CORRELATION BETWEEN CHROMATOGRAPHIC AND DIFFUSIONAL BEHAVIOUR OF SUBSTANCES IN BEDS OF PORE CONTROLLED GLASS

CONTRIBUTION TO THE MECHANISM OF STERIC CHROMATOGRAPHY

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(Received October 9th, 1967)

Chromatographic columns filled with grains of pore-controlled glass display elution spectra which, except for somewhat sharper resolution, are similar to those shown by the widely used hydrogel or organogel columns. Fig. 1 illustrates the elution behaviour of dextrans of various molecular weights on columns of porous glass with water as the eluant. Fig. 2 shows the peak positions of various polystyrenes *versus* their molecular weights on two glasses of different pore sizes with an organic solvent as eluant. The data of Fig. 1 were obtained in our laboratory, and the data of Fig. 2 were obtained by Dr. J. C. MOORE of Dow Chemical Corporation on glasses which our laboratory had prepared and supplied[1].

When steric chromatography on pore controlled glass was first developed in our laboratory[2], the fact that elution spectra in this new type of chromatography were similar to those in gel chromatography and the evidence of very narrow range of pore size in the glasses[3], seemed to contradict current volume-exclusion theories all of which assume a broad distribution of pore sizes[4–8]. Because of its mechanical

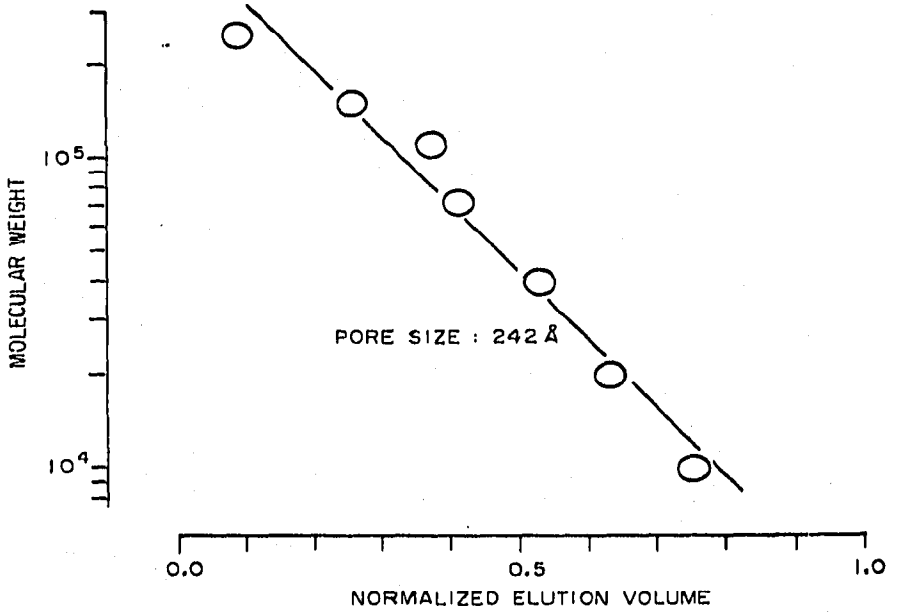

Fig. 1. Chromatography of dextrans on pore controlled glass. Peak elution volumes normalized: TMV ≡ 0, BA ≡ 1.0. Column: 1 × 50 cm; eluant: water; flow rate: 1 cm³/min.

