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APPLICATION OF A GENERAL EQUATION FOR CONTROLLED PORE GLASS PERMEATION CHROMATOGRAPHY TO AN AGGREGATING, SPHERICAL VIRUS

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SUMMARY

A semi-empirical permeation chromatography equation relating pore size, species size and elution coefficient, which had previously been derived from chromatographic data of narrow-molecular-weight dextrans on controlled pore glass, was applied to the chromatography of an aggregating virus particle. A large number of chromatographic runs on columns of different pore sizes were combined and statistically evaluated. The resulting diameter distribution *versus* infectivity curve for the virus particle population shows distinct maxima at multiples of 50 nm. This unit size agrees with electron microscopical observations and confirms the applicability of the chromatography equation.

INTRODUCTION

In a previous paper[1], one of the authors derived a permeation chromatography equation from chromatographic data of narrow-molecular-weight fractions of dextrans on controlled pore glass (CPG) columns of narrow pore size distribution. The equation presented in this previous paper is semi-empirical in nature. It relates the pore diameter, P_e, of the CPG substrate, determined by mercury intrusion[2], to the permeation chromatography elution coefficient, K (also called normalized elution volume), to diameter, D, of the molecular species studied. In the case of the previous study[1], D was the diameter of the dextran molecules, which was derived either from their measured exclusion pore size on CPG columns by a technique described elsewhere[3], or their equivalent sphere diameters calculated from intrinsic viscosity values based upon a random flight coil type model. The sphere diameters calculated by both techniques coincide very closely, indicating near-ideal solution behavior of dextran

and a low degree of electrostatic interaction between the dextran molecules and the surface of the controlled pore glass.

Eqns. 1, 3 and 4 on p. 393 of the previous paper[1] can be rearranged to

$$D = P\,(1 - K)^{2.66\,P^{-0.164}} \tag{1}$$

whereby D and P are in Å and K is the elution coefficient or normalized elution volume[4] defined by

$$K = (V_e - V_0)/(V_t - V_0) \tag{2}$$

where V_e is the measured elution volume at peak maximum and V_0 and V_t are the exclusion and total free volumes of the used columns.

The above eqns. 2 and 1 were applied to the results of a large number of chromatographic runs of infective cell culture fluids on CPG columns of different pore diameters. Chromatographic peak positions were determined by incremental infectivity measurements of eluent fractions. One of the first noteworthy results of the study was the appearance of discrete infectivity peaks in the individual eluograms which suggested the presence of several infective particles of different sizes. This conclusion had important virological implications and justified the extensive further work on a substance which is tedious to produce, time-consuming to assay and potentially infective to man.

MATERIALS AND METHODS*

Virus suspensions

Lymphocytic choriomeningitis (LCM) virus strain WE[5] was plaque purified three times[6]. It was propagated in L-cell monolayer cultures, sonicated, ultracentrifuged and the supernatant ether used immediately or after storage at −70°C. Details of the procedure are described in another publication[7].

Infectivity assay

The number of infectivity units (IU) per ml in the original virus suspension and also in the eluent were performed on cultures of L-cells as previously described[8].

Chromatography on controlled pore glass

Procedural details for filling and operating CPG columns have been published[1,2,9–11]. For experiments described here, CPG (Electro-Nucleonics, Fairfield, NJ, U.S.A.; or H. Hölzel, Dorfen, G.F.R.) with the following mean pore sizes was used (figures in parentheses give the deviations from pore size in % and the specific pore volume in cm^3/g): 33.2 nm (± 5.4, 1.59); 41.1 nm (± 5.04, 1.32); 43.7 nm (± 6.7, 1.37); 47.5 nm (± 3.04, 1.04); 67.7 nm (± 7.7, 0.89); 122.0 (± 10.0, 0.79); 142.2 nm (± 4.5, 102); 199.0 nm (± 9.8, 0.90); 257.4 nm (± 5.6, 1.44); 312.5 nm (± 7.6, 0.91). Data were supplied by the manufacturer.

* The mentioning of trade names and suppliers does not constitute endorsement by the authors or their organizations.

