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Evaluation of morphological structure of packings by gel permeation chromatography

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ABSTRACT

Evaluation of several matrices by gel permeation chromatography (GPC) revealed that the chromatographic characteristics of packings are sensitive to the morphological structure. When closed morphology granules are used, a novel, non-classical chromatographic profile is observed. This profile is seen in chromatograms of polymeric standards and also in the calibration graph for the packing and has been termed the "skin effect". Gel filtration of polymeric standards was used to evaluate granule surface permeability and GPC process equilibrium. The use of closed morphological structure matrices for GPC is problematic because of the narrow fractionation interval and possible non-equilibrium GPC processes. Among the closed morphological structure packings, membranous granules are notable for their denser but sufficiently permeable surface. Such packings fractionate macromolecules according to molecular mass into two groups.

INTRODUCTION

Among several available means for the investigation of packings, gel permeation chromatography (GPC) is notable for the information it provides [1]. In addition, this method is valuable for its simplicity, as it can be carried out using standard chromatographic apparatus. In addition to the common characteristics of packings such as porosity and exclusion limits, it is also possible to determine the most important parameters of matrices, namely average pore size, polydispersity and specific pore surface area, by means of gel chromatographic porosimetry [2]. However, if the morphology of the granules is not fully investigated, then all of the above factors do not fully reflect the complexity of the matrix structure, or their complete chromatographic characteristics.

The morphological uniformity of a porous structure may be evaluated by means of electron microscopy [3,4]. However, there are difficulties in the preparation of soft hydrogel samples. Essential changes in porous structure may occur when the samples are dried. In order to minimize the drying effect, cryogenic preparation methods are employed for soft cellulose hydrogels (freeze-drying at the critical point). Samples may also be washed with volatile organic solvents and dried under vacuum [5].

Samples which are not dried, however, are more acceptable for the analysis of matrix porous structure. One of these methods is known as the "interuption" method [6], by which the granule morphology of ion exchangers can be qualitatively characterized. Special equipment is necessary, however, for the investigation of ion-exchange kinetics.

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Further, the application of this method is problematic with neutral matrices.

It is worth mentioning with regard to the GPC method that it is generally accepted that the matrix morphology has a significant effect on chromatographic characteristics. However, there have been no detailed studies on the determination of the relationship between chromatographic characteristics and granule morphology, except for the work of Motozato, *et al.* [7]. They prepared granules with a denser external layer and determined their chromatographic properties.

The evaluation of the effect of matrix granule morphology on gel size-exclusion properties is reported in this paper.

EXPERIMENTAL

Apparatus

A liquid chromatograph (Kovo, Czechoslovakia) consisting of an HPP 5001 precision electromechanical pump, RIDK 102 differential refractometer, LCD 2563 UV–VIS detector and TZ 4620 linear recorder was utilized.

Materials

For packing of columns, Granocel-4, Granocel-8, Granocel-14, Granocel M-8 and Granocel MB-8 (0.07–0.2 mm fraction) were used. These matrices were developed in our laboratory. We also employed commercial packing materials: open morphological structure gel Toyopearl HW-55F (Toyo Soda, Japan) and closed morphological structure ion exchangers Ostsorb-DEAE (Spolchemie, Czechoslovakia) and DEAE-cellulose (Reanal, Hungary). The dextrans listed in Table I were used as standard polymers.

TABLE I

CHARACTERISTICS OF STANDARD POLYMERS

M_w = Mass-average molecular mass; M_n = number-average molecular mass.

Dextran	Manufacturer	M_w	M_w/M_n
T5	Serva	4700	–
T10	Pharmacia	9000	1.73
T20	Ferak	23 000	1.30
T40	Pharmacia	35 000	1.80
T70	Pharmacia	74 000	2.05
T100	Serva	129 000	1.41
T500	Pharmacia	484 000	2.46
T2000	Pharmacia	2 000 000	–
T5000–40 000	Serva	5 000 000–40 000 000	–

D-(+)-Glucose (Reakhim, USSR) was used as a low-molecular-mass (low-M_r) chromatographic standard. Bovine serum albumin (BSA) was obtained from Reanal.

