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# SIZE EXCLUSION AT THE EXTERNAL SURFACES OF SPHERICAL PACKINGS USED IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Experimental observations from several laboratories, including our own, have demonstrated that the elution volume of very large macromolecules or particles shows a small dependence on size even when the packing gels are impermeable to these substances. We call this process "external size exclusion", but it has also been termed "hydrodynamic chromatography".

We have derived a quantitative expression for the phenomenon based on an equilibrium process in which spherical macromolecules of radius $r$ pass through a column filled with uniform spheres of radius $R$ in hexagonal close packing. The final expression

$$V_e/V_g = 1 - \frac{\pi}{3\sqrt{2}} - \frac{\pi}{\sqrt{2}}\frac{r}{R} + \sqrt{2}\,\pi\left(\frac{r}{R}\right)^2 + \frac{5\pi}{3\sqrt{2}}\left(\frac{r}{R}\right)^3$$

gives the ratio of the elution volume $V_e$ of a sphere of radius $r$ to the total volume of the column $V_g$. The range of practical usefulness is $0.01 < r/R < 0.1$. "Premature" elution of aggregated tobacco mosaic virus is explained by the use of this equation.

## INTRODUCTION

Early theories of Sephadex gel filtration were based on the concept that next to any surface, there is an element of volume from which molecules are excluded, and that the thickness of this element of volume for spherical macromolecules is equal to the radius. One need only to accept the principle that the elution volume for a given macromolecule is equal to the volume available to that macromolecule within the column in order to understand the size exclusion separation process. These principles were clearly described in terms of simple geometrical models in which the interior of the gel beads was treated as an assembly of cones[1] as well as one of rods, cones and cylinders[2]. This general mechanism of separation is now widely[3] if not universally

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accepted. Other mathematical relationships between elution volume and molecular weight have been reviewed[3].

It is a common practice to define the elution volume of a macromolecule that is too large to enter the gel as $V_0$, and to consider this as equal to the interstitial volume between the spherical packing materials. This practice was well justified when dealing with Sephadex, where the practice originated, since the swollen beads typically have diameters in excess of 100 $\mu$m. New packing materials for size exclusion chromatography (SEC) are much smaller. In fact, we have learned that spherical packings of 3 $\mu$m diameter are being tested for high-performance liquid chromatographic (HPLC) applications prior to marketing. For small packing spheres, the external surface area, while still small compared with the internal surface area, may no longer be negligible and size exclusion at these external surfaces may begin to contribute to the separation process.

We have recently developed[4] a new method of analysis of SEC data which gave better linearity between parameters of elution volume and molecular size, at least for the Toyo Soda G-3000 SW analytical column we were using, and also appeared to give additional insight into the size exclusion process. When we applied this method of analysis to data[5] from a Toyo Soda G-5000 PW preparative column, we found that tobacco mosaic virus (TMV) eluted substantially earlier than three other viruses which we presumed to be adequate probes of $V_0$. It seemed possible that external size exclusion was responsible for this early elution. In order to test this hypothesis, and in view of the more general applications referred to above, we have developed theoretical equations for external size exclusion and present them here in the expectation that they might prove useful.

The phenomenon to which we refer as external size exclusion was probably first recognized by Pedersen[6] who obtained separations of proteins by passing solutions through columns packed with solid glass beads. More recently, Small[7] has shown that polystyrene latex spheres of different sizes have different elution volumes when passed through columns packed with small spheres which are impermeable to the latex particles. He has proposed the term hydrodynamic chromatography for the process. While our treatment is based on an equilibrium process, his is based on the velocity profile of the liquid passing through the beads. Regardless of the mechanism involved, it is clear that we are referring to the same phenomenon.

## THEORY AND METHODS

Our calculations are based on a model consisting of a column packed with spheres of radius $R$ in hexagonal close packing. It is our objective to calculate the elution volume of a spherical macromolecule of radius $r$ which is too large to enter the gel. Thus, for the purpose of this calculation we can treat the spheres as though they were solid. We define[5] $V_g$ = the geometrical volume of the empty column, $V_t$ = the volume of the packed column available to water, $V_s$ = the volume occupied by the solid components of the packing matrix and unavailable to water, $V_i$ = the volume inside the spheres which is available to water, $V_0$ = the interstitial volume outside the packing spheres, $V_e$ = the elution volume of a macromolecule or stable supramolecular aggregate of radius $r$.

Thus

$$V_g = V_0 + V_i + V_s \text{ and } V_t = V_0 + V_i$$

$V_g$ can be measured unambiguously as the volume of the empty column, or more precisely, the volume between the injector and the detector. For measurement of $V_t$, $^3H_2O$ or $NaN_3$ may be used and even an injection of $H_2O$ provides a signal in buffers containing electrolyte, but these do not[5] always provide identical values and judgements must be made. Appropriate probes of $V_0$ also present a problem which we propose to treat here.

