Resolution obtainable with the gel permeation chromatography method applied to polymers and proteins

A. A. Gorbunov
All-Union Research Institute for Highly Pure Biopreparations, 197110 Leningrad, USSR

and A. M. Skvortsov
Chemical-Pharmaceutical Institute, 197022 Leningrad, USSR
(Received 30 August 1990; accepted 12 October 1990)

The resolution obtainable with the gel permeation chromatography (g.p.c.) method in separating macromolecules and globular proteins has been analysed. A relationship has been established between the optimum sizes of separated macromolecules and the width of the chromatographic packing. It has been shown that for all monoporous sorbents the polymer molecular weight logarithm separation range is the same and amounts to ~1.5 decade. For proteins the g.p.c. resolution is 1.5 to 1.8 times poorer than that for flexible chain polymers, and their separation range is broader by the same factor.

(Keywords: macromolecules; proteins; gel permeation chromatography; resolution; selectivity)

INTRODUCTION

Evaluation of the resolution obtainable with the gel permeation chromatography (g.p.c.) method is required in practically all principal applications of this method. Thus, in fractionation, it would be necessary to compare the degree of separation of the components of a polymer or protein mixture. High resolution is also essential for analysing the molecular weight distribution (MWD) and determining the polydispersity of polymers.

The resolution provided by the g.p.c. method in polymer applications has been discussed in numerous studies[1-5]. However, most of these studies are based on simplified semi-empirical concepts, specifically on the concept assuming calibration linearity in g.p.c.

The object of this work is to examine the resolution problem from the standpoint of a rigorous theory of polymer g.p.c. Attention will mainly be focused on establishing the general principles of resolution, valid for various polymers and packing materials, so that we should be able to formulate the conditions for optimizing the separation and analysis of macromolecules and proteins.

SEPARATION COEFFICIENT

The key concept for the determination of resolution in chromatography is the peak separation coefficient[1-4]:

$$R = \frac{V_2 - V_1}{2(\sigma_1 + \sigma_2)} \quad (1)$$

where V_1 and V_2 are retention volumes corresponding to the elution of two individual polymeric components of molecular weights M_1 and M_2 while σ_1 and σ_2 are the standard widths of the respective chromatograms. Retention volume V_i is normally measured from the position of the maximum of the chromatographic peak or, where the chromatogram is asymmetrical, from its first moment. The second (central) moment (peak dispersion) is equal to σ_i^2.

Equation (1) is also used in chromatography for estimating the peak separations of polydisperse samples. In this case, the dispersion of each peak comprises two components, one associated with the polydispersity of the sample, the other independent of the polydispersity and known as instrumental broadening. Subsequently, while examining the resolution, we shall discuss the separation of monodisperse components in the assumption that σ_1 and σ_2 in equation (1) are dependent on instrumental broadening only. It is normally assumed that for adjacent peaks $\sigma_1 \approx \sigma_2 \approx \bar{\sigma}$.

Peak separation patterns corresponding to different separation coefficient R values can be found in reference 1. Separation is generally considered to be good at $R \geqslant 1$.

Peak-to-peak distance $\Delta V = V_2 - V_1$ obviously depends upon the difference between the molecular weights of the separated components. Therefore the separation coefficient as such is not sufficient to characterize the resolution obtainable with the g.p.c. method. For a more adequate determination of resolution, reference 1 suggests standardization of the difference between the molecular weights of the components and introduces the concept of a normalized separation coefficient:

$$R_s = \frac{R}{\Delta \log M} \quad (2)$$

Numerically, R is equal to the separation coefficient for two components with a decade difference in molecular weight. From equations (1) and (2) it follows that:

$$R_s = \frac{1}{4\bar{\sigma}} \frac{\Delta V}{\Delta \log M} \quad (3)$$

Figure 1 illustrates schematically how the MWD values

0032-3861/91/163001-05

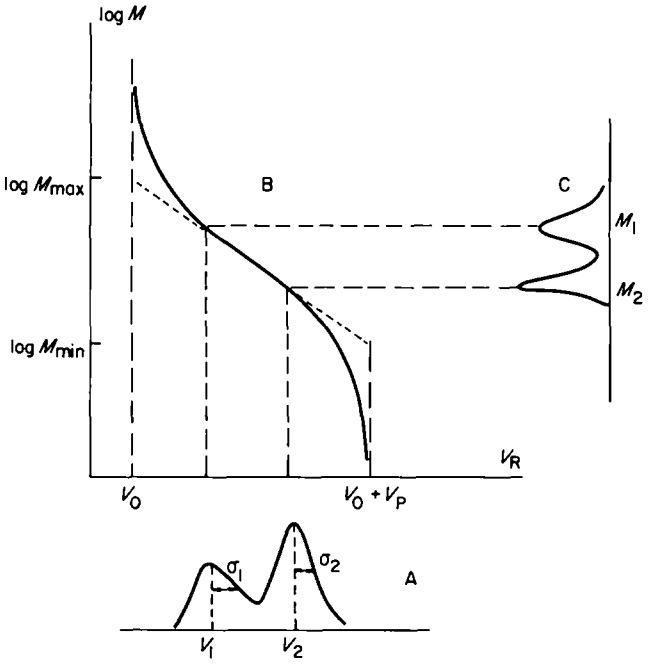

Figure 1 Schematic view of a chromatogram corresponding to two closely similar polymer homologues (A), a calibration relationship (B), and the MWD of polymer homologues of molecular weights M_1 and M_2 (C). Marked on the calibration curve (B) is the log M_{min} to log M_{max} working range

for samples (*Figure 1C*) are obtained from a chromatogram (*Figure 1A*) using a calibration relationship (*Figure 1B*).

The use of normalized separation coefficient R_s to characterize the resolution assumes the linearity of the calibration curve in the log M *versus* V coordinates (*Figure 1*). The parameter $\Delta V/\Delta \log M$ is then constant and equal to the reciprocal slope of this curve. At the same time, it is well known that the calibration relationship may be considered approximately linear, within a limited MW range. Strictly speaking, the calibration slope varies continuously; the difference in slope in the centre and at the ends of the calibration curve is quite substantial[1-5]. For the purposes of resolution analysis, we shall therefore introduce a differential normalized separation coefficient:

$$\tilde{R}_s = \frac{1}{4\bar{\sigma}} \frac{dV}{d \log M} \tag{4}$$

OPTIMUM SEPARATION

Let us pass over from retention volume V to distribution coefficient $K = (V - V_0)/V_p$ where V_0 and V_p are the mobile and stationary phase volumes. Then:

$$R_s = \frac{V_p}{4\bar{\sigma}} \frac{dK}{d \log M} \tag{5}$$

The first cofactor $V_p/4\bar{\sigma}$ in equation (5) is called the peak capacity[4,5] and is numerically equal to the maximum number of individual components (polymer homologues) separated within V_0 to $V_0 + V_p$, with the separation coefficient $R_s = 1$. The peak capacity increases with increasing packing, pore volume and with decreasing instrumental broadening $\bar{\sigma}$. Instrumental broadening in g.p.c. depends on a number of factors, the main ones being non-uniform chromatographic column packing, particle size of packing, eluent flow rate, geometrical column dimensions and spreading of the sample in the extra-column lines[5]. These are all known factors, and their influence is usually accounted for.

The instrumental broadening value can be estimated experimentally from the width of polymer standard chromatograms. This procedure is not quite precise, however, because there is still some polydispersity inherent in polymer standards and fractions. One other method of determining instrumental broadening experimentally, which does not require strictly monodisperse samples, is that used by Tung[6].

Thus, the first term in equation (5) is non-universal, being dependent on the design of a specific chromatographic system (column geometry and operating parameters). However, it can be measured and, in a measure, is amenable to optimization[5]. The dependence of this term upon distribution coefficient, pore width, etc. has been frequently discussed in the literature yet no satisfactory explanation has been found so far.

The object of this work will be to consider the second cofactor introduced in references 7 and 8, as well as its dependence on the parameters K, d and R and on the solvent quality. Thus the discussion will not be on the resolution capability as a whole, but rather on the selectivity of the g.p.c. method.

Using the universal dependence of distribution coefficient K on the macromolecule-to-pore size ratio R/d obtained by Casassa[9] for the theory of g.p.c., it was found possible to ascertain that the ψ parameter ($\psi = dK/d \ln M$), i.e. the calibration curve slope, is dependent on the distribution coefficient only and is given by an equation[7,8]:

$$\psi(K) \approx \begin{cases} (1-K)/2 & \text{at } K > 0.5 \\ K \ln\left(\dfrac{8}{\pi^2 K}\right) & \text{at } K < 0.5 \end{cases} \tag{6}$$

Experimental verification of equation (6) has shown[8,10,11] that this relationship holds good for various polymers and various types of chromatographic packing.