Other equipment consisted of Chromodule columns Type 0.8 (H. Hölzel), Guldener Vario-Perpex II peristaltic pump (W. Meyer, Luzern, Switzerland), Uvicord II ultra-violet monitor (LKB, Stockholm, Sweden), PM 8100 recorder (Philips, Eindhoven, The Netherlands) and Liniar II fraction collector (Serva, Heidelberg, G.F.R.). Polyethylene glycol of molecular weight 20,000 (PEG 20,000) for column treatment was obtained from Electro-Nucleonics or H. Hölzel. A burette, graduated in 0.2-ml steps, with a light beam-controlled switch-off mechanism (H. Hölzel) was used to measure elution buffer volumes.

Exclusion volume and total free buffer volume for each column were determined both by calculation from filling data and chromatography of a mixture of tobacco mosaic virus (gift of Professor H. L. Sänger, Giessen, G.F.R.) and tryptophan. Peak positions could be reproduced within 0.2 ml using flow-rates of 1.0–5.0 ml/min. Before use, columns were washed with dilute ammonium hydroxide, concentrated nitric acid and water. They were then treated with PEG 20,000 to prevent absorption of virus to the inner surface of CPG[12]. Undiluted fractions were assayed with columns or other equipment, sterilized either by autoclaving or by pumping 70% sterile ethanol through the system followed by sterile buffer. For chromatography of LCM virus, a high ionic strength buffer at pH 8.5, chosen to minimize surface interactions and to stabilize the infectivity, was used.

RESULTS

Chromatography

When CPG columns were first used for chromatography of the virus suspension, they were not treated with PEG 20,000. With pore sizes 43.7 nm or less not more than 10% of the initial infectivity was lost by adsorption to the untreated glass. In contrast, if CPG of slightly larger pore size, *i.e.*, 47.5 nm, was employed, freshly prepared virus preparations often lost more than 90% of their infectivity. If the virus had been kept for a week or more at 4°C prior to chromatography, the titer would drop 10-fold or more, but nearly 50% of the remaining infectivity was excluded by the untreated CPG of pore sizes 475, 122 and 199 nm.

For all subsequent experiments, an alkaline buffer with high ionic strength and CPG treated with PEG 20,000 was used. Under these conditions, measurable quantities of infectious virus did not adsorb to the glass.

A 2-ml volume of cell culture fluid diluted to contain $10^{3.5}$ IU/ml were passed through CPG columns at flow-rates of either 2.5 ml/min or 5.0 ml/min, corresponding to total chromatography times of 25 min or 2 h, respectively. This variation of experimental conditions had no effect on the results. When CPG of pore sizes 33.2, 41.1 or 43.7 nm were employed, infectious virus never penetrated into the pores. Also, with CPG of pore size 47.5 nm, all infectivity remained in the exclusion volume.

In contrast, an increase in pore size above 47.5 nm produced new discrete symmetrical peaks, in addition to an exclusion peak. The number of these new peaks increased as larger pore size CPG was used. For each pore size a minimum of five runs were titrated. For illustration, four chromatograms obtained with CPG of four different pore sizes are shown in Fig. 1. The appearance of several symmetrical infectivity peaks in the chromatographic runs is strong evidence for the presence in the culture fluid of a population of several types of infective particles with different discrete sizes.