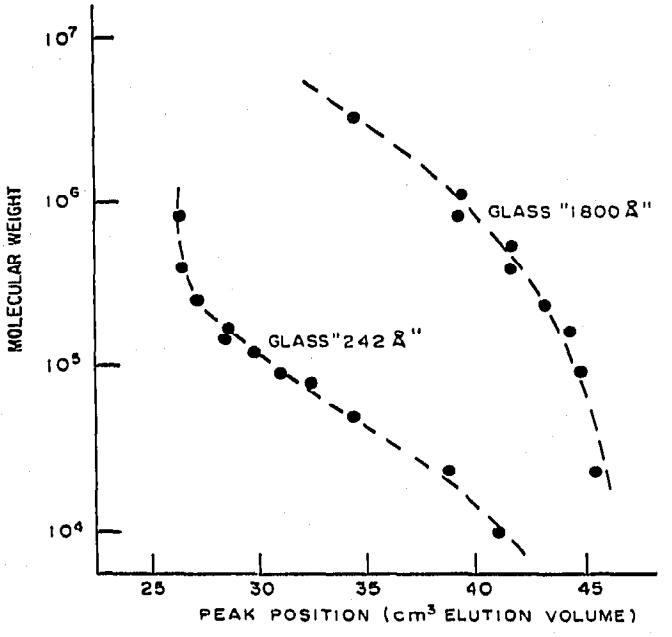

Fig. 2. Chromatography of polystyrenes on pore controlled glass. Peak elution volume *versus* molecular weight. Column: 0.78 × 122 cm; eluant: butanone–isopropanol (6:1, by vol.); flow rate: 1 cm³/min. Figures next to curves indicate pore diameter of glass used.

rigidity and the absence of swelling, the actual pore size of porous glass can be well determined by electronmicrography, intrusion, and adsorption techniques; and columns with known pore and void volume can be prepared. These features seemed to make porous glass particularly suitable for theoretical investigations of the mechanism of chromatography. Of further advantage are the facts that columns of porous glass afford extremely high flow rates and that glass grains settle rapidly from suspensions. Rapid settling permits sampling of the supernatant almost immediately after a suspension is stirred, a technique that is useful in diffusion experiments.

Fundamentally, the present study consisted in first performing elution experiments on a porous glass column of measured pore and void volume over a wide range of flow rates. Then, suspensions of the same porous glass in the same liquids as the eluants were prepared. These suspensions were vigorously stirred and measured quantities of substances were added. The concentrations of the substances in the exterior liquid were measured as a function of exposure time and thus the kinetics of uptake was determined. The results of these, theoretically simple, diffusion experiments were compared with the actual chromatographic behaviour.

A porous glass of 620 Å nominal pore diameter was used in the entire study. The mercury intrusion and related pore-distribution curve of the glass are shown in Fig. 3. The methods for these measurements have been described elsewhere[2]. The mercury-intrusion pore volume of the glass was 0.4 cm³/g. This is smaller than the pore volume of the glass we usually employ in this laboratory. It was chosen because of the possibility (later not substantiated) that glass with larger pore volume would break under the vigorous stirring of the diffusion experiments.

Substances investigated were: benzyl alcohol (BA) as a representative small molecule, tobacco mosaic virus (TMV) as a substance larger than the pores of the glass, and the *coli*-phage MS2 as a species which elutes in the middle of the chromato-

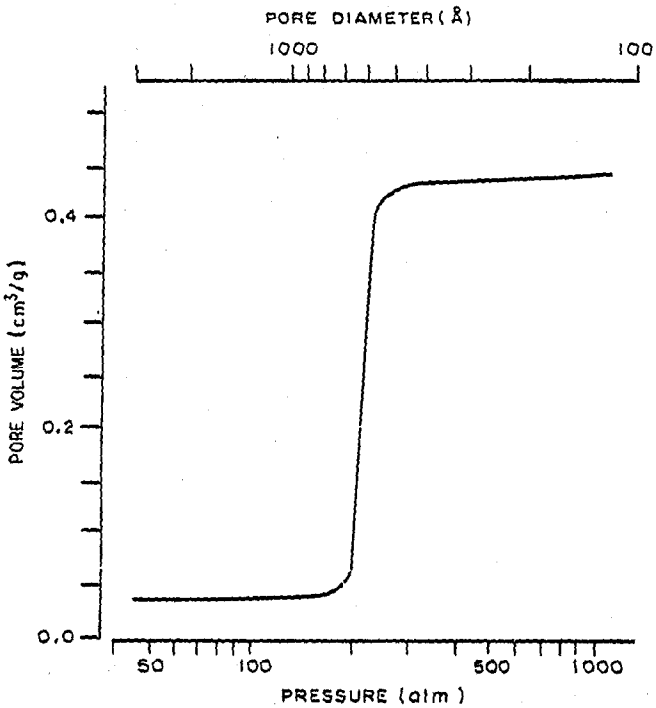

Fig. 3. Mercury intrusion and related pore size distribution of glass of 620 Å pore diameter.