Conditions

The concentration of injected sample solutions was in the range 0.3–2.0 mg/ml. The flow-rate was 7.1–42.6 ml/cm^2 · h. Chromatography of BSA was performed in 0.05 *M* Tris–HCl buffer (pH 8.35).

RESULTS AND DISCUSSION

In order to determine effect of the morphology of granules on the chromatographic characteristics of packings, cellulose matrices with a closed morphological structure, *viz.*, Granocel-4, Granocel-8, Granocel-14, Granocel M-8 and Granocel MB-8, were investigated. It was observed that the gel chromatograms of standard dextrans (Fig. 1a) obtained under normal GPC conditions (flow-rate 21.2 ml/cm^2 · h, sample concentration 2.0 mg/ml) when using a column packed with neutral cellulose matrix Granocel-8 differ from those obtained when typical gels were employed. As shown in Fig. 1, dextrans T2000 and T500 do not penetrate into the pores of Granocel-8 and are eluted with the column void volume (v_0). Lower-M_w dextrans, T70 and T40, partially penetrate into the packing. However, their elution curves are different from typical chromatograms, which exibit Gaussian curves. In spite of the monomodal M_r distribution of dextrans T70 and T40 (Pharmacia data), the chromatograms are elongated and have two more or less noticeable peaks. Further, the first chromatographic peak corresponds to the column void volume (v_0).

According to the ratio of the areas under these peaks, the amount of sample retained increases with decreasing M_r of the dextran. Injected samples of standard dextrans T20 and T10 and also glucose were almost completely retained.

Analogous separations of standard dextrans were obtained with the more porous Granocel-4 and the less porous Granocel-14 packings (data not presented). The calibration graph for Granocel-8 (Fig. 1b)

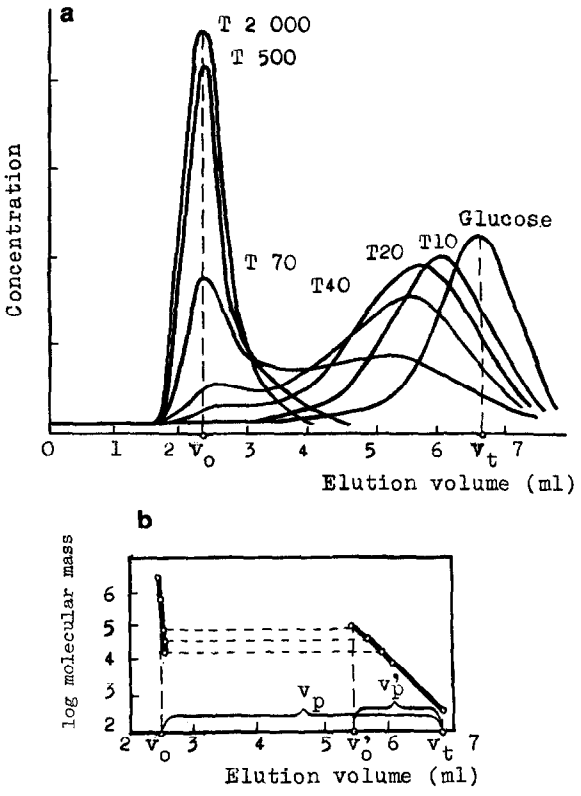

Fig. 1. (a) Chromatograms of standard dextrans obtained on the closed morphological structure cellulose packing Granocel-8 and (b) calibration graph for the packing. Flow-rate, 0.1 ml/min; detector, refractometer; column, 250 × 6 mm I.D.; sample, 0.3 ml, 2 g/l; eluent, distilled water.

shows the typical curve obtained for closed morphological structure packings. As can be seen in Fig. 1b, fractionation of polymeric standards, which is usually observed for conventional gels, does not occur within the whole interval $[v_0; v_t]$. The monotonous dependence of the elution volume v_e on the logarithm of M_w $[v_e = f(\log M_w)]$ is observed only within the interval $[v'_0; v_t]$. This interval represents only part of the whole packing pore volume v_p. Therefore, the efficiency of application of such packings for gel chromatography is always less than the efficiency of application of typical gels. In each particular case this depends on the ratio v'_p/v_p (Fig. 1b). When the magnitude of v'_p/v_p approaches unity, the "skin effect" manifests itself more subtly. As this occurs, the morphological structure and chromatographic properties of the packing differ very little from the characteristics observed for typical gels.