Previous treatments of SEC, including a recent one of our own[5], have confined themselves to processes involving spherical macromolecules of radius $r < r_c$ where $r_c$ is the radius of a macromolecule of a critical size barely excluded from the gel. This value can be calculated quite precisely from the intersection of two lines when the data are calculated as we have proposed[5]. It is our object here to calculate the elution volume of spheres of $r > r_c$. We seek an expression of the form

$$V_e/V_g = 1 - \frac{V_s + V_i}{V_g} - \frac{V_{ex}}{V_g} \tag{1}$$

where $V_{ex}$ is the volume at the external surfaces of the spheres from which spheres of radius $r > r_c$ are excluded. The term $(V_s + V_i)/V_g$ is simply the volume fraction occupied by spheres in hexagonal close packing. Its value is $\pi/3\sqrt{2}$ and is well known from crystallography and appears in standard textbooks of Physical Chemistry (*e.g.* ref. 8). It can be simply calculated from a tetrahedral model of $n$ spheres on a side where $n$ is a very large number and edge effects can be ignored.

The additional volume which is unavailable to a macromolecule of radius $r > r_c$ is calculated in two steps. In step 1, we recognize that for each sphere, there is an unavailable volume equal to that of a sphere of radius $R + r$. Thus the volume lost per sphere due to external size exclusion is

$$\frac{4\pi}{3}(R + r)^3 - \frac{4\pi}{3}R^3 = 4\pi R^2 r + 4\pi R r^2 + \frac{4\pi r^3}{3}$$

This is equivalent to increasing $V_s + V_i$ by the factor

$$\frac{4\pi}{3}(R + r)^3 \Big/ \frac{4\pi}{3}R^3 = \left(1 + \frac{r}{R}\right)^3$$

and increasing $(V_s + V_i)/V_g$ to $\pi(1 + r/R)^3/3\sqrt{2}$. We now have

$$V_e/V_g = 1 - \frac{\pi}{3\sqrt{2}}(1 + r/R)^3 \tag{2}$$

In this equation we have neglected the fact that the spheres are in contact. We correct for this in step 2. At each point of contact between two spheres, the increase in

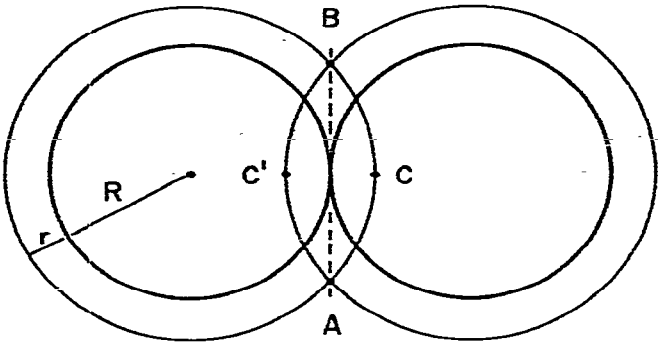


Fig. 1. Surrounding the contact point between two spheres of radius $R$, there is a lens-shaped volume of overlap ACBC′ in the excluded volume elements of thickness $r$ around each sphere.

radius from $R$ to $R + r$ creates an element of overlapping volume which has the shape of a lens, *i.e.* lens ACBC′ in Fig. 1. The volume of the half-lens, ABC, is given by

$$\int_R^{R+r} \pi\{(R + r)^2 - x^2\}dx = \pi\left(Rr^2 + \frac{2r^3}{3}\right)$$

Since each sphere touches 12 others in hexagonal packing, the volume correction for overlap, expressed relative to the volume of the packing sphere is

$$\frac{\Delta V}{V_{sph}} = \frac{12\pi\left(Rr^2 + \frac{2r^3}{3}\right)}{4\pi R^3/3}$$

Since $\Delta V/V_g = \Delta V/V_{sph} \cdot V_{sph}/V_g = \Delta V/V_{sph} \cdot \pi/3\sqrt{2}$, we have

$$V_e/V_g = 1 - \frac{\pi}{3\sqrt{2}}(1 + r/R)^3 + \frac{9\pi}{3\sqrt{2}}\left(\frac{r^2}{R^2} + \frac{2r^3}{3R^3}\right)$$

Expanding and collecting terms we have

$$V_e/V_g = 1 - \frac{\pi}{3\sqrt{2}} - \frac{\pi}{\sqrt{2}}\frac{r}{R} + \sqrt{2}\,\pi\left(\frac{r}{R}\right)^2 + \frac{5\pi}{3\sqrt{2}}\left(\frac{r}{R}\right)^3 \qquad (3)$$

This equation has a convenient form. It is a function only of $r/R$, and therefore is dimensionless. Since $r/R < 0.1$, the final term can be omitted. In the limit of small $r/R$, $V_e/V_g = 0.2595 = 1 - \pi/3\sqrt{2}$ and at $r/R = 0.1$, we have $V_e/V_g = 0.0855$. In other words, the apparent void volume would be reduced by 70% by using a spherical macromolecular probe of a radius one-tenth that of the packing spheres. We consider this to be near the upper theoretical limit of the contribution due to this effect. In practice, macromolecules of sizes approaching this value might be trapped due to packing irregularities and might not pass through the column at all.

