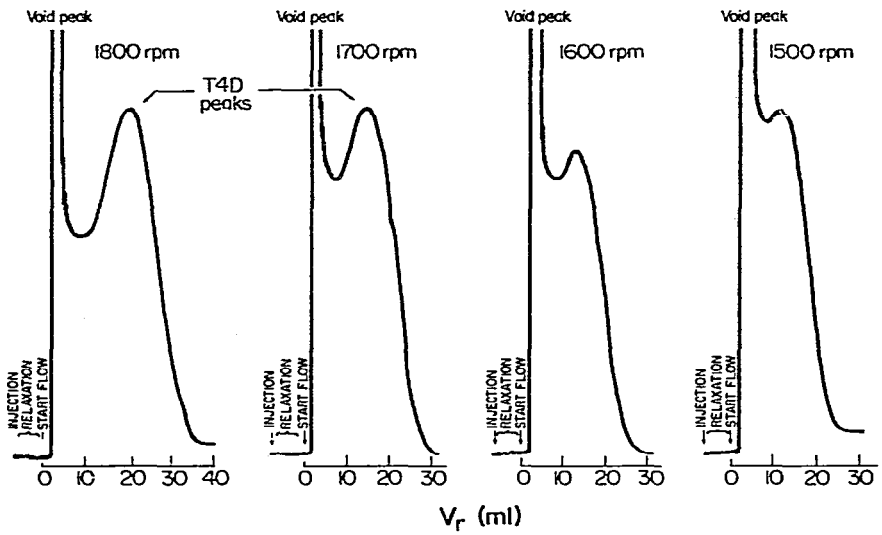



Fig. 3. Changing retention of T4D virus at different field strengths. Horizontal axis represents elution volume. Flow-rate *ca.* 39 ml/h.

*Overall consistency of molecular weight determinations.* As a check of self-consistency, the average effective molecular weight determined under all FFF conditions (variable field strength, solvent velocity, sample size and different centrifuged channels) was calculated together with its standard deviation. The value is $(100 \pm 1) \cdot 10^6$, which is satisfactory.

We conclude that sedimentation FFF is an excellent technique for the determination of molecular weights of virus particles, yielding consistent values under many different experimental conditions. Unfortunately, values in the literature for the true molecular weight of the T4 virus are scattered in the range $180 \cdot 10^6$–$300 \cdot 10^6$, and offer little aid in judging the accuracy of our experimentally derived values[9–12].

The book *Comprehensive Virology* lists $220 \cdot 10^6$ daltons as an approximate value for the molecular weight of T-even bacteriophages[13]. The same book lists a value for the volume of the virus head, $3.5 \cdot 10^5$ nm$^3$, which was obtained from electron microscopic measurements. If the particle density is assumed to be 1.47 g/ml, the virus head alone would account for a molecular weight of $318 \cdot 10^6$ daltons. Only an unrealistically low particle density of 1.01 g/ml would make the reported values for head volume and molecular weight compatible with one another. A proper accounting for the virus tail would consequently require an even lower density value in order for volume and molecular weight values to correspond to one another.

A recent paper[14] lists an even larger volume for the T2 phage ($5.10 \cdot 10^5$ nm$^3$), which was determined by the resistive pulse technique.

Much attention has been given over the years to the two forms of T2 phage which can be distinguished by their different sedimentation behavior in the ultracentrifuge under different conditions of pH and divalent metal ion concentration[15]. Whereas some workers ascribe these differences to changes in molecular weight[10] or head shape[9], others suggest that the differences are due to conformational changes in the viral tail[16].

The T4B phage is also reported to exist in forms with different sedimentation coefficients[17].

The current estimate of molecular weight for T4 phage differs considerably from a value for T2 phage determined earlier in this laboratory[5]. The earlier determination was performed using a carrier solution which contained EDTA. The sample was a commercial preparation supplied as a suspension in the same type of EDTA-containing buffer. As the presence of $Mg^{2+}$ ions stabilizes the viral structure, the $Mg^{2+}$ complexing agent (EDTA) was eliminated from all carriers in the present study in which buffers were instead made 5 m*M* with respect to magnesium chloride. A partial disruption of virus particles in the T2 preparation would show in sedimentation FFF as a lower average molecular weight.

The wealth of scattered information on the molecular weight of the T-even phages indicates that additional work is needed in the comparison of diverse techniques and the assessment of relative accuracy.

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