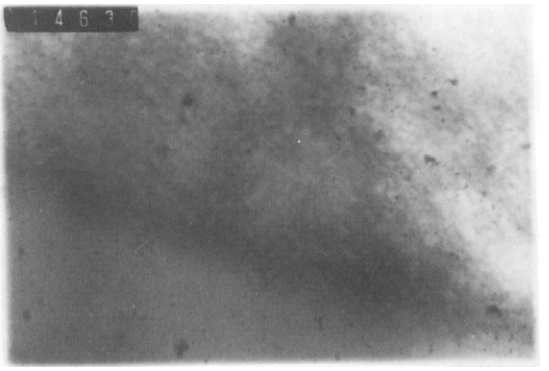

Fig. 2. Electron micrograph of Granocel-8 sphere cross-section (× 23 800).

The "skin effect" is a reflection of a specific chromatographic property of the packing which is due to the density gradient on the exterior of the granules and should be understood as a qualitative characteristic of granule morphology. The reflection of the density gradient quantitative parameters of the surface of the granules was not determined from gel chromatographic characteristics. On the other hand, qualitative determination of the morphological structure of the packing granules by means of GPC is superior because of its sensitivity. This sensitivity is observed both when there is a gradual densification of the outer layer of the granules which is indistinct and unobservable by other methods (Fig. 2). This effect is also observed when there

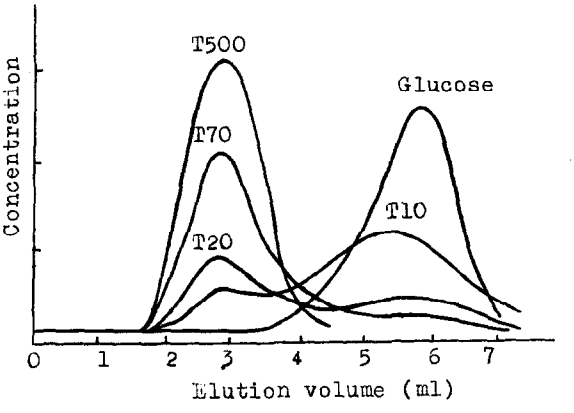

Fig. 3. Chromatograms of standard dextrans obtained on the closed morphological structure cellulose packing Granocel M-8. Flow-rate, 0.1 ml/min; detector, refractometer; column, 190 × 6 mm I.D.; sample, 0.3 ml, 2 g/l; eluent, distilled water.