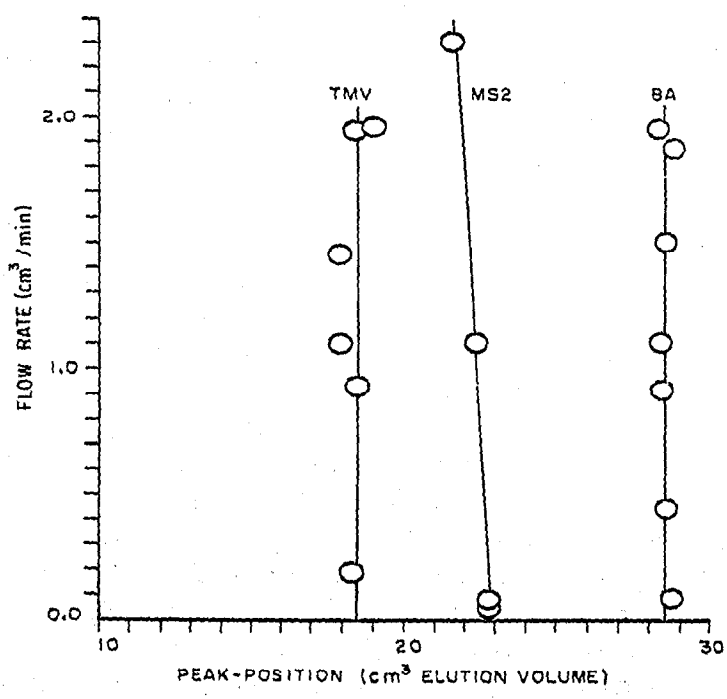

Fig. 4. Peak positions of tobacco mosaic virus, MS2-virus and benzyl alcohol at various flow rates. Column: 1 × 50 cm; pore diameter: 620 Å; pore space: 10.0 cm³ (0.4 cm³/g glass); void space: 18.5 cm³ (loosely packed), total fluid volume in column: 28.5 cm³.

graphic spectrum of this particular glass. All three substances absorb in the ultraviolet, providing easy detection and quantitative assay. The MS2-virus was chosen for its theoretically-pleasing spherical shape, for its uniform diameter[9] of 260 Å and because this laboratory had previously developed purification procedures for this virus[10].

CHROMATOGRAPHIC EXPERIMENTS

A glass envelope of 50 cm length and 1 cm inner diameter was filled under light vibration with a suspension of the glass powder in water. The column contained 24.9 g of glass (dry weight) and 28.5 g of water. From these weights and the determined mercury-intrusion volume of 0.40 cm^3/g of the glass one can calculate a pore volume of 10.0 and a void volume of 18.5 cm^3 for the column.

A peristaltic pump was used to move the eluant from a burette through a surge-supressor into the head of the column. An ultraviolet absorption monitor (2537 Å) behind the column detected the presence of substances in the effluent stream. In all cases 0.5 cm^3 of sample was injected through a rubber diaphragm into the head of the column. Flow rate and peak positions were determined by observing the burette and marking the volume of spent eluant on the strip chart recording the U.V.-absorption curve. Fig. 4 shows a plot of the observed peak positions for the three substances *versus* flow rate in cm^3. The elution volumes were corrected by adding one half of sample volume and by substracting the dead volume in monitor and tubing. This dead volume was determined by injecting a small U.V. absorbing sample into the end of the column and metering its arrival at the monitor.