We next inquire into the consequences of applying this calculation to results

obtained from SEC experiments carried out in columns packed with spheres that are neither uniform in size nor hexagonally close packed. If one is reasonably confident that he has measured the elution volume of a macromolecule of a size equal to or not much larger than the critical size too large to enter the gel, he can use this value as a provisional estimate of $V_0$. If one should also find that $V_0/V_g$ is not greatly different from 0.2595, he can probably assume that perhaps due in part to compensating effects the properties of the column are equivalent to that of the model, and calculations of the type illustrated in eqn. 3 can be made with reasonable confidence. We next illustrate the use of this equation in the analysis of the data[5] that provided the primary motivation for deriving an expression for the external size exclusion effect.

*Application of theory to published data*

In an earlier publication[4], we derived two equivalent equations, numbers 3 and 4, and pointed out that preference would depend on whether $V_0$ or $V_t$ could be determined with greater accuracy. Experience has shown that this is true of $V_t$. As a consequence, we intend[9] to use eqn. 4 in the future. Thus our analysis is based on the equation

$$\frac{V_e^{1/3} - V_t^{1/3}}{V_0^{1/3} - V_t^{1/3}} = \frac{(t_e/t_t)^{1/3} - 1}{(t_0/t_t)^{1/3} - 1} = F_{(v)} = \frac{M^{1/3} - M_A^{1/3}}{M_C^{1/3} - M_A^{1/3}} = \frac{r - r_A}{r_C - r_A} \qquad (4)$$

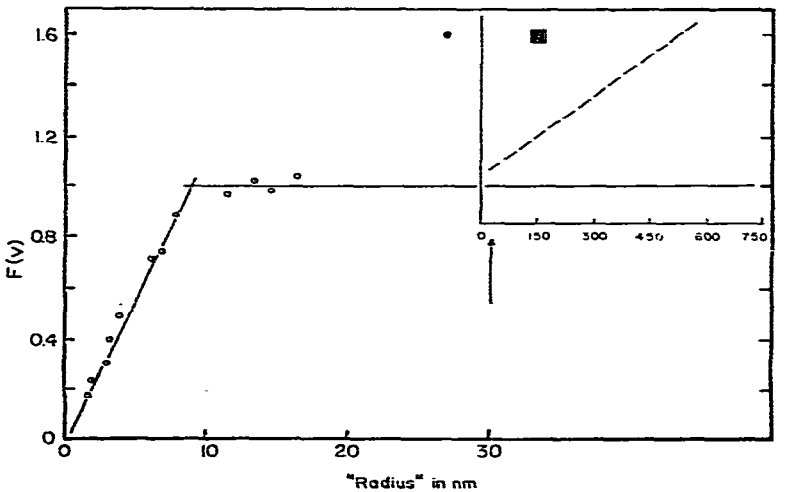


Fig. 2. F(v) as calculated by eqn. 4 is plotted against "molecular radius" which in turn, has been calculated in several ways. For symbols indicated by circles, we have calculated the radius expected for a compact sphere of average hydration using the equation[6] $r(\mu m) = 0.0882\ (M\bar{v})^{1/3}$ where $\bar{v}$ is the partial specific volume. The square refers to half the length of the TMV particle. The diagonal line was calculated from least squares analysis, and the horizontal solid line at F(v) = 1 is that expected for $V_e = V_0$ if external exclusion is neglected. The dashed line is calculated from the equation $V_e/V_g = 0.478 - 2.22\ r/R + 4.44(r/R)^2 + 3.7(r/R)^3$ using the value $R = 8.5\ \mu m$ as the average radius of the packing spheres (data supplied by Toyo Soda, Japan). We have substituted the value $V_0/V_g = 0.478$ for the term $1 - \pi/3\sqrt{2}$ in eqn. 3. This substitutes a term calculated from experimental data for the corresponding term calculated for hexagonal close packing. In calculating F(v) theoretical values of $V_e/V_g$ were converted to $V_e/V_t$ by use of the identity $V_e/V_t = V_e/V_g \cdot V_g/V_t$.