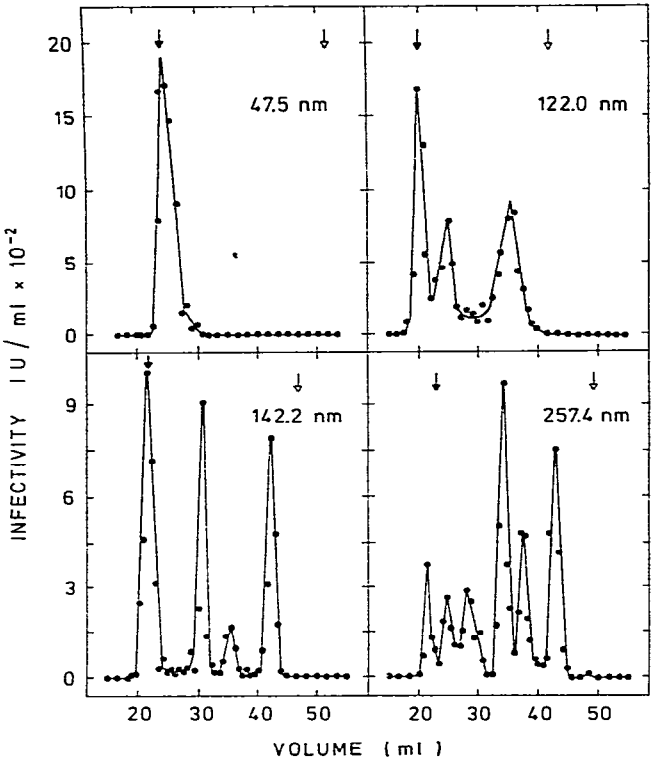

Fig. 1. Infectivity profiles of LCM virus chromatographed on columns of CPG with pore sizes between 47.5 and 257.4 nm. Data from different experiments are shown. A 2-ml volume of crude cell culture fluid, diluted to a concentration of $10^{3.5}$ IU/ml, was eluted with flow-rates of 2.5 ml/min. Total infectivity recovered was over 90% of the infectivity applied to the columns. Exclusion volumes and total buffer volumes of the individual columns employed are indicated by arrows.

Analysis of chromatographic data

To obtain quantitative size data, the above eqns. 1 and 2 were applied. In order to minimize experimental errors inherent in infectivity titrations, the data from a large number of chromatographic runs were treated by a statistical summation procedure. This also compensates, for each new culture fluid preparation, for the possible differences in the relative abundance ratios of particles in the different size classes. First, the elution volumes of the titrated fractions of 36 chromatographic runs on six different columns were converted to the normalized elution volume, K (also called elution coefficient), by eqn. 2. Sample volume correction of V_e was made uniformly on calibration and separation runs.

The normalized elution volumes, K, of the titrated fractions were then converted to D, using eqn. 1. No corrections for P were made for a possible reduction of the CPG pore diameter by adsorbed PEG 20,000 molecules which were used in column pretreatment. Such a correction would only introduce a slight systematic shift of all calculated particle diameters. For the reasons previously stated[1], eqn. 1 is applicable for $0.15 < K < 1.00$ and therefore titration data only within this range were used for further statistical treatment.

After converting the IU results from elution volume to particle size using eqn. 1, the data of each chromatographic run were distributed into a size raster having 1-nm wide units. Since this width is below the width of the collected fractions, IU of a given fraction were equally divided over the raster units within the size limits of the fraction. Subsequently, the IU within identical size units of the raster for all the chromatographic runs were summed up and displayed in an IU *versus* particle size graph. Since the chosen raster unit width of 1 nm was obviously below the resolution which one could expect from fraction width and experimental errors, statistical noise in the data was suppressed by centroid averaging over 11-nm raster units. The resulting computer plot of the summation curve of all 36 runs is shown in Fig. 2. It represents a size-distribution curve showing six discrete peaks. The experimental curve (heavy line) was further decomposed by computer least-mean-square treatment into six Gaussian curves (dotted lines). The accuracy of the decomposition was tested by summing up the six computer-produced curves into a reconstruction curve (fine line). The agreement is excellent. The computer-determined peaks of the six Gaussian curves are at 49, 104, 147, 199, 239 and 276 nm particle diameter. The assayed infectivity of the chromatographed preparations was associated with particles measuring approximastely 50 nm or multiples of this value.

DISCUSSION

The mathematical evaluation of the data obtained by CPG chromatography shows that the assayed infectivity is associated with particles of a basic size unit and its multiples. This suggests that the infectious particle forms aggregates leading to a constant increase in the effective radius when two, three or more units adhere to each other. The theoretical number of peaks was obtained for each pore size used. Furthermore, when the experimental K values were used to calculate particle diameters and all IU were summed in their respective size classes, the overall profile (Fig. 2) shows the six distinct regions into which the maxima of all chromatographic runs will fall. Computer analysis of these data shows a size close to 50 nm for the basic unit. The

