SOME RECENT DEVELOPMENTS IN GEL CHROMATOGRAPHY, WITH SPECIAL REFERENCE TO THIN LAYERS

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The term "gel chromatography" is not yet very familiar. Originally one spoke of "gel filtration" without indicating that the method is in fact a chromatographic technique and that its efficiency rests upon the principle of chromatography. The principle of all chromatographic separations, on a column consisting of a two-phase system, is the difference in the accessibility to substances of the stationary phase. Substances of lower affinity will travel faster than those implicated to a greater degree. To the classical principles of adsorption and of partition, the penetrability of a gel has now been added. Smaller molecules, which more readily find access to this phase, will move more slowly than larger molecules.

Nowadays many different gels are in use. There are gels with hydrophilic properties, Sephadex probably being the best known. Sephadex is a dextran of bacterial origin cross-linked by 1,3-glyceryl ether bridges. The different types from G-10 to G-200 differ in their degree of cross-linking. The greater the cross-linking, the smaller is, of course, the average diameter of the mesh or pores and, simultaneously, the capacity for swelling and their accessibility for higher molecular weight substances decreases. The same is true for another hydrophilic preparation, a cross-linked polyacrylamide, originally prepared by HJERTÉN[1] and manufactured in the U.S.A. (Biogel). Gels of agarose, a polysaccharide made up of alternating L- and D-galactose residues, are cross-linked only by hydrogen bridges and are therefore suitable for the separation of extremely high molecular weight particles[2]. Of the lipophilic gels, only Styragel is commercially available. It consists of a copolymerizate of styrene and divinylbenzene and is used in a chromatographic device for determining the molecular weight distribution of synthetic polymers (Waters Assoc.). In our laboratory beads of a copolymer of methacrylate and glycol bis(methacrylate) have been studied since 1962; this will be discussed later.

Finally Sephadex LH (lipophilic–hydrophilic) is mentioned. It has both properties and can be used in aqueous and in organic solvent systems. In Fig. 1, a separation in alcohol of polyethyleneglycols $(—O—CH_2—CH_2—)_n$ of different molecular sizes is shown.

This paper is a report on some of the applications of the Sephadex gels mainly regarding our special field of research, amino acids, peptides and proteins. Gel chromatographic separation of low molecular weight peptides can be carried out in water on the strongly cross-linked Sephadex G-10. Fig. 2 shows the different elution volumes with water (V_e) of a di-, tri-, and tetra-peptide registered by an automatic ultraviolet recorder.

By plotting V_e/V_0 (reduced elution volumes) against log M (molecular weight),

a straight line is obtained. The same holds for $\sqrt[3]{K_d}$ *versus* $\sqrt[2]{M}$. (K_d is defined in very much the same way as the distribution coefficient in partition chromatography; K_d is directly proportional to V_e/V_0.) Both of these linear correlations are also valid for molecular weights of proteins and their rates of movement. A compilation was obtained by DETERMANN AND MICHEL[3], of the elution volumes of 26 proteins, from data from several laboratories. Plots of the reduced elution volumes against log M resulted in straight lines with different slopes for all types of Sephadex investigated.