Figure 2a shows experimental data[12] on calibration relationships for dextrans on porous glasses with different pore diameters. The same data are given in *Figure 2b* in the universal coordinates of ψ *versus* K. It can be seen that for all five packings the points fit the same curve which coincides with the theoretical relationship in equation (6). *Figure 2* also presents data from references 10 and 11 for polystyrene on modified porous silica SW 3000 with a pore diameter of ~15 nm.

The ψ *versus* K relationship in *Figure 2b* testifies once again against the concept of calibration curve slopes being invariably constant and indicates that there exists a maximum value of ψ_{max} corresponding to optimum separation conditions in g.p.c. According to the theory advanced in reference 7, $\psi_{max} \approx 0.3$ at $K \approx 0.3$. Under these conditions the reduced separation coefficient reaches the maximum value:

$$(\tilde{R}_s)_{max} \approx 0.69 \frac{V_p}{4\bar{\sigma}} \tag{7}$$

Let $2d$ denote the packing pore diameter, and $\bar{R}$ the mean radius of gyration of the separated macromolecules. As previously mentioned, according to the g.p.c. theory in reference 9, the distribution coefficient K is a universal

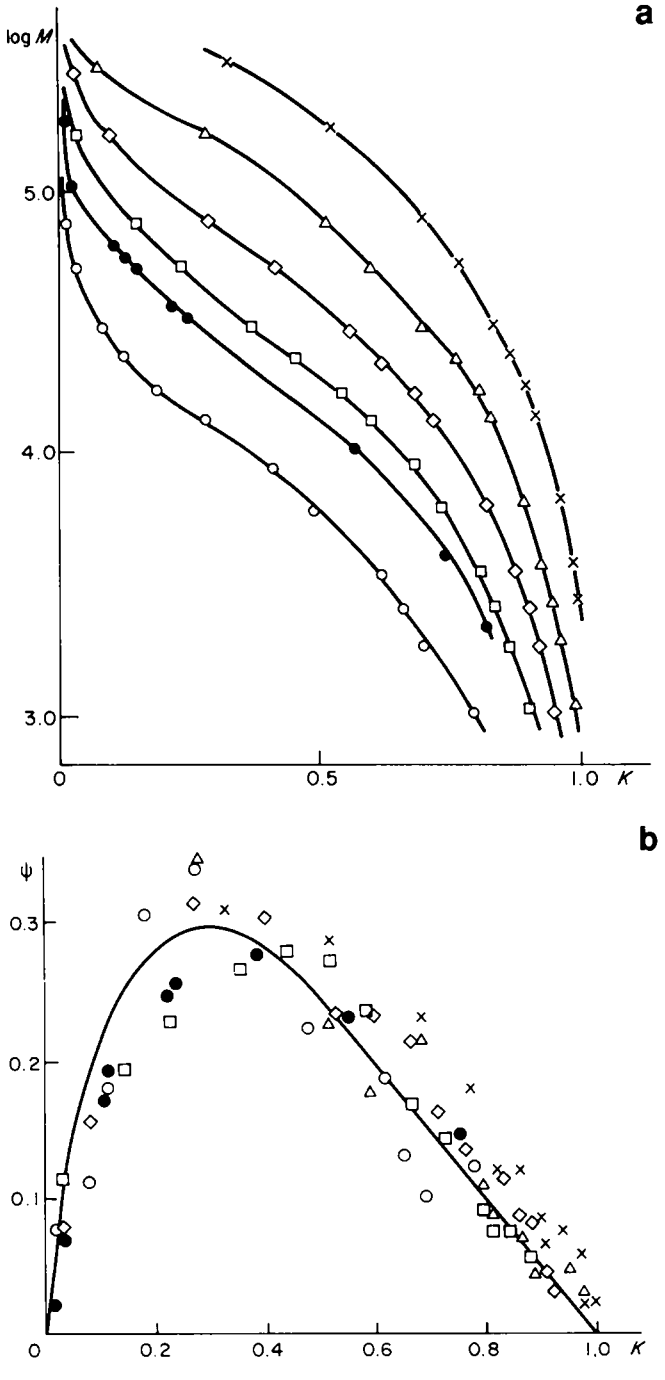

Figure 2 (a) Calibration relationships for g.p.c. of narrow MWD dextrans on porous glasses with pore diameters $2d$ equal to: ○, 8.4 nm; □, 15.9 nm; ◇, 22.7 nm; △, 31.4 nm; +, 51.7 nm (according to reference 12); ●, calibration relationships for g.p.c. of polystyrene standards on porous silica SW 3000 with $2d \approx 15$ nm (according to references 10 and 11). (b) The same data in the universal coordinates of $\psi = dK/d \ln M$ *versus* K. Solid line shows theoretical relationship (6) for slit-like pores