The benzyl alcohol sample was a 1 % solution in water. The TMV sample was a purified fraction kindly supplied by Dr. L. STEERE. The MS2 sample was virus which has been isolated in this laboratory from virus-infected, precipitation-concentrated *E. coli* culture by steric chromatography on a 1 × 100 cm column of glass having pore diameter of 222 Å. The raw concentrate was injected in 10 cm^3 portions into the column. The eluant was collected, pooled, and used without further concentration. Details of the purification procedure are described elsewhere[10].

All experiments were carried out within a week after purification of the virus. Purified virus, when stored in excess of one week at 4°, developed a secondary peak in the elution spectrum, suspected to be due to virus crystals or aggregates. No such aggregation was observed on storage of virus contaminated with its host-proteins.

BA and TMV were eluted with water. The MS2 was eluted with saline (0.1 *M*) TRIS–HCl (0.05 *M*) buffer of pH 7.6, to which EDTA (0.001 *M*) had been added[11]. The columns were operated at room temperature.

DIFFUSION EXPERIMENTS

The penetration of the substances into the porous glass was measured by suspending 15 g of dry glass in enough de-aerated water or buffer (~ 30 cm^3) to penetrate all of the pores and to afford a shallow pool of supernatant after brief standing. An amount of 1–3 cm^3 of virus or BA solution were then measured into the suspension and the container was immediately vigorously agitated by a motor-driven device. At various intervals, the mixing was stopped and a sample of supernatant was re-

moved to determine the concentration of the added substance. In order to compensate for turbidity effects caused by abraded glass particles, bottles with glass and solvent were simultaneously agitated on the same device and the supernatant from these blanks was placed into the reference beam of the spectrophotometer. A double beam recording spectrophotometer (Cary 14) was used to measure substance concentration. Calibration curves for the substances were prepared over the concentration ranges which were anticipated in the diffusion experiments. To further suppress turbidity effects the differences between the extinctions at two wavelengths were used for the determinations. These were 260 and 320 nm for TMV, 257 and 280 nm for BA, and 260 and 300 nm for MS2.

The penetration of the glass pores by BA occurred so fast that no measurements of its stages could be made. The terminal concentration in the supernatant was very slightly below that expected for full penetration, indicating that BA penetrated all the pore volume and that in addition possibly a very small amount of BA was adsorbed on the surface of the glass. If due to adsorption and not due to experimental error, the adsorption amounted to far less than one monolayer.

The TMV concentration in the supernatant did not change with time and indicated complete exclusion from the water in the pores. Calculation showed that per gram of glass 0.405 cm^3 of water was not accessible to TMV. This agrees within experimental error with the mercury-intrusion volume (0.40 cm^3/g) and shows that even for long exposure time virtually none of the virus penetrates the pores of the glass.

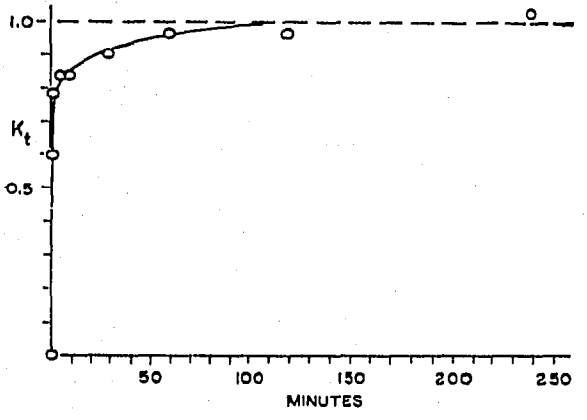

Fig. 5. Diffusion of MS2-virus (260 Å diameter) into porous glass of 620 Å pore diameter. Exposure time *versus* apparent fraction of pore volume which is penetrated.