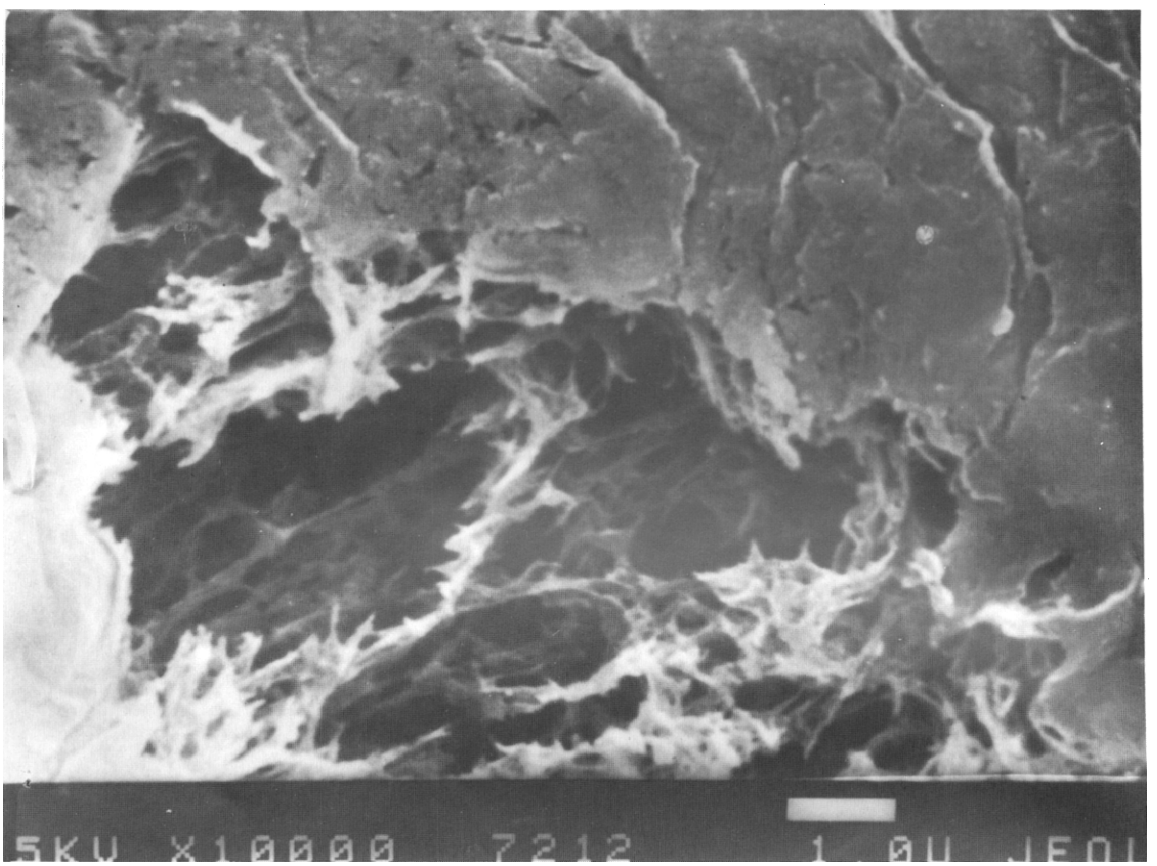

Fig. 4. Electron micrograph of Granocel M-8 granule surface and inner layers (× 10 000).

is distinct film on the granules. This film has quantitative characteristics that can be observed by microscopic methods (Figs. 3 and 4).

The "skin effect" may be observed in cellulosic matrices other than the present Granocel materials (Figs. 3, 5 and 6 in ref. 9) and also with synthetic packings, *e.g.*, Toyopearl HW-65 and Toyopearl HW-75 (Fig. 7B and C in ref. 9). A closed morphological structure is characteristic also for the ion exchangers Ostsorb-DEAE and Reanal DEAE-cellulose (Fig. 5).

The closed morphological structure of the matrices may be easily verified when the granules are subjected to mechanical grinding, *i.e.*, destroying the outer layer and obtaining easily accessible porous structure particles. After the grinding of Granocel-8 granules, for example (Fig. 6), the character of the elution curves of standard dextrans is entirely different (Fig. 7a). Typical Gaussian curves are now observed. As can be seen in Fig. 7b, the upper exclusion limit of the ground granules is increased and a linear dependence $v_e = f(\log M_w)$ is observed throughout almost the whole $[v_0; v_t]$ interval.

It is necessary to carry out this grinding procedure when there is doubt about the possible adsorption interaction of the matrix with standard polymers. As two different results are obtained under analogous conditions, the possibility of adsorption interaction may be excluded as a factor in the chromatographic behaviour of the packing. It is important to note that the gel chromatographic conditions for both intact and ground particles were identical.

In order to explain the nature of the chromatographic "skin effect", it is worth remembering that based on general GPC principles [10], every macro-