function of the $\bar{R}/d$ ratio. This permits equation (6) to be transformed as follows:

$$\psi(\bar{R}/d) \approx \begin{cases} \pi^{-1/2}\bar{R}/d & \text{at } \bar{R} < 0.45d \\ 2(R/d)^2 \exp\left[-\left(\dfrac{\pi R}{2d}\right)^2\right] & \text{at } \bar{R} > 0.45d \end{cases} \quad (8)$$

The ψ *versus* $\bar{R}/d$ relationship is shown in *Figure 3* (curve A). From equation (8) and *Figure 3* it follows that the maximum of ψ and, consequently, the maximum of resolution obtainable with the g.p.c. method in macromolecular applications are realized at:

$$\bar{R}_{opt} \approx \frac{2}{\pi} d \quad (9)$$

Equation (9) serves to establish the optimum ratio between the mean size of the separated macromolecules and the packing pore size.

To generalize equation (9) for the arbitrary pore shape case, we introduce, in keeping with reference 9, a specific pore surface area Σ equal to the ratio of the total area, S_p, of all pores to their total volume V_p. For pores of regular shape, $\Sigma = \alpha/d$ where $\alpha = 1$ for the slit-like pore shape, $\alpha = 2$ for the cylindrical pore shape, and $\alpha = 3$ for the spherical pore shape. We shall have then:

$$\bar{R}_{opt} \approx \frac{2}{\pi} \Sigma^{-1} \approx 0.64 \frac{V_p}{S_p} \quad (10)$$

In addition to the optimum size of macromolecules, one other important factor is the width of the working molecular weight (or size) range, wherein adequate separation is possible based on the use of a given chromatographic packing. To determine the working range, the middle portion of the calibration curve is approximated by a linear relationship which is extended until it intersects vertical straight lines $V_R = V_0$ and $V_R = V_0 + V_p$. As a result, $\log M_{max}$ and $\log M_{min}$ values are obtained, and the working range width is expressed as the number of decades: $n = \log(M_{max}/M_{min})$. It is easy to see that the working range width $n = \log M_{max} - \log M_{min}$ is linked to the maximum value ψ_{max}. In fact, according to *Figure 1*:

$$n = V_p\left(\frac{d \log M}{dV_R}\right)_{max} = \left(\frac{dK}{d \log M}\right)^{-1}_{max} = (2.3\psi_{max})^{-1} \quad (11)$$

Since $\psi_{max} \approx 0.3$ at any d, then according to equation (11) the working range width is the same for any monoporous packing and is equal to $n \approx 1.46$ MW decades.

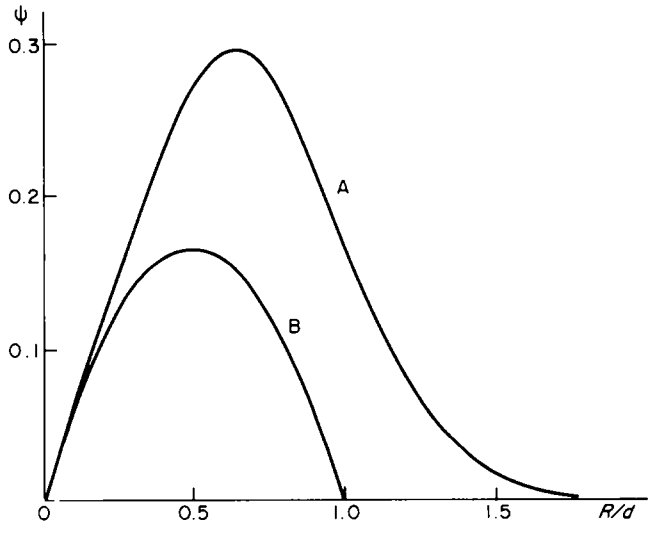

Figure 3 Calibration curve slope ψ *versus* the macromolecule-to-pore size ratio for Gaussian coils (curve A) and for proteins (curve B)

