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ARTIFICIAL PEAKS IN GEL PERMEATION CHROMATOGRAPHY DUE TO COMBINING COLUMN SUPPORTS WITH DIFFERENT PORE SIZES

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SUMMARY

Contrary to the elution pattern of bovine eye lens proteins chromatographed on a TSK GEL G5000 PW type column, an additional peak, representing $4 \cdot 10^6$ dalton aggregates, was observed on a combined G5000 PW–G4000 SW type system. This extra peak, found between the G5000 PW total exclusion fraction and α-crystallin ($M_r = 1.1 \cdot 10^6$), appeared to correspond to the G4000 SW void volume. Simulation calculations substantiated that this peak is the result of coupling the two columns and does not originate from a distinct sub-population of molecules. Such artificial peaks may be expected whenever a broad molecular weight distribution extends beyond the fractionation limits of at least one of the constituents of a combined gel permeation system. It is inferred that the hypothetical exclusion limit for compact, globular, symmetrical proteins on TSK GEL G5000 PW is near 10^8 dalton.

INTRODUCTION

Semi- and non-rigid column packings allowing high-pressure and high-speed gel permeation chromatography (GPC) which have been used in non-aqueous applications for more then 10 years, have recently become popular in aqueous (bio)polymer characterization[1–12]. Two types of rigid hydrophilic column packings are available: chemically modified macroporous silica supports and microspheres consisting exclusively of hydrophilic polymer. The first type is preferred when proteins are investigated and the latter gives higher resolution for synthetic polymers[5]. The application of silica-based TSK GEL SW type high-pressure GPC in combination with a molecular weight-dependent detection system, using a low-angle laser light scattering photometer, greatly improved the size characterization of biological macromolecules[8]. In a previous paper we described[13] the chromatography of eye lens proteins, crystallins, on TSK GEL G3000 SW and G4000 SW columns. Since the fractionation range of the largest pore size of SW type columns is limited to molecules having radii below 20 nm (inferred from the exclusion limits and molecular dimensions for dextrans[4,7,14]), these supports are less appropriate than the conventional

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agarose matrices for the separation of high-molecular-weight (HM)-crystallin with dimensions up to 500 nm[15]. Compared with the SW type columns, the polymer-based TSK GEL G5000 PW and G6000 PW type columns have larger pores[5,7]. Himmel and Squire[9] used a TSK GEL G5000 PW type column for size exclusion of. large proteins and viruses and Ozaki et al.[6] were able to fractionate human serum lipoproteins on G5000 PW or G6000 PW columns. Moreover, to obtain an optimal one-step fractionation of the lipoproteins, the latter authors used several combinations of PW and SW type columns.

The present communication deals with the results of fractionating lens proteins on a TSK GEL G5000 PW column and a combination of a G5000 PW and a G4000 SW type column. In the latter case an additional peak was found between the void volume and the α-crystallin peak, suggesting a discrete sub-population of inter-mediately sized HM-crystallin. However, because no indication whatever of such peak could be obtained using only the G5000 PW type column, simulation calcu-lations were done and showed that the extra peak is artificial.

EXPERIMENTAL

High-pressure GPC was carried out at room temperature in prepacked col-umns containing TSK GEL SW and PW type columns which were, when combined, connected in sequence of descending pore size: GPWP (precolumn, 10 × 0.75 cm), G5000 PW and G4000 SW (60 × 0.75 cm each) (Toyo Soda). The carrier buffer was composed of 0.10 M sodium sulphate and 0.02 M sodium phosphate pH 6.9 and was Millipore-filtered and degassed under vacuum before use. Elution was performed at constant flow-rate of 0.4 ml/min for the PW type alone and 0.8 ml/min for the combined system (solvent delivery: Beckman/Altex Model 100A). Detection was ac-complished with an ultraviolet absorbance detector at 212 nm (Hitachi/Altex). Sam-ples of 100 μl, obtained from nuclei of calf lenses as described earlier[13,16] and diluted in elution buffer to a concentration of 2 mg/ml, were applied using a Valco loop injector.

Molecular weight determination was done with a flow-through low-angle laser light scattering photometer (Chromatix: KMX-6) and a differential refractive index detector (Melz: LCD 201) according to the relative method[8] and using a Hewlett-Packard 3353 data system as described previously[13].

RESULTS

GPC of high-molecular-weight lens proteins
Fig. 1 shows the result of applying a diluted extract from the innermost part (nucleus) of a calf lens to a TSK GEL G5000 PW type column (upper trace) and a G5000 PW–G4000 SW type combination (lower trace). The upper elution pattern shows that a good separation of the crystallins is obtained, especially between the largest HM-crystallin aggregates eluting in the void volume and the α-crystallin peak. Due to the pore characteristics of the G5000 PW support, the resolution of the other crystallins is limited. The peaks were identified by comparison of the profiles with the patterns obtained from SW type columns and by molecular weight determinations using the molecular weight-dependent low-angle laser light scattering device; the data
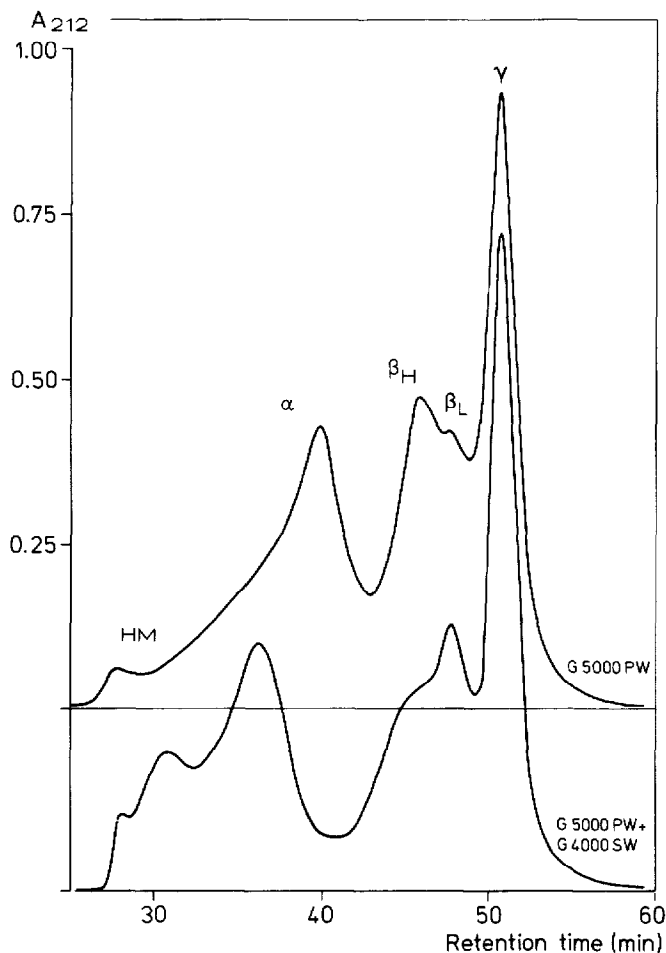
Fig. 1. High-performance GPC of calf nuclear water-soluble lens proteins. The upper elution profile was obtained using a TSK GEL G5000 PW type column (flow-rate 0.4 ml/min) and the lower one represents the pattern obtained from elution on a TSK GEL G5000 PW–G4000 SW column combination (flow-rate 0.8 ml/min).

were in accordance with those presented earlier[13] and are included in Table I.

To obtain a better resolution in the β- and γ-crystallin range we coupled a TSK GEL G4000 SW type column to the PW type column and a typical elution pattern is shown in the lower trace of Fig. 1. When both drawings are compared, the decreasing overlaps between α- and β- and β- and γ-crystallin indicate the improvement in resolution. The ratio of peak heights for $\beta_{H(igh)}$- and $\beta_{L(ow)}$-crystallin changes markedly; however, on comparing the areas under the curves for both chromatograms it was found that the proportions of β_H- and β_L-crystallin are similar in both analyses. Because of its heterogeneity[13], the β_H-crystallin group is eluted as a broader peak in the better resolving system, with a lower peak height.

The presence of the additional peak between the void volume and the α-crystal-

TABLE I

MOLECULAR WEIGHTS AND GPC ELUTION PARAMETERS FOR TSK GEL G5000 PW

t_e = Retention time of a particular fraction; t_t = retention time for totally permeated molecules; t_0 = retention time at which the void volume elutes; $K_d = (t_e - t_0)/(t_t - t_0)$; $F_{(v)} = (\sqrt[3]{t_e/t_t} - \sqrt[3]{t_0/t_t})/(1 - \sqrt[3]{t_0/t_t})$.

Compound	M_r	K_d	t_e/t_t	$F_{(v)}$	$\sqrt[3]{M_r \bar{v}}$
Tobacco mosaic virus	$39.4 \cdot 10^6$*	0	0.495*	0	306.4*
HM-crystallin	$>20 \cdot 10^6$**	0	0.495**	0	>245.5**
Sea worm chlorocruorin	$2.9 \cdot 10^6$*	0.343	0.668*	0.398	130.2*
α-Crystallin	$1.1 \cdot 10^6$**	0.442	0.718**	0.500	93.37**
Thyroglobulin	670,000*	0.477	0.736*	0.535	78.43*
Dimeric serum albumin	136,000*	0.636	0.816*	0.686	46.00*
β_H^s-crystallin	130,000**	0.640	0.818**	0.690	45.40**
Monomeric serum albumin	68,000*	0.699	0.848*	0.744	36.51*
β_L^2-crystallin	50,000**	0.711	0.854**	0.755	33.02**
γ_L-crystallin	20,000**	0.824	0.911**	0.854	24.33**
Cytochrome c	12,500*	0.871	0.935*	0.894	20.79*

* Molecular weight and elution data were taken from ref. 9.

** Elution data correspond to Fig. 1; molecular weights were determined by low-angle laser light scattering and agree with those reported previously[13]. For α- and HM-crystallin we used a partial specific volume, $\bar{v}$, of 0.74 ml/g based on experimental data[20,21]; for β- and γ-crystallin we used a $\bar{v}$ value of 0.72 ml/g based on estimated values from amino acid compositions[22,23].

lin fraction was more puzzling. At first we thought of superior resolution of a discrete sub-population of intermediately sized HM-crystallin aggregates. Such a population has never been found as a peak fraction in a one-step GPC procedure[15-17]. Although van Kleef and Hoenders[18] isolated these aggregates ($s_{20,w}$ near 40 S) as a peak fraction on a Bio-Gel A-50m column, they obtained this by rechromatography of collected Bio-Gel A-5m void volume fractions.

We found molecular weight values near $4 \cdot 10^6$ by the low-angle laser light scattering device for the material eluting in the extra peak seen in the lower trace of Fig. 1. It thus seems possible that the aggregates eluting in this peak represent the earlier characterized HM2[18], high α-[15] and oligomeric α-crystallin[16]. However, the suggestion that this fraction represents a discrete sub-population of aggregates is uncertain. Since this material could never be isolated as a peak fraction on one-step agarose GPC, no indication of a corresponding peak in the elution pattern from the G5000 PW type column could be found and its molecular weight value coincides possibly with the G4000 SW exclusion limit for these proteins, we looked for an artificial origin of this peak. The results of simulation calculations, presented below, confirm the artificial character of this peak caused by coupling the two different columns.

The G5000 PW exclusion limit for compact globular protein

Although the literature concerning TSK GEL SW fractionation of proteins is rapidly expanding, few papers have described the elution of proteins on TSK GEL PW type supports[2,5,6,9]. To our knowledge Himmel and Squire[9] are the only authors to mention the fractionation range for native high-molecular-weight proteins chroma-
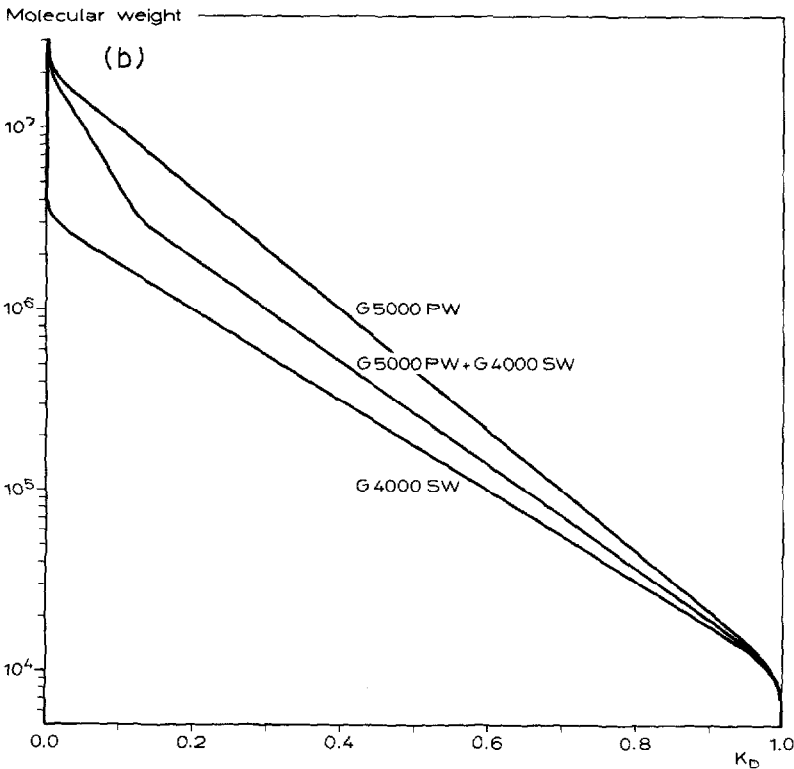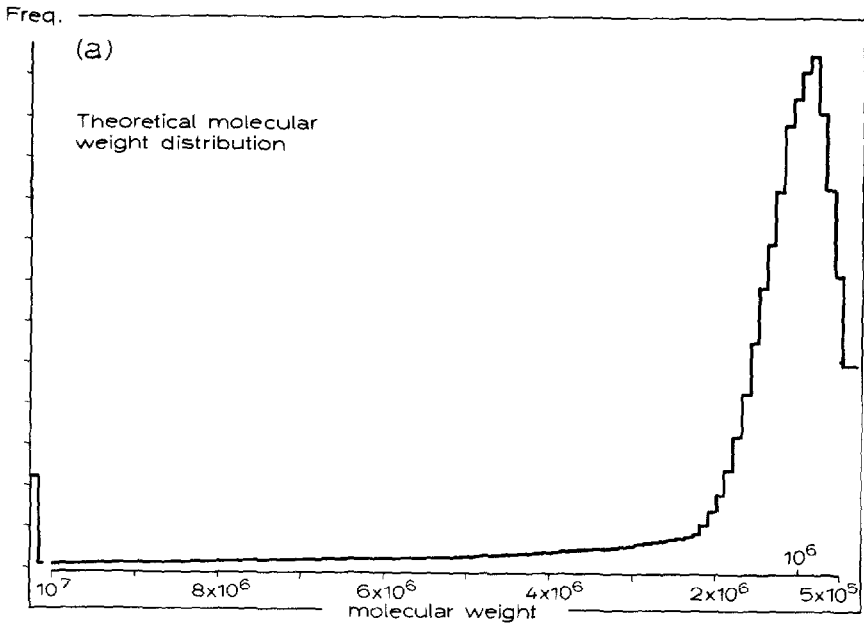
Fig. 2. Theoretical molecular weight distribution for calf lens nuclear HM- and α-crystallin (a) and calibration plots for the G5000 PW and G4000 SW type columns alone and in combination (b).

tographed on a TSK GEL G5000 PW column. Since the reported exclusion limit of $1.4 \cdot 10^6$ dalton is much lower than our estimate, we compared our results with a selection of elution data given by the previous authors. Table I lists several parameters of the elution shown in the upper part of Fig. 1 and those reported in ref. 9: M_r; distribution coefficient, K_d; the t_e/t_t value; the elution volume parameter, $F_{(v)}$, and the molecular weight function $\sqrt[3]{M_r \bar{v}}$. It can be seen that there is a good agreement between the elution parameters K_d and t_e/t_t for crystallins and other proteins of comparable size analyzed by Himmel and Squire[9].

It appears that the inconsistency between our exclusion limit estimate and that of Himmel and Squire[9] can be attributed to the assignment of the void volume peak. The latter authors considered most viruses and the sea worm chlorocruorin to be eluted in the void volume ($t_e/t_t = 0.642–0.668$) and tobacco mosaic virus to behave anomalously ($t_e/t_t = 0.495$). Moreover, in their elution pattern for the $5 \cdot 10^5$ dalton dextran sample a small void volume peak can also be found with a t_e/t_t value of 0.495. Whatever the structure of tobacco mosaic virus or the high-molecular-weight dextran component may be, it is theoretically impossible for them to be eluted earlier than the void volume. The reason why the three other viruses and the sea worm proteins elute at practically the same time remains to be established. We are convinced that the largest HM-crystallin aggregates elute in the totally excluded volume. Since the tobacco mosaic virus peak elutes at exactly the same position, our further calculations are based on the t_e/t_t value of 0.495 representing the void volume.

Fitting all data from Table I with the least-squares method revealed an excellent linear correlation between the distribution coefficient and the logarithm of the molecular weight (correlation coefficient 0.999). The exclusion limit, obtained by extrapolation of K_d to zero, amounts to $100 \cdot 10^6$ dalton. This value is higher than our estimate of $20 \cdot 10^6$ for HM-crystallin obtained using high-pressure GPC/low-angle laser light scattering and trypsin-treated HM-crystallin[19]; we found a weight average molecular weight of $30 \cdot 10^6$ for the smallest aggregates still eluting in the void volume and $15 \cdot 10^6$ for the aggregates which eluted immediately after the void volume peak. The discrepancy between both estimates may be explained by the structure of HM-crystallins which feature chain-like polymers mainly composed of α-crystallin entities[16].

Extrapolation using the method of Himmel and Squire[9,10], based on a linear relationship between the elution volume function, $F_{(v)}$, and $\sqrt[3]{M_r \bar{v}}$, yields a considerably lower value for the exclusion limit: $13 \cdot 10^6$ dalton (correlation coefficient 0.97).

Simulation of elution patterns

To investigate whether an extra peak may be generated when two columns with different pore sizes are coupled, simulation calculations were performed. Fig. 2a shows a theoretical molecular weight distribution for HM- and α-crystallin. Although precise data are still lacking, we think that this pattern represents a reasonable approximation of the actual distribution. The distribution profile is sliced into fractions of width 10^5 dalton. In Fig. 2b, theoretical calibration curves are presented for a TSK GEL G4000 SW column, a G5000 PW column and a combination of both. The curves for the G5000 PW and G4000 SW columns were constructed considering
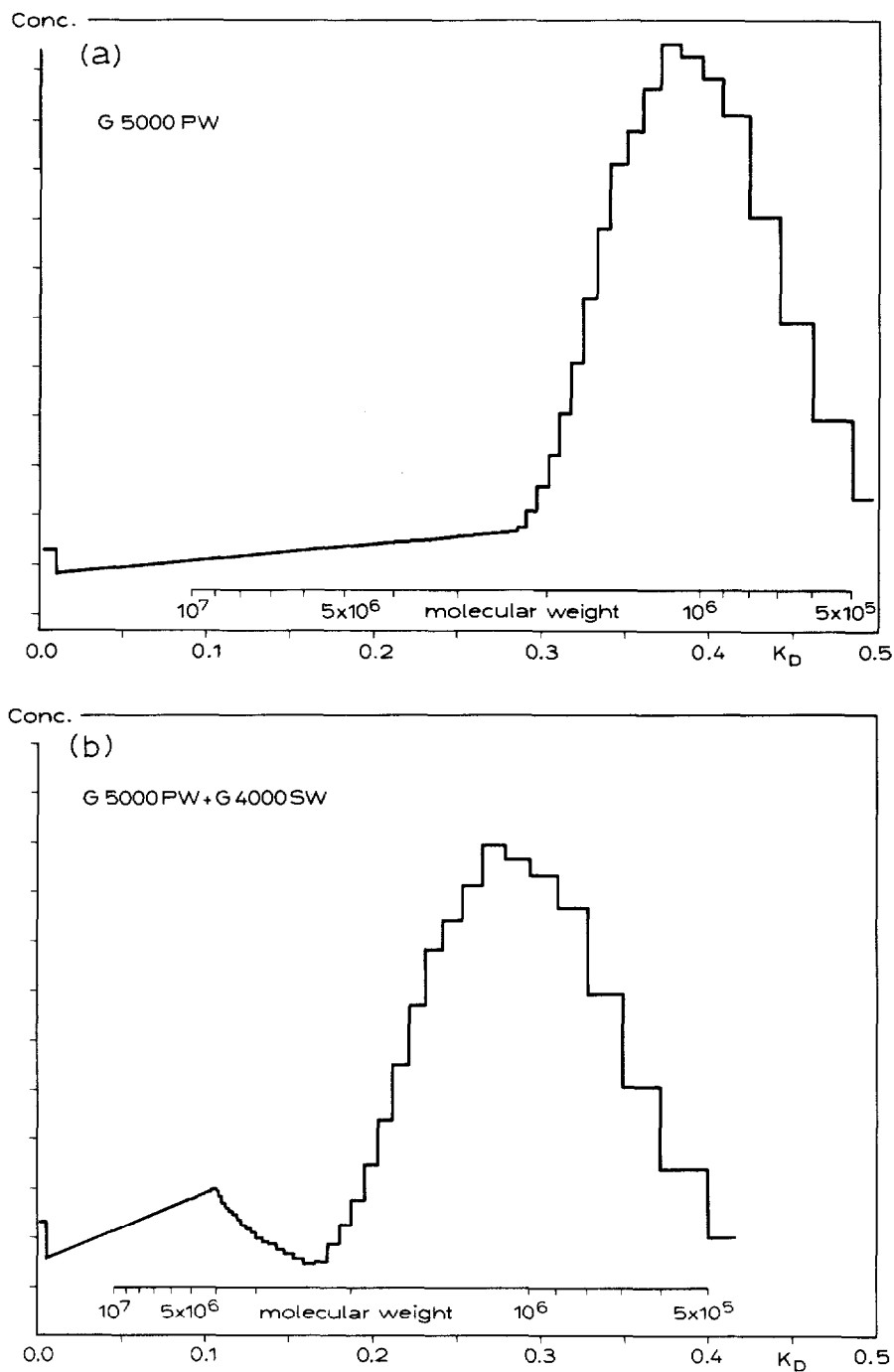
Fig. 3. Simulated chromatograms calculated from the molecular weight distribution and calibration plots given in Fig. 2. The pattern for a G5000 PW type column alone (a) and the profile corresponding to the coupled G5000 PW–G4000 SW type columns (b) are given.

exclusion limits of $4 \cdot 10^6$ and $20 \cdot 10^6$, respectively, and a total permeation limit of 10^4 for both supports. We think that a reasonable description is provided for the actual situation in the case of crystallins. The curve for the combined columns was obtained by plotting for each molecular weight the average distribution coefficient for the separate columns. This is permitted since both columns are of exactly the same size and appear to have similar pore volumes. The inflection point appearing at the upper exclusion limit of the G4000 SW type column is marked.

In fig. 3 simulated chromatograms are shown which were calculated from the theoretical molecular weight distribution and calibration curves. For each slice in Fig. 2a, representing molecules within a range of 10^5 dalton, the distribution coefficients at the molecular weight limits were determined from Fig. 2b. The simulated chromatograms were constructed using the minimal and maximal distribution coefficients for each slice (representing the slice width), and considering the areas of the different slices to be proportional to those in the molecular weight distribution plot (Fig. 2a). So, the slice heights were obtained from the ratio of the slice areas and the slice widths. The extra peak in the simulated elution pattern of the combined columns arising at the upper exclusion limit of the G4000 SW column is obvious.

It has to be stressed that the shape of the chromatograms is rather arbitrary; in fact we calculated Fig. 2a from Fig. 3a based on a linear increase of the chromatogram for eluting molecules between $20 \cdot 10^6$ and $2.1 \cdot 10^6$ dalton. If only straight lines were used in the theoretical calibration plot a saw-tooth shape for the artificial peak was obtained. For the sake of simplicity we neglected diffusion and/or band spreading effects in the simulation.

DISCUSSION

Whenever a broad continuous molecular weight distribution extends beyond the fractionation limits of the constituents of a combined GPC column system, additional peaks may be observed caused by the chromatographic system. This effect is observed with high-molecular-weight eye lens proteins on a combined TSK GEL G5000 PW and G4000 SW column system. While investigating the suitability of the semi-rigid TSK GEL Toyopearl packings for GPC analysis of dextrans, Barker et al.[12] also found an additional peak for which they had no explanation. Since it occurred only when columns were packed with certain mixtures of HW55S and HW65S and appeared to be related to the exclusion limit of the HW65S packing, we are convinced that they observed the same effect.

Using different column combinations, Mori[24] compared the average molecular weight distribution curves for polystyrene NBS 706, NBS 705 and a commercial polystyrene with a broad MW distribution. Although the average molecular weights were not affected by the column combinations, he found that the integral molecular weight distribution curves showed definite differences among several column sets. For a broad-range calibration column systems, Yau et al.[25] suggested the combination of two GPC columns having about one decade difference in pore size and approximately equal pore volumes for the two pore sizes. The desired situation is one in which the linear portions of the individual column molecular weight calibration graphs are substantially non-overlapping and the pore volume of each packing is such that the linear parts of the calibration graphs are essentially parallel. Unfortunately, such

supports are not yet available for aqueous high-pressure GPC; it appears that the TSK GEL SW and PW type columns principally differ in their upper exclusion limits and to a lesser extent in the lower ones, thus resulting in non-parallel calibration curves.

The accuracy of molecular weight determinations using empirically derived calibration plots is influenced by many factors. Besides the development of high-performance instrumentation, attention has been focused on obtaining linear calibration curves, thus reducing the number of necessary calibration points. However, it is obvious that for the determination of molecular weights on combined column systems, many calibration samples are needed properly to construct the calibration curves. An alternative would be the use of a molecular weight-sensitive detection system.

It should be clear that it is not strictly relevant to determine the exact value for the exclusion limit of TSK GEL G5000 SW type supports for globular and compact high-molecular-weight proteins since there are very few proteins which meet these demands; both HM-crystallin and tobacco mosaic virus are asymmetrical. However, the difference between the value of $1.4 \cdot 10^6$ of Himmel and Squire[9] and our estimate of $100 \cdot 10^6$ is too large. An intermediate value of $13 \cdot 10^6$ may be found if one re-calculates the data of the previous authors using the method based on the $F_{(v)}$ vs. $\sqrt[3]{M_r \bar{v}}$ relation[9,10] and a different void-volume fraction. Support for the $100 \cdot 10^6$ value can be obtained if we consider the GPC elution behaviour of dextrans on TSK GEL G4000 SW and G5000 PW; the exclusion limits were experimentally found to be $0.5 \cdot 10^6$ (ref. 4) and $7 \cdot 10^6$ (ref. 3), respectively. Using the data compiled by Kuga[7], these values correspond with equivalent sphere radii of 17 and 50 nm, respectively; according to Senti et al.[14] ($R = 0.066 M_r^{0.43}$), radii of 19 and 58 nm, respectively, can be found. Since the approximately three-fold increase in radius corresponds with a 27-fold increase in volume and considering the exclusion limit of compact globular proteins on TSK GEL G4000 SW, $7 \cdot 10^6$ (ref. 4), the estimate of $100 \cdot 10^6$ for such proteins on G5000 PW type columns seems more reasonable than the lower values. For the chain-like HM-crystallin protein clusters a value of $20 \cdot 10^6$ is postulated for the molecular weight exclusion limit.

In conclusion, we would like to emphasize that checking of the elution behaviour on separate columns is needed to decide whether additional peaks or shoulders, observed in a high-resolution system consisting of supports with different pore sizes, represent discrete sub-populations of molecules or column artifacts. Although the method of Himmel and Squire[9] seems to have advantages over the logarithmic treatment for molecular weight estimations by interpolation of the data obtained from standard proteins, extrapolation of these data to obtain the exclusion limit should preferably be performed according to the logarithmic method.

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