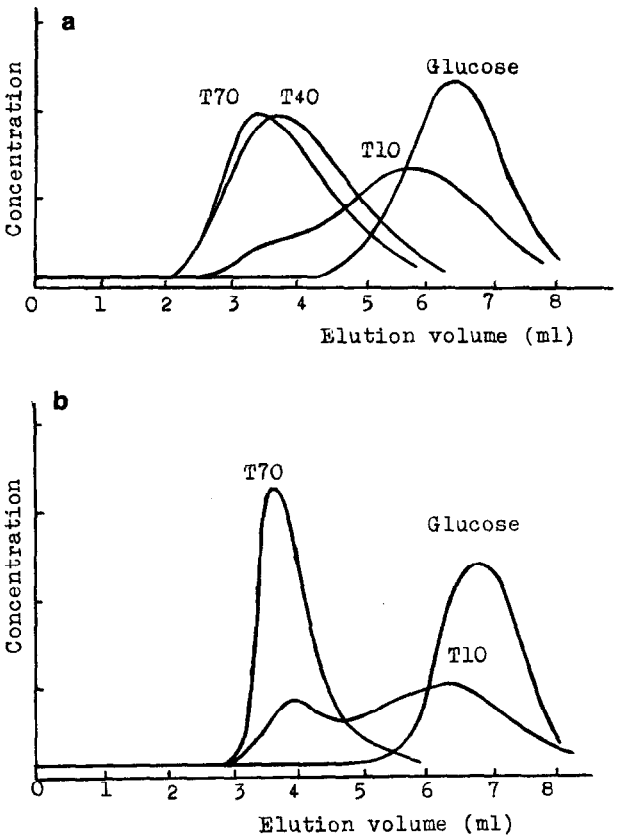

Fig. 5. Chromatograms of standard dextrans obtained on cellulose anion-exchangers: (a) Ostsorb-DEAE (column, 210 × 6 mm I.D.) and (b) Reanal DEAE-cellulose (column, 270 × 6 mm I.D.). Flow-rate, 0.1 ml/min; detector, refractometer; sample, 0.3 ml, 2 g/l; eluent, distilled water.

Fig. 6. Optical micrograph of ground Granocel-8 packing granules (× 90).

molecular sorption–desorption process has the following stages: (1) random macromolecules wandering in the canals of the mobile phase, due to thermal motion and hydrodynamic conditions; (2) transfer of macromolecules from the mobile phase into the stationary phase, *i.e.*, their entry into packing pores; (3) macromolecular diffusion back and forth from the external surface of the packing granules into internal canals, *i.e.*, random movement back and forth in the porous space; and (4) desorption of macromolecules, *i.e.*, their transition from the surface of the packing granules into the mobile phase.

In the process of GPC, there is no adsorption interaction between the macromolecules and the packing matrix. Therefore, stages 2 and 4 are relatively shorter than stages 1 and 3, and the kinetics of the GPC process are determined by random macromolecular motion in the mobile phase and their diffusion within granules. The denser layer on the

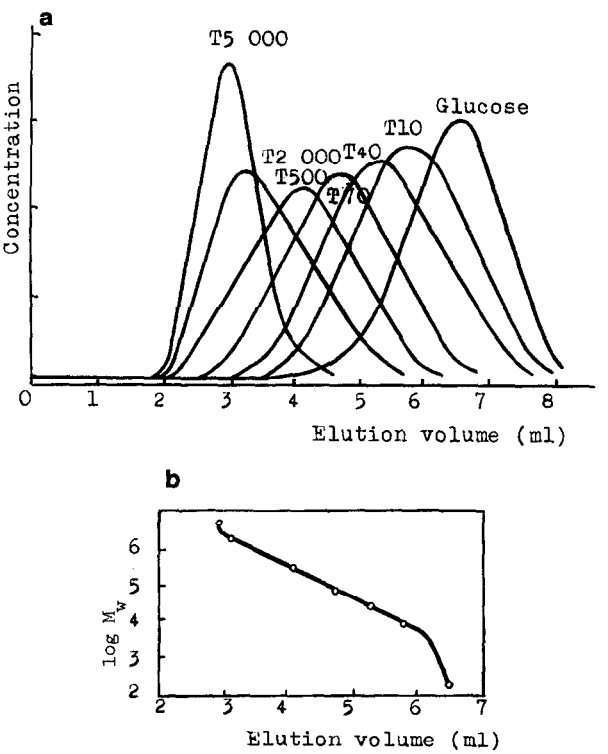

Fig. 7. (a) Chromatograms of standard dextrans obtained on the ground cellulose packing Granocel-8 and (b) calibration graph for the packing. Flow-rate, 0.1 ml/min; detector, refractometer; column, 250 × 6 mm I.D.; sample, 0.3 ml, 2 g/l; eluent, distilled water.

granule surface makes diffusion of macromolecules in and out of particles much more difficult. For this reason, the stages outlined above are prolonged, causing the chromatographic process to change.

Fig. 8 illustrates the dynamics of the separation of a macromolecular standard with a monomodal M_r distribution into two zones. This profile is not chracteristic of open morphological structure packings.