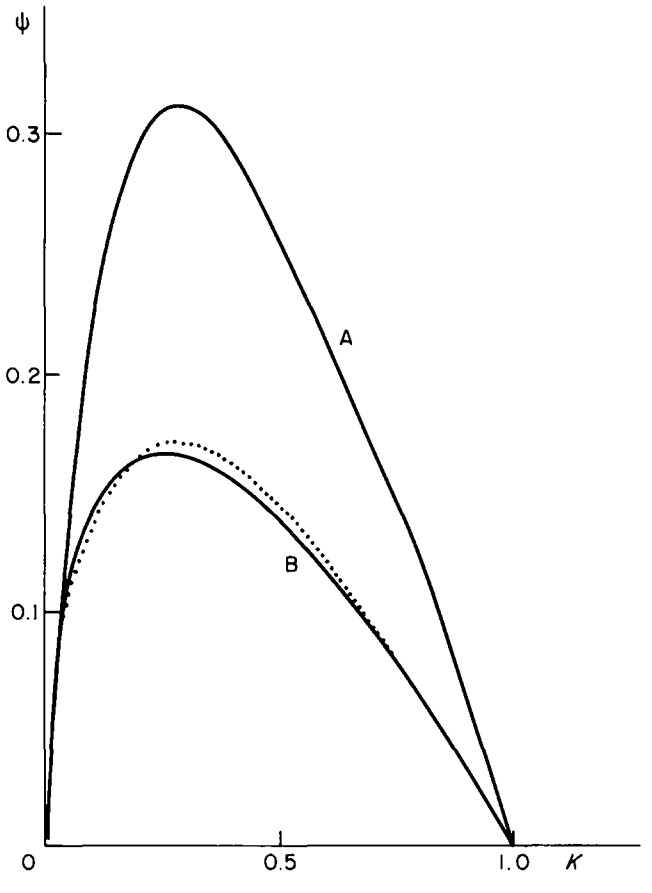

Figure 4 Relationship of ψ *versus* K for flexible-chain polymers in a good solvent with $\nu = 0.6$ (curve A) and in a poor solvent with $\nu = 1/3$ (curve B). Dotted line shows calculation from equation (17) for spherical particles (proteins) in cylindrical pores ($\alpha = 2$)

SEPARATION OF NON-GAUSSIAN MACROMOLECULES AND PROTEINS

Strictly speaking, the above analysis of the resolution obtainable with the g.p.c. method is valid only for ideal (θ) solvents, in which $\bar{R} \sim M^{\nu}$, and $\nu = \nu_\theta = 0.5$. In practice, use is more frequently made of good solvents with $0.6 > \nu > 0.5$. At the present time, there is no rigorous g.p.c. theory for macromolecules in a good solvent (with volume effects), but it has been shown experimentally that the universal Benoit calibration[13], i.e. the universal K *versus* R relationship, holds for both good and θ solvents. It may therefore be supposed that the general concepts regarding the g.p.c. resolution for macromolecules will remain valid if the solvent quality is accounted for by means of parameter ν. Then:

$$\psi = \frac{dK}{d \ln M} = \frac{\nu}{\nu_\theta}\psi_\theta = \frac{\nu}{0.5}\psi_\theta \qquad (12)$$

$$n \approx \frac{0.5}{\nu} n_\theta$$

According to equations (12), in good solvents with $\nu = 0.6$, the resolution obtainable with the g.p.c. method is 20% higher than in a θ solvent, while the working range is narrower.

Equations (12) also allow one to estimate the resolution obtainable with the g.p.c. method for macromolecules in a precipitating agent at $\nu_{gl} = 1/3$, i.e. practically globular proteins.

There is also another way to obtain the ψ *versus* K relationship for proteins. It is known[14,15] that the distribution coefficient K_{pr} for spherical molecules of radius R is equal to:

$$K_{pr} = \left(1 - \frac{R}{d}\right)^{\alpha} \qquad (13)$$

where $\alpha = 1, 2, 3$ for slit-like, cylindrical, or spherical pores, respectively. By modelling protein molecules as solid spheres with $R \sim M^{1/3}$, we obtain from equation (13):

$$\psi_{pr}(K) = \frac{dK_{pr}}{d \ln M} = \frac{\alpha}{3}(1 - K^{1/\alpha})K^{(\alpha-1)/\alpha} \qquad (14)$$