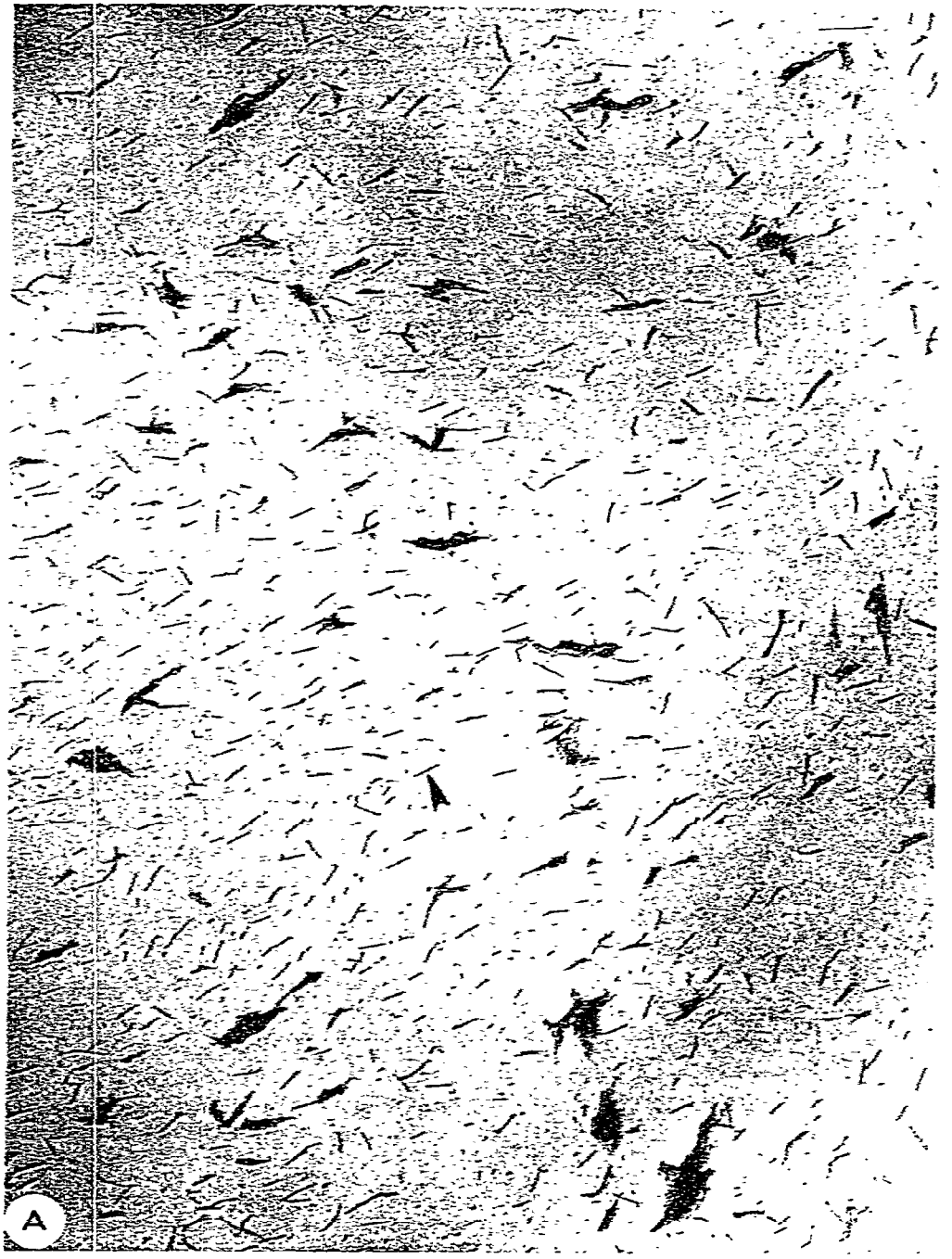


Fig. 3.