When closed morphological structure packings are used in GPC, the process may not undergo complete equilibrium owing to the poor permeability of the granules surface. This is confirmed by the experimental results for the chromatography of monodisperse protein on Granocel-8 packing (Fig. 9). Chromatography of BSA, which exibits normal chromatographic behaviour on the open morphological structure gel Toyopearl HW-55F, shows a distribution into two chromatographic peaks and separation of the protein into a wide zone when Granocel-8 was used. Also, when the concentration of the injected sample is decreased, the character of the chromatographic curve changes, *i.e.*, the relationship between the peak areas and also the position of the second peak.

The chromatograms of the polydisperse standard T70 ($M_w/M_n = 2.05$) are also essentially changed (analogous to the chromatographic curves of BSA) when different concentration samples are injected.

It should also be noted, however, that even during a non-equilibrium GPC process the sample may undergo partial fractionation according to M_r. As analysis of the collected fractions from the first and second chromatographic peaks (Fig. 10a) obtained using Toyopearl HW-55F gel has shown (Fig. 10b) these fractions are not pure with respect to M_r. The first chromatographic peak contains the higher-M_r fraction and the second contains the lower-M_r fraction. As the chromatographic process on Granocel-8 is non-equilibrium, however, refractionation of the collected fractions results in separation again into two zones (Fig. 10a). It was found that when the flow-rate was changed from 7.1 to 42.6 ml/cm^2 · h, the elution of the intermediate molecular mass

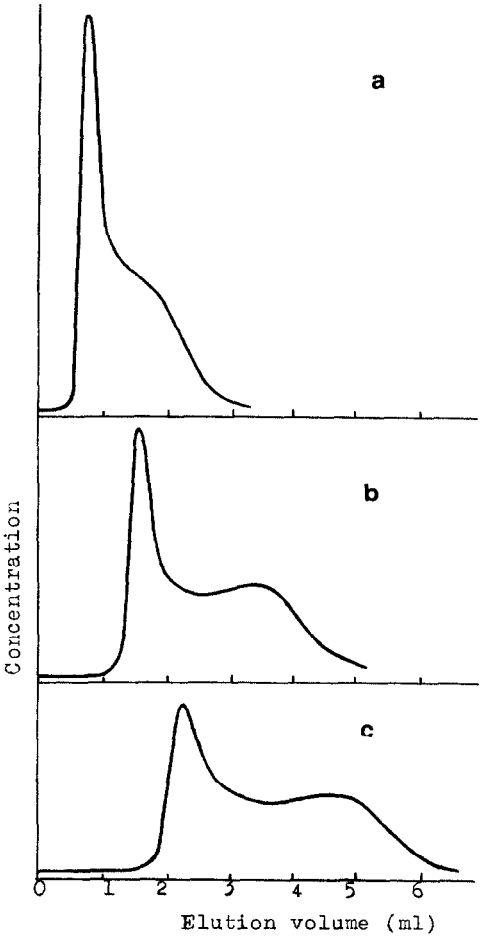

Fig. 8. Dynamics of resolution of standard dextran T70 into two chromatographic zones obtained on the cellulose packing Granocel-8 when columns of (a) 56 × 6 mm I.D., (b) 116 × 6 mm I.D. and (c) 170 × 6 mm I.D. were used. Flow-rate, 0.1 ml/min; detector, refractometer; sample, 0.3 ml, 2 g/l; eluent, distilled water.

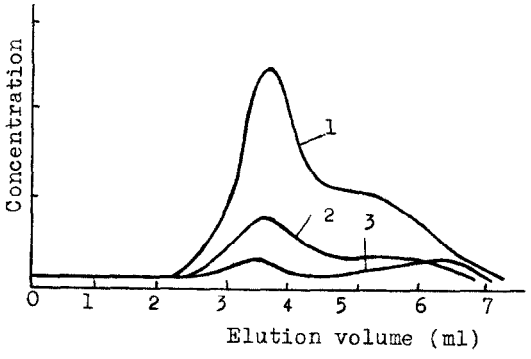

Fig. 9. Resolution of BSA when the closed morphological structure cellulose packing Granocel-8 was used. Flow-rate, 0.1 ml/min; detector, UV (254 nm); column, 250 × 6 mm I.D.; sample (0.3 ml) concentration, (1) 10 g/l, (2) 3 g/l and (3) 1 g/l; eluent, 0.05 *M* Tris–HCl buffer (pH 8.35).

dextran standards on the Granocel-8 packing occurred in different way. The change in the character of the chomatographic profiles is a same as that observed when the concentration of the injected sample is increased.