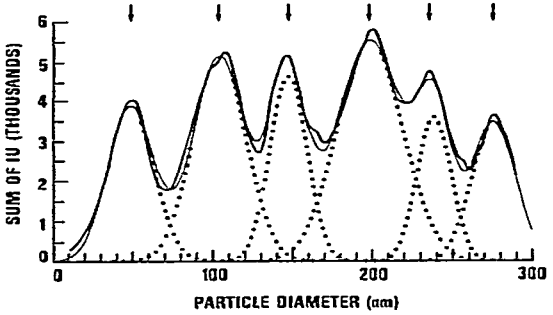

Fig. 2. Summation curve of infectivity distribution of LCM virus of 36 chromatographic runs on CPG columns of six different pore sizes (67.7, 122.0, 142.2, 199.0, 257.4 and 312.5 nm). The elution volumes of the individual runs were converted to particle diameter before summation. The experimental curve (heavy solid line) was computer-decomposed by statistical treatment into six Gaussian curves (dotted lines). The fine line shows the computer summation of the six Gaussian curves. The maxima of the six curves are at 49, 104, 147, 199, 239 and 276 nm.

possible penetration of infectious particles into pores measuring 47.5 nm, but never into pores measuring 43.7 nm, indicates that the actual size of the small particles is slightly below 50 nm. This indicates that the assayed infectivity is not associated with the pleomorphic arena virus particle as characterized by the LCM virus[13] but with an additional infective virus. The chromatographic data also show that in the small aggregates the alignment of the unit particle is linear or chain-like. For spherical particle aggregating under isodirectional adhesion forces this is an unusual arrangement. The linear aggregation of the particles would require special structural parameters for their orientation.