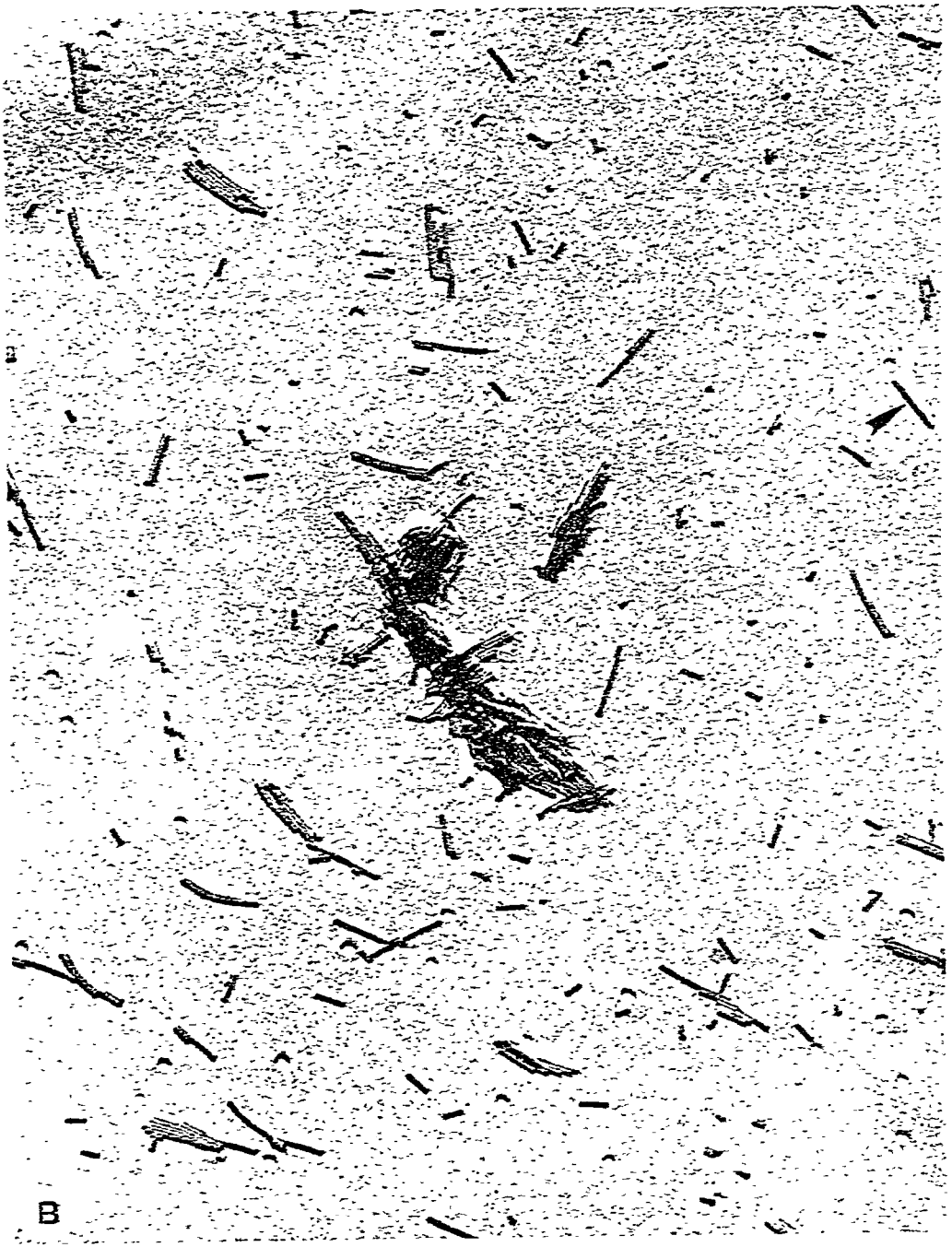


Fig. 3. The original TMV preparation was diluted 300X in the same buffer used for HPSEC. A 2.5-$\mu$l volume of this solution was placed on 200-mesh copper grids coated with 0.5 mg/ml polylysine (> kdaltons) and 0.1 mg/ml PEG (>20 kdaltons). The final preparation was shadowed with Pt at 25°C, 175 Hz. Magnification is 11,000 × (A) and 37,000 × (B).

The function F($v$) is calculated from the ratio, $t_e/t_t$, of the elution time of a protein of molecular weight $M$ to that of a small molecule[4] and $t_0/t_t$ is the corresponding ratio for a molecular probe of $V_0$. The two expressions on the right hand side relate F($v$) to molecular parameters, thus $M_A$ and $r_A$ denote the limiting value of molecular weight and radius of molecular probes of $V_t$ and $M_C$ and $r_C$, the limiting values corresponding to $V_0$. These parameters are determined graphically[9]. The original data[5] have been plotted according to eqn. 4 in Fig. 2. The data fall into three classes. The data for a series of 8 globular proteins ranging from cytochrome $c$, 12,400 daltons to apoferritin $0.96 \cdot 10^6$ daltons fall on or near a straight diagonal as predicted by eqn. 4. Three globular viruses, turnip yellow mosaic virus (TYMV) of $5.4 \cdot 10^6$, southern bean mosaic virus (SBMV) of $6.6 \cdot 10^6$ and tomato bushy stunt virus (TBSV) of $8.9 \cdot 10^6$ and sea worm chlorocruorin $2.9 \cdot 10^6$ daltons, respectively, all appear to scatter randomly around the horizontal line suggesting that any one of them might be an adequate probe for $V_0$, and we took the mean value of the four as our best estimate for that parameter. The final data point corresponds to TMV 0.018 $\mu$m in diameter and a length which can depend on the past history of the sample, but for freshly and carefully prepared samples, lengths of 300 nm have by far the highest of frequency. This corresponds to a molecular weight of $39.4 \cdot 10^6$ daltons.