It follows from the analysis of equation (14) that the maximum of $\psi_{pr}(K)$ is attained at $K_{opt} = [(\alpha - 1)/\alpha]^{\alpha}$ and is equal to $(\psi_{pr})_{max} = [(\alpha-1)/\alpha]^{(\alpha-1)}/3$. *Figure 4* shows a ψ *versus* K relationship calculated for the cylindrical pore shape and for macromolecules in a good solvent at $\nu = 0.6$ (curve A) and in a poor solvent at $\nu = 1/3$ (solid line curve B). The dotted line shows a $\psi_{pr}(K)$ relationship calculated from equation (14) for the cylindrical pore model (at $\alpha = 2$). Comparison of dotted and solid lines B shows that the ψ *versus* K relationship for globules and the ψ_{pr} *versus* K relationship for globular proteins are practically identical. Comparison of curves A and B, *Figure 4*, shows that the separation behaviour of globules and globular proteins in g.p.c. is poorer than that of flexible chain polymers. At the same time the working MW logarithm separation range for proteins is wider than for polymers and, according to equation (12), constitutes over two decades.

Equation (14) also enables determination of the optimum ratio between the size of the protein molecule (its radius R) and the packing pore width. For cylindrical pores:

$$R_{opt} = 0.5d$$

The ψ *versus* R/d relationship for proteins is given in *Figure 3* (curve B). The protein separation principles in g.p.c. as described above are in qualitative agreement with the experimental observations.

It would be interesting to compare experimentally the calibration curve for a series of globular proteins with a similar curve for the same proteins in a denatured coil-like state. After differentiating these relationships numerically, they could be compared with curves A and B in *Figure 4*.

References 16 and 17 cite the molecular weights and Stokes radii $r_s(A)$ of many globular proteins. Processing these data has shown[17] that:

$$r_s \approx 0.49M^{0.378} \qquad (15)$$

Frigon *et al.*[16] carefully measured the distribution coefficients K of these proteins and advanced an empirical relationship between K and r_s:

$$r_s = 85.7 - 144.4K + 111.9K^2 - 47.6K^3 \qquad (16)$$

By regarding equations (15) and (16) as an experimental calibration curve for globular proteins, it is easy to obtain the following relationship for the slope of this curve:

$$\psi(K) = 0.38\left(\frac{85.7 - 144.4K + 111.9K^2 - 47.6K^3}{144.4 - 223.8K + 142.8K^2}\right) \qquad (17)$$

This relationship is shown in *Figure 5* (curve B).

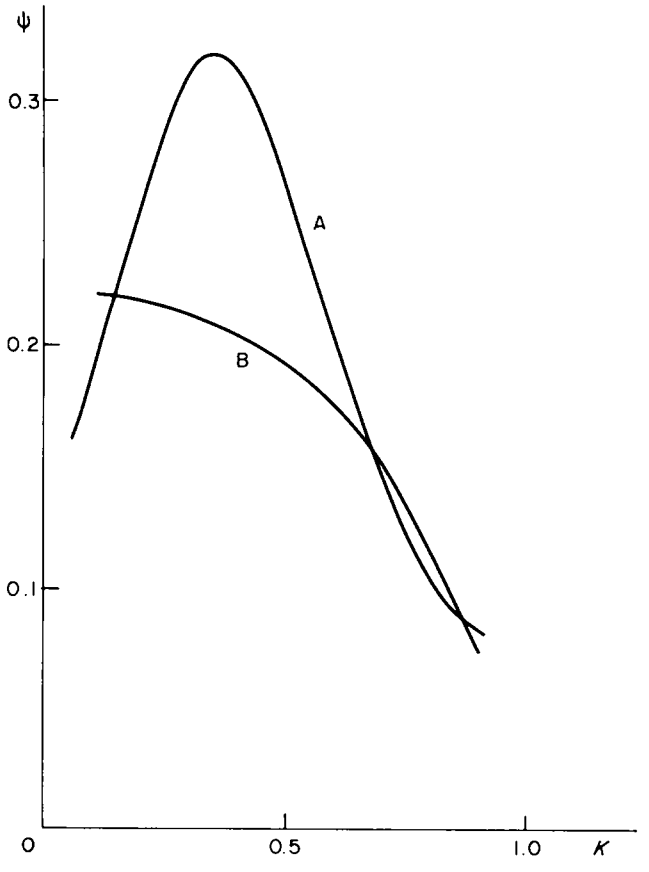

Figure 5 Relationships of ψ *versus* K for dextrans (curve A) and for globular proteins (curve B), plotted in accordance with empirical equations (16) and (18) describing the experimental data of reference 16