The results for the penetration of the MS2-virus into the suspended glass are shown in Fig. 5. Within 1 min an amount of virus had left the external solution equivalent to 60 % equilibrium penetration of the total pore space in the glass. Ultimately, the external solution depleted to a concentration which was equivalent to complete equilibration between external fluid and the total internal pore space. In the figure, shaking time of the virus and glass suspension is plotted *versus* the apparent fraction of pore volume which is penetrated (k_t). To compute k_t, the apparent non-accessible volume x_t was first calculated, using the equation:

$$C_t = \frac{v_s}{v_0 + v_s - x_t} \qquad (1)$$

where C_t is the ratio of the concentration of virus in the supernatant solution to that in the stock solution,

v_0 is the volume of buffer which was first added to the glass (28 cm^3),

v_s is the volume of virus stock-solution added (3 cm^3).

From this, k_t was calculated according to:

$$k_t = \frac{v_p g - x_t}{v_p g} \qquad (2)$$

where g is the amount of glass (15 grams) used,

v_p is the pore volume of the glass (0.405 cm^3/g, from the TMV exclusion experiment).

For this formulation, $k \equiv 0$ if no penetration takes place and $k \equiv 1$ for complete penetration. The accuracy of the experiments was limited by the low ratio of glass to liquid. However, the accuracy was sufficient to indicate the rapid initial uptake, far exceeding that expected from the chromatographic experiment if volume-exclusion mechanism is assumed to be valid. To ascertain that the terminal uptake was really governed by the total pore volume and was not a fortuitous coincidence, the suspensions were observed beyond the time shown in Fig. 5. After 20 and even 28 h k_t had not further increased. As a matter of fact, a small decrease was noted which could have been bacterial breakdown or experimental error due to increased scattering-background produced by glass-abrasion over the extended stirring period.

DISCUSSION

The experiments with the two substances at the extremes of the elution spectrum of the porous glass used are in agreement with the generally accepted view that in steric chromatography the "oversized" molecules elute at the void volume of the column and very small substances at its total free volume. The experiments, in our case carried out with rigid columns of precisely determinable characteristic volumes, confirm what had previously been concluded from gel-chromatographic experiments, where void and pore volume are not always well known but where a wealth of experience has been available. The diffusion experiments with the two substances further confirm the correctness of the assumed effective pore volume. It is interesting to note that, not only in the chromatographic experiment but also in the diffusion experiment of up to 42 min duration, all of the tobacco mosaic virus was excluded from the pores of 620 Å diameter. The rod-shaped TMV is 150 Å in diameter and 3000 Å long[12]. This confirms STEERE's view[13] that its stiffness and its tumbling motion in the solution prevent its entering pores which are smaller than its length but larger than its diameter.

Within experimental limit, there was no change in the elution volume of TMV, even at extremely high flow rates. This indicates that there is no "pocket" effect[14] from the voids between the irregularly shaped grains as was observed by PEDERSEN[15] with columns filled with glass spheres. Similarly, no flow-rate dependence was observed with benzyl alcohol, which is to be expected for a rapidly diffusing small molecule. The position of the BA-peak at the total volume of the column hardly allows an alternative to the assumption that diffusion equilibrium is reached within the

column at all stages of passage and that the pore volume indeed determines the peak position of very small molecules.

The MS2-virus was eluted approximately in the middle of the chromatographic spectrum of the column. There was a slight flow-rate dependence of the peak position, with a variation from $v_e = 22.8$ for 0.055 cm³/min to $v_e = 22.6$ for 2.3 cm³/min. Extrapolation to zero flow rate gives a $v_e = 22.9$ cm³. When normalized in the usual way, whereby substances eluting at the void volume have $k_e \equiv 0$ and eluting at the total free volume have $k_e \equiv 1$, a k_e of 0.44 is obtained.

The diffusion experiment with the MS2 confirmed our suspicion about the failure of the volume exclusion theory. As electron micrographic and mercury-intrusion evidence[3] had already shown, and as the diffusion experiments further proved, the pores of the glass are so uniform that the total pore volume is accessible to a species of intermediate size. The fact that, in spite of this, the MS2 displays on the column a relative "intrusion volume" of $k_e = 0.44$ speaks strongly against a volume exclusion mechanism in this case and most likely in "gel"-chromatography as well. However, little doubt exists that, for smaller species, the pore volume becomes important. Otherwise, the elution volume of very small substances would not coincide with the total volume of columns.