We next inquire as to whether our calculations based on hexagonal close packing can be safely applied to these data. The total volume $V_g$ and the void volume $V_0$ were found[5] to be 228 and 109 ml, respectively. Thus $V_0/V_g = 0.48$, rather poor agreement with the value 0.26 expected for hexagonal close packing. In contrast the TSK G 3000 SW column which we have used, had values of $V_0 = 8.11$, $V_g = 26.50$ and $V_0/V_g = 0.31$ (ref. 4). Calculations we have made from Sephadex columns have repeatedly yielded approximately the same ratio. Thus it is evident that the column used in these experiments is somewhat loosly packed, and errors will be introduced by using calculations based on hexagonal close packing, but since these are the only data available of the type we require and which indicate size exclusion, we will proceed with the analysis.

As seen in Fig. 2, treating TMW as a compact sphere (the circle) gives poor agreement with the theoretical predictions indicated by the dashed line. If we consider the rod as rotating rapidly due to Brownian motion and assume that it behaves hydrodynamically as a sphere of radius half its length (the square) the agreement is improved somewhat, but a macromolecule which behaved hydrodynamically as a sphere of radius 500 nm would be required for agreement with theory.

At this point, chronologically, we were curious as to whether the TMV in the sample might actually be present as very large aggregates with dimensions of approximately 1 $\mu$m. The sample had been prepared by Professor H. S. Loring in 1974 and was made available to us by Dr. A. T. Tu. It had been stored in phosphate buffer since its preparation. Electron micrographs of the sample are shown in Fig. 3A and B. In Fig. 3A, we see many rods with the expected length of 300 nm, and a few fragments, but most important observation is that approximately half of the virus is in fact in the form of large aggregates. One of these aggregates is shown in higher magnification in Fig. 3B.

While no extensive calculations have been attempted, it seems likely that the theory of size exclusion plotted in Fig. 2 permits us to interpret the elution diagram in Fig. 4 in terms of the size distribution shown in Fig. 3A. Thus the peak appearing at

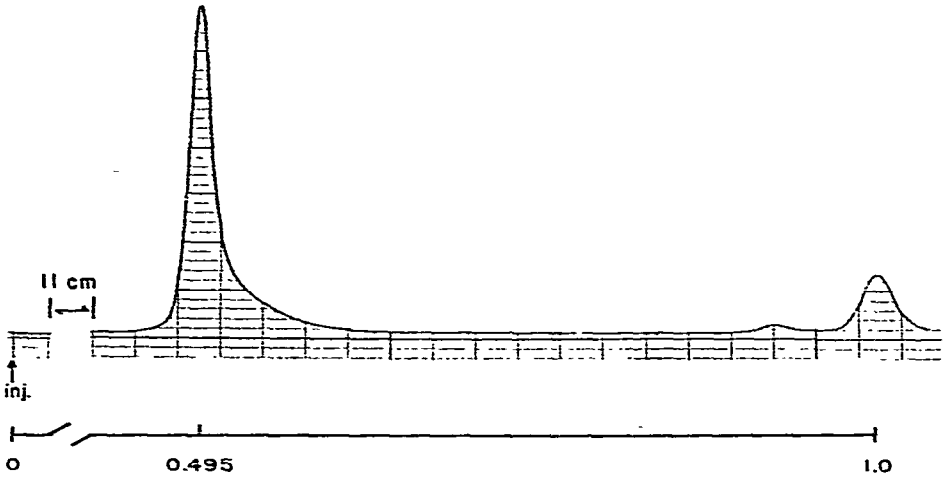


Fig. 4. Elution diagram of TMV on a Toyo Soda G5000 PW preparative column measuring 600 × 21.5 mm I.D. at a flow-rate of 0.96 ml $min^{-1}$. The buffer was 0.010 *M* phosphate pH 7 in 0.1 *M* KCl.

F(*v*) = 1.60 corresponding to radii of about 500 nm may be attributed to the large aggregates that comprise about half of the sample, while the trailing edge might plausibly reflect a partial resolution of an almost continuous distribution of smaller particle sizes by a rather inefficient process of external size exclusion. In order to determine whether fractionation according to size was actually occurring within the confines of the "$V_0$ peak", the chromatography experiment was repeated, yielding an elution diagram virtually identical to the one in Fig. 4. A sample, designated fraction A, was drawn from the rising portion of the curve, from about 0.3–0.7 of $H_{max}$ and a second sample, designated fraction D, from the descending edge between about the same limits. These samples were then examined by electron microscopy. As seen in Fig. 5A and B, the prediction of fractionation within the $V_0$ peak was confirmed. Fraction A, Fig. 5A contains a large number of rods of length about 300 nm, but about half of the sample consists of rods of nearly twice this length. Some rods shorter than 300 nm are also seen. While fraction D also contained 300-nm rods, very few longer rods were seen, and roughly 80% of the particles were shorter than 300 nm. Even after scanning numerous fields, we were unable to find any of the very large aggregates, which we call "rafts", as shown in Fig. 3B. Evidently these "rafts" are disaggregated during chromatography.