For denatured proteins, no such relationships exist. Use can be made, however, of experimental data obtained from g.p.c. of narrow MWD dextrans on modified porous silica SW 3000. According to reference 16, these data are well approximated by a third-degree polynomial:

$$\log M = 5.11 - 3.26K + 5.17K^2 - 4.69K^3$$

By differentiating this expression, a relationship can easily be obtained for coil-like dextrans:

$$\psi(K) = [2.3(3.26 - 10.34K + 14.07K^2)]^{-1} \quad (18)$$

This relationship is likewise given in *Figure 5* (curve A).

Comparison of the experimental data for proteins and dextrans (*Figure 5*) with the theoretical relationships for globules and coils (*Figure 4*) bears witness to the agreement between theory and experiment. It follows from *Figure 5* that optimum separation of coil-like macromolecules and globular proteins can be attained at distribution coefficient $K = 0.3$. Separation of polymeric coils in this case will be 1.5 times better than that of globular proteins.

In conclusion, we shall touch upon the question of g.p.c. resolution for branched polymers obtained by polymerization of functionally active monomers. Valence bonds are formed in this case randomly, by any pair of monomers containing free radicals, and the resulting structures have the so-called lattice animal form[18]. The mean radius of gyration of such macromolecules obeys the ratio $\bar{R} \sim M^{1/4}$ irrespective of the solvent quality[19]. According to equations (12), the resolution obtainable with the g.p.c. method for such randomly branched chains is two times lower than for linear Gaussian macromolecules of the same molecular weight while the working range for them is twice as broad. It would be interesting to verify the theoretical predictions experimentally.

REFERENCES

1 Yau, W. W., Kirkland, J. J. and Bly, D. D. 'Modern Size-Exclusion Liquid Chromatography', John Wiley, New York, 1979
2 Deyl, Z. (Ed.) 'Separation Methods', Elsevier, Amsterdam, 1984, p. 275
3 Balke, S. T. 'Quantitative Column Liquid Chromatography', Elsevier, Amsterdam, 1988
4 Nefyodov, P. P. and Lavrenko, P. N. 'Transportnyye Metody w Analiticheskoj Khimii Polimerow' (Transport Methods in the Analytical Chemistry of Polymers), Khimiya, Moscow, 1979
5 Belenky, B. G. and Vilenchik, L. Z. 'Khromatografija Polimerow' (Polymer Chromatography), Khimija, Moscow, 1978
6 Tung, L. H., Moore, J. C. and Knight, G. W. *J. Appl. Polym. Sci.* 1966, **10**, 1261
7 Gorbunov, A. A. and Skvortsov, A. M. *Vysokomol. Soedin. (A)* 1987, **29**, 920
8 Gorbunov, A. A., Skvortsov, A. M. and Tennikov, M. B. *Vysokomol. Soedin. (A)* 1989, **31**, 1306
9 Casassa, E. F. and Tagami, Y. *Macromolecules* 1969, **2**, 14
10 Tennikov, M. B., Gorbunov, A. A. and Skvortsov, A. M. *Vysokomol. Soedin. (A)* 1989, **31**, 1328
11 Tennikov, M. B., Gorbunov, A. A. and Skvortsov, A. M. *J. Chromatogr.* 1990, **509**, 219
12 Haller, W. *Macromolecules* 1977, **10**, 83
13 Grubisic, Z., Rempp, P. and Benoit, H. *J. Polym. Sci. (B)* 1967, **5**, 7531
14 Porath, J. *Pure Appl. Chem.* 1963, **6**, 233
15 Giddings, J. C., Kucera, E., Russell, C. P. and Myers, M. N. *J. Phys. Chem.* 1968, **72**, 4397
16 Frigon, R. P., Leypoldt, J. K., Uyeji, S. and Henderson, L. W. *Anal. Chem.* 1983, **55**, 1349
17 Andrews, P. *Meth. Biochem. Anal.* 1970, **18**, 1
18 Grosberg, A. Yu. and Khokhlov, A. R. 'Statisticheskaja fizika makromolekul' (Statistical Physics of Macromolecules), Nauka, Moscow, 1989
19 Irzhak, V. I., Rosenberg, B. A. and Enikolopyan, N. S. 'Setchatyye Polimery' (Polymer Networks), Nauka, Moscow, 1979