The results, although not encouraging for the volume-exclusion theory, do not spell instant victory for the restricted diffusion theory[16] unless a very effective flow-rate-cancelling term is discovered. One can roughly estimate the exposure time of a given grain of column material to the substance by assuming that the injected sample maintains its initial sharp concentration boundary. A 0.5 cm³ sample, as in our case, will then surround the grain for 13 sec at our highest flow rate and for 10 min at our lowest flow rate. Comparing these exposure times with the uptake figures in the diffusion experiments (Fig. 5), one finds that after 13 sec the actual fractional uptake must have been below a value of 0.44, although the k_e found in the chromatographic experiment was 0.44. On the other hand, after 10 min the fractional uptake in the diffusion experiment was $k_t = 0.84$, yet the chromatographic peak position shifted only very slightly over this range. It is conceivable that a storage effect is responsible for the insensitivity of the peak positions to flow rate in steric chromatography. At higher flow rates less substance penetrates less deeply into the stationary phase and thus takes less time to be released again into the faster moving eluant, causing a retardation of about the same volume at lower flow rates. Such an effect could easily operate when no substance reaches the center of the grain and diffusion into and out of an infinite solid governs the process. This may be true at the very high flow rates or with very large grains. Obviously our high flow rates fall into this range. At very slow flow rates, however, the grain completely equilibrates with the surrounding and the release time from the grain will be the same if it had saturated at a slow or at a very slow flow rate. It is in this transition range where the constancy of peak position is most surprising.

Another interesting result of the diffusion experiment is the fact that the MS2-virus apparently does not completely behave as a hard sphere. The geometric center of a hard sphere of radius r within a cylindrical pore of radius R cannot come closer than r to the wall of the pore. Thus the volume available for the spheres is, by a factor of $(1 - r/R)^2$ smaller than the total volume of the cylinder. For a sphere of 260 Å diameter and a cylinder of 620 Å diameter, this means that only 60 % of the volume

should be available for the spheres. The diffusion experiment shows that 100 % was available for the virus. Since the pore diameter of the glass was measured by mercury intrusion, however, the 620 Å is the value for the narrowest part of the pores. Looking at electron micrographs one can roughly assume that the average pore diameter which may be effective in an equilibrium experiment is roughly three times the diameter of the access-limiting passages. This leads to an available volume of approximately 74 % which differs sufficiently from 100 % that it still would have been observed in the diffusion experiment. The failure to observe it indicates either soft-sphere behaviour or the compensating effect of a slightly increased transient population of spheres in the neighborhood of the walls. In any case, its magnitude is small and it enforces rather than weakens the drawn conclusions.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. L. STEERE of the U.S. Department of Agriculture for supplying the purified tobacco mosaic virus and Dr. C. R. MERRIL of the National Institute of Mental Health for supplying the MS2-culture. He further wishes to thank Mr. D. H. BLACKBURN, Mrs. R. J. RAKOWSKY and Mr. J. W. WEST for their technical help.

SUMMARY

Chromatography and diffusion experiments were performed with columns and suspensions of grains of pore-controlled glass of 620 Å pore diameter. The elution positions of tobacco mosaic virus and of benzyl alcohol coincide with the actual void volume and the total fluid volume of the column. A spherical virus (MS2) of 260 Å diameter eluted at a normalized elution volume of $k_e = 0.44$ and its peak position shifted only very slightly over a wide range of elution rates.

The diffusional uptake of virus (MS2) by the porous glass grains from a well stirred suspension indicated that the total pore volume of the glass was accessible to the virus. It took approximately 30 sec for one half of the ultimate amount of virus to penetrate the glass. In the light of these results it is very unlikely that a volume exclusion mechanism determines the elution position of the virus. Since porous glass columns of uniform and controlled pore size display elution spectra similar to those of gel columns, it is also unlikely that volume exclusion governs the elution spectrum in steric chromatography on gels.

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