The possibility that the "rafts" were trapped in the column was eliminated by the fact that the yield, as determined by UV absorption was 97%. We have also repeated the experiment in order to perform rechromatography on freshly isolated chromatographic fractions. In this experiment fraction A, taken from the rising side of the peak, had a value of $t_e/t_t$ of 0.475 in the preparative run, and 0.473 on rechromatography while the corresponding parameter for fraction D increased from 0.491 to 0.500. These data further support the concept that a fractionation is occurring as we traverse the major peak and that the individual subfractions retain their chromatographic characteristics, at least for a few hours.

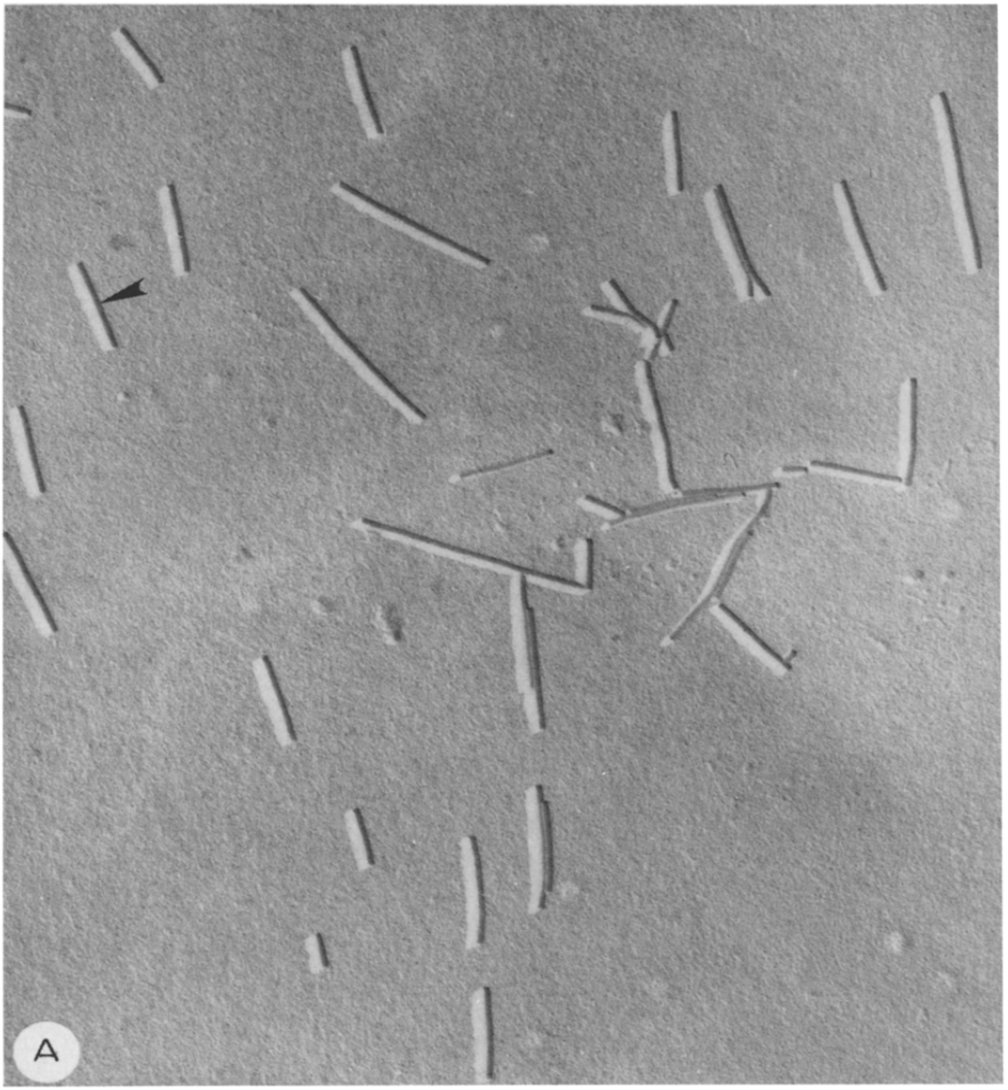


Fig. 5.

## CONCLUSIONS

We were gratified to note that the quantitative theory of size exclusion which we have presented here, resulted in two predictions which we subsequently verified. First of all, the high value of $F(v)$ suggested the presence of aggregates larger than the 300 nm particle which is accepted as the infectious unit, and is the value usually quoted. Electron microscopy of the original sample demonstrated the presence of very large three dimensional aggregates which we refer to as "rafts" of the size predicted by our equation. These "rafts" are evidently partially dissociated during chromatography, however, since they were not observed in the column effluent. End

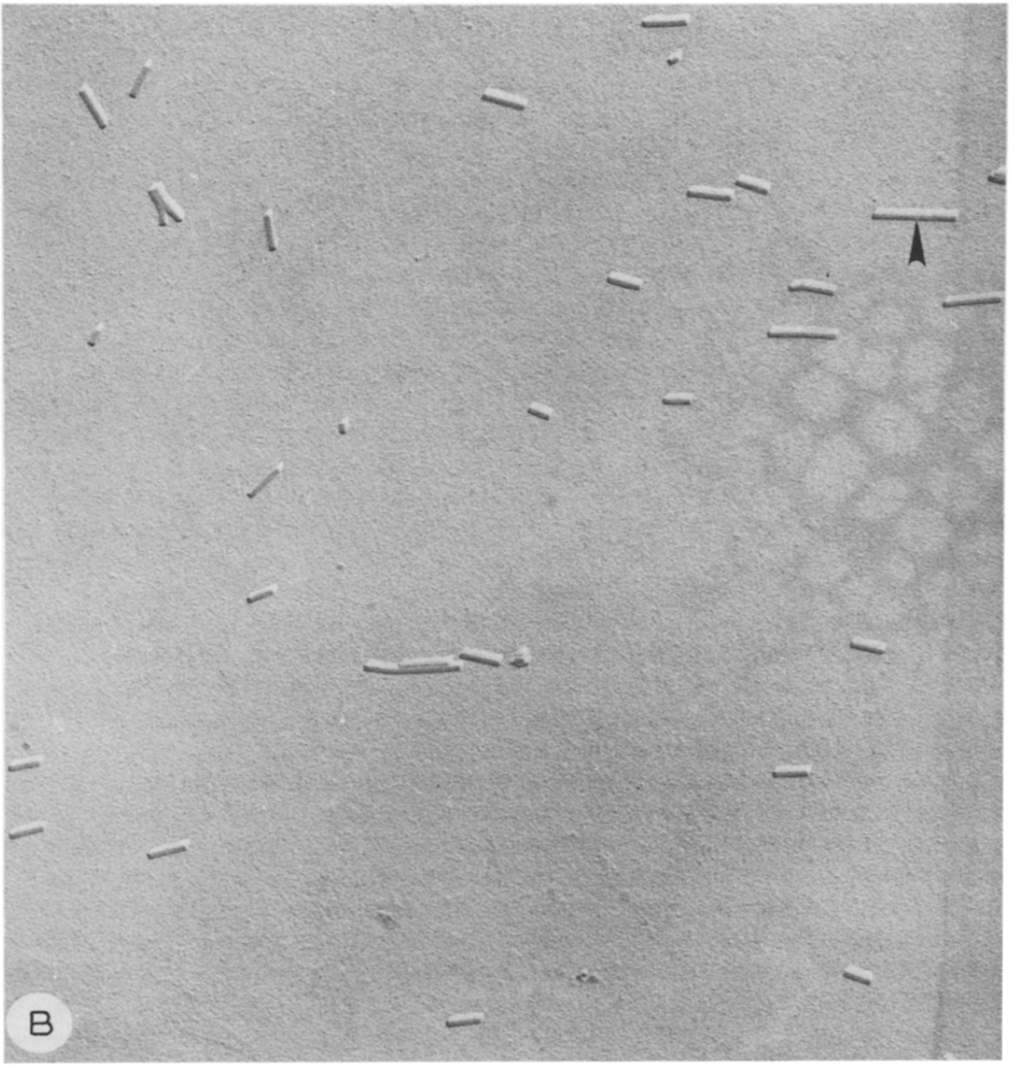


Fig. 5. A was taken from fraction A, the rising portion of the "$V_0$ peak" shown in Fig. 4, and B was from fraction D, the descending edge of the same peak at 39,600 ×. To allow for changes in magnification during reproduction, a rod of 300 nm length is indicated by an arrow in each photograph.

to end aggregates having lengths equal to the diameters of spheres of a size predicted by eqn. 3 were observed, however, in the leading edge of the $V_0$ peak. The second prediction was that fractionation would occur across the $V_0$ peak. This prediction was also confirmed, as it had previously been done by Small[7] with polystyrene latex spheres.

Whether long rods of length $2r$ behave hydrodynamically as spheres of radius $r$ during transport through the interstitial space between the packing spheres may well be open to question, but it is evident that the length plays the determining role in the

fractionation illustrated in Fig. 5. It is clear from the photographs as well as from the known properties of the virus, that essentially all the rods have the same diameter. Thus, by making appropriate assumptions one can argue that the molecular dimensions observed by electron microscopy agree with those predicted by theory, but it is also possible that we may have forced the fit.

An alternative explanation must therefore be considered. Our model is based on equilibrium conditions, and we believe that this is the predominent effect in external size exclusion as it is internal size exclusion[3], and yet the velocity flow profiles which are the basis of the mechanism developed by Small[7] very likely exist in the interstitial space where flow occurs and may well make an additional contribution which would probably depend on flow-rate up to the point of turbulence. Hjertén[10] has reported a substantial effect of flow-rate on the elution profile when ribosomes are passed through a column packed with beads of 1 % agarose.

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