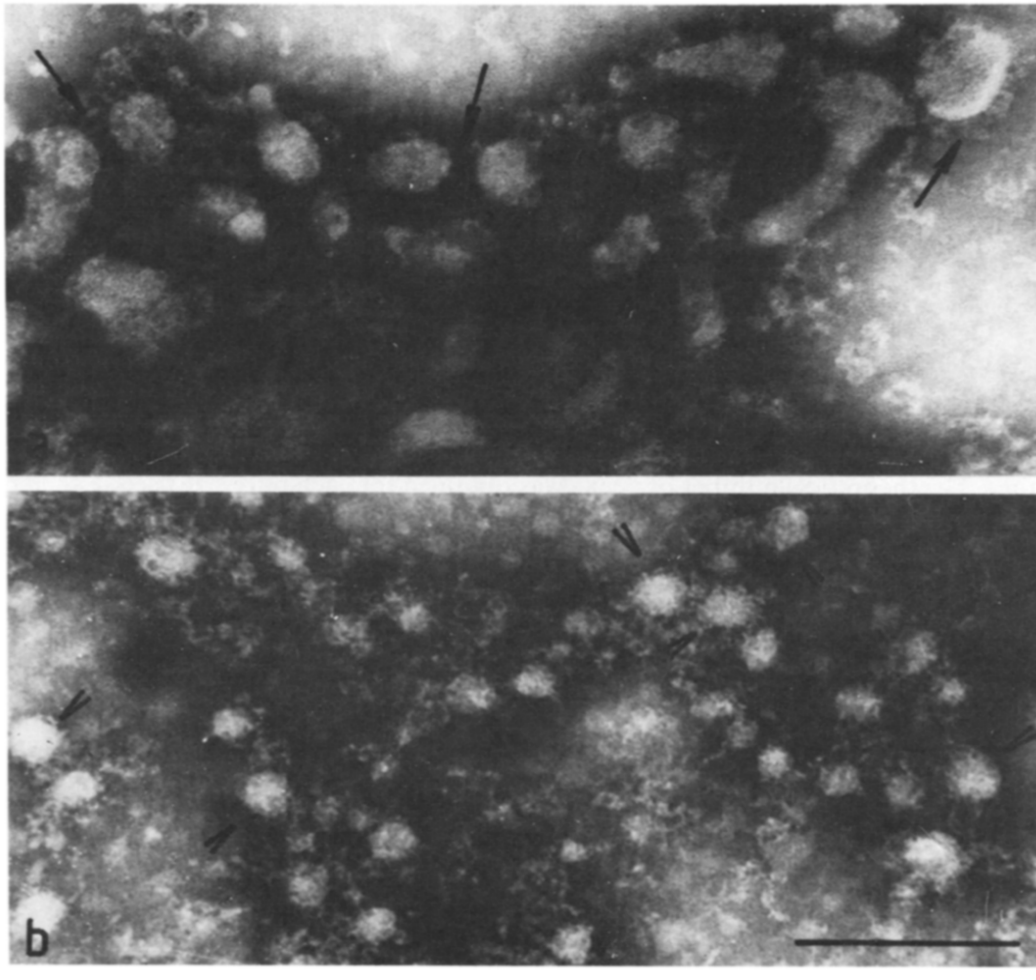

Fig. 3. Immuno precipitates of virus formed by an anti-LCM virus antiserum (the bar represents 200 nm). a. Precipitates of the pleomorphic arena particles, found in the medium of LCM virus infected cell cultures. At high magnification a surface projection layer is seen on some particles (arrows) below the immunoglobulin coat. Particle size is approximately 100 nm. b. Precipitates of small particles which always found next to the pleomorphic particles which were purified by the described procedure. Particle perimeters are obscured by a dense immunoglobulin coat. Largest particles (triangles) *ca.* 50 nm diameter. Smaller presumably disrupted particles can be seen within the immuno precipitate.

In order to supplement the results of the chromatographic study, electron microscopy (EM) of the infective culture fluid and the CPG-purified infective fraction of suspension was performed. Details of the EM preparation procedures are described elsewhere[14]. Fig. 3 shows two different immuno precipitates obtained with an anti-LCM virus antiserum produced in rabbits inocculated with culture fluid of LCM-infected rabbit kidney cell (RK3) cultures which were grown in rabbit serum-containing medium. One precipitate (a) contains the well described arena particles coated with surface projections (arrows) and is represented in varying sizes around 100 nm. The other precipitate (b) contains small particles of approximately 50 nm in size (arrow heads). This type of particle precipitate is the only particle found in purified virus suspension or in the CPG frctions (Fig. 1). While actual particle chains could not be found in the EM pictures, possibly because the chains do not survive the preparation procedure, the pictures suggest that the smallest unit particles are not smooth spheres but polyhedra. They are often seen with hexagonal or pentagonal circumferences which are characteristic for icosahedral particles. Icosahedrons, a basic shape of virus nucleocapsids[15], reveal a simple parameter for linear aggregation in the three-fold symmetry axis.

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REFERENCES

1 W. Haller, A. M. Basedow and B. Konig, *J. Chromatogr.*, 132 (1977) 387–397
2 W. Haller, *Nature (London)*, 206 (1965) 693–696
3 W. Haller, *Macromolecules*, 10 (1977) 83–86
4 R. M. Wheaton and W. C. Bauman, *Ann. N.Y. Acad. Sci.*, 57 (1953) 159
5 T. M. Rivers and T. F. M. Scott, *J. Exp. Med.*, 63 (1936) 415–432.
6 M. Popescu and F. Lehmann-Grube, *J. Gen. Virol.*, 30 (1976) 113–122.
7 H. H. Gschwender, M. Brummund and F. Lehmann-Grube, *J. Virol.*, 15 (1975) 1317–1322.
8 H. H. Gschwender and F. Lehmann-Grube, *J. Gen. Virol.*, 26 (1975) 205–208.
9 H. H. Gschwender, W. Haller and P. H. Hofschneider, *Biochim. Biophys. Acta*, 190 (1969) 460–469.
10 H. H. Gschwender, M. Brummund and F. Lehmann-Grube, *J. Virol.*, 15 (1975) 1317–1322.
11 T. Darling, J. Albert, P. Russell, D. M. Albert and T. W. Reid, *J. Chromatogr.*, 131 (1977) 383–390
12 C. W. Hiatt, A. Shelokov, E. J. Rosenthal and J. M. Galmore, *J. Chromatogr.*, 56 (1971) 362–364
13 K. Mannweiler and F. Lehmann-Grube, in F. Lehmann-Grube (Editor), *Lymphocytic Choriomeningitis Virus and Other Arena Viruses*, Springer, Berlin, Heidelberg, New York, 1973, pp. 37–48.
14 J. D. Almeida and A. P. Waterson, *Advan. Virus Res.*, 15 (1969) 307–338.
15 D. L. D. Caspar and A. Klug, *Cold Spring Harbor Symp. Quant. Biol.*, 27 (1962) 1–24.