By repeated fractionation and analysis of the collected fractions of dextran standards, it was established that the equilibrium process may be partialy reached when the flow-rate and concentration of the sample are decreased. Theoretically, however, the direct cause of the non-equilibrium GPC process should be the insufficient accessibility of the granule porous structure. This depends on the ratio of the open pore surface area to the total granule surface area. The application of closed morphological structure matrices in GPC is therefore problematic not only owing to the narrow fractionation interval, *i.e.*, lower selectivity, but also to possible non-equilibrium GPC processes. In this instance the results of chromatography are very dependent on the sample concentration and the elution flow-rate. If in a common gel chromatographic process changing the sample concentration or flow-rate causes a change in the width and the height of the chromatographic peak (*i.e.*, a change in the efficiency of elution), then in a non-equilibrium gel chromatographic process a further change is observed (*i.e.*, a change in the position of the peak).

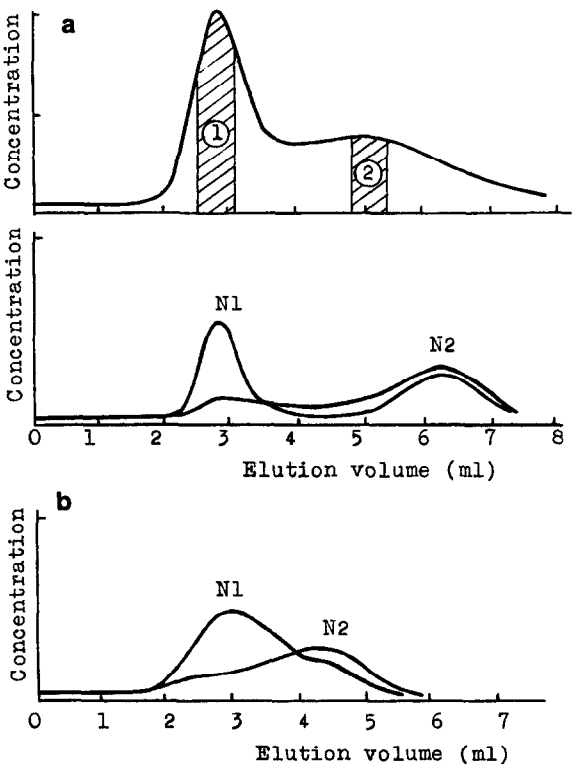

Fig. 10. (a) Collection of standard dextran T70 fractions N1 and N2 and their repeated fractionation. Packing, Granocel-8; flow-rate, 0.1 ml/min; eluent, distilled water. (b) Analysis of collected fractions N1 and N2 on the typical gel Toyopearl HW-55F. Flow-rate, 0.1 ml/min; detector, refractometer; column, 250 × mm I.D.; sample, 0.5 ml; eluent, distilled water.

Interesting chromatographic properties are characteristic of closed porous structure packings with denser but still sufficiently permeable membranes on the granule surface. In this instance macromolecules are fractionated into two groups, of lower and higher M_r the elution volumes of which correspond to the chromatographic zones v_t and v_0 (total volume and void volume), respectively. Such a chromatographic process is more similar to ultrafiltration, because the macromolecular fractionation does not show a linear dependence $v_e = f(\log M_w)$, whereas the upper and lower exclusion limits become so narrow that they almost coincide. Their value is dependent on the pores size of the granule membrane. Because of the easily accesible porous structure of the packing, this case may be treated as a limiting case. Therefore, it is worth studying this situation in more detail.

Fig. 11 illustrates chromatograms of dextrans obtained using the membranous cellulose packing Granocel MB-8. Polydisperse polymeric probes with a wide M_r distribution may be separated into two zones, as is also obtained when using Granocel-8. In this instance, however, the granule packing surface is sufficiently permeable and, as seen from repeated elution of collected fractions N1 and N2, the chromatography using this packing is an equilibrium process. It was determined by analysis of the collected fractions that they are very similar with respect to molecular mass and would be very difficult to separate using typical gels. When the typical gel chromatographic packing Toyopearl HW-55F is used, the chromatograms of fractions N1 and N2 show substantial overlapping and the peaks are separated with very low selectivity. However, when the membranous packing Granocel MB-8 is used, they are separated with maximum selectivity, *i.e.*, are eluted in the zones which correspond to the void volume and total volume of the column (Fig. 11).

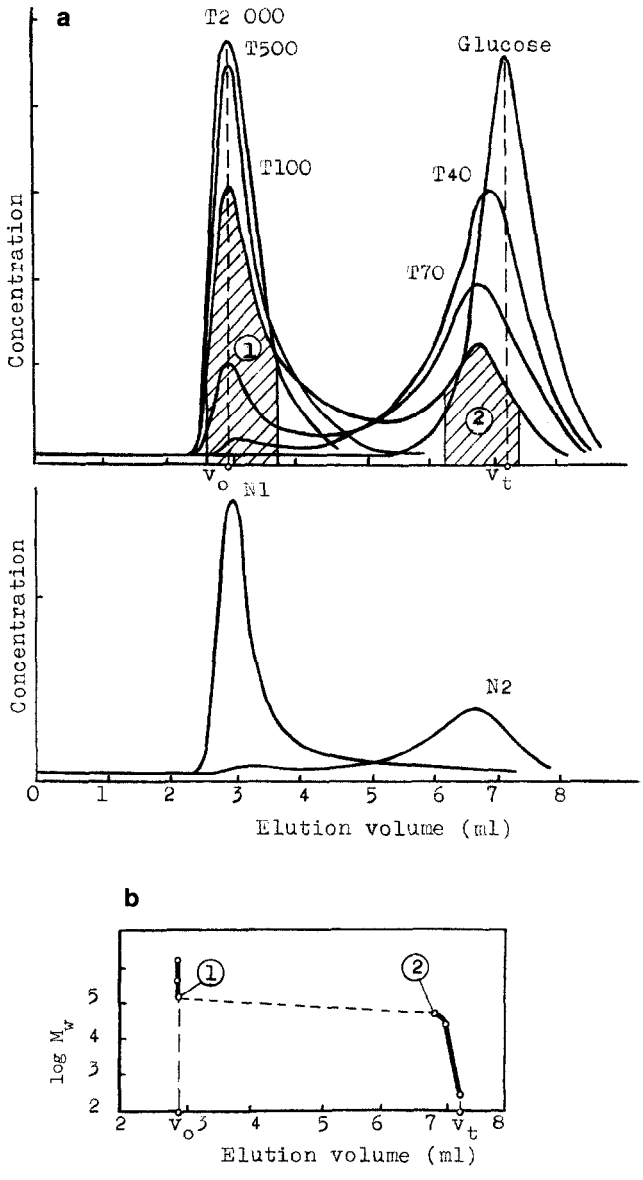

Fig. 11. (a) Chromatograms of standard dextrans obtained when the membranous cellulose packing Granocel MB-8 was used and resolution of collected standard dextran T100 fractions, and (b) Calibration graph for the packing. Flow-rate, 0.1 ml/min; detector, refractometer; column, 250 × 6 mm I.D.; sample, 0.5 ml, 2 g/l; eluent, distilled water.

CONCLUSIONS

Gel chromatographic packing characteristics are sensitive to the granule morphological structure. A closed or open morphology of the packing granules can therefore be easily determined by GPC. A closed granule morphological structure causes essential changes in the chromatographic process and the "skin effect" is reflected in chromatograms of standard polymers and also in the column packing calibration graph.

Application of a closed structure packing in GPC depends on the intensity of the "skin effect", *i.e.*, on the GPC process equilibrium, which is due to the accessibility of the porous structure, and also on the decreased fractionation selectivity.

Among the closed morphological structure packings, membranous granules are notable for their special chromatographic characteristics. These packings fractionate macromolecules according to M_r into two groups with maximum selectivity. This is due to the denser but sufficiently permeable granule surface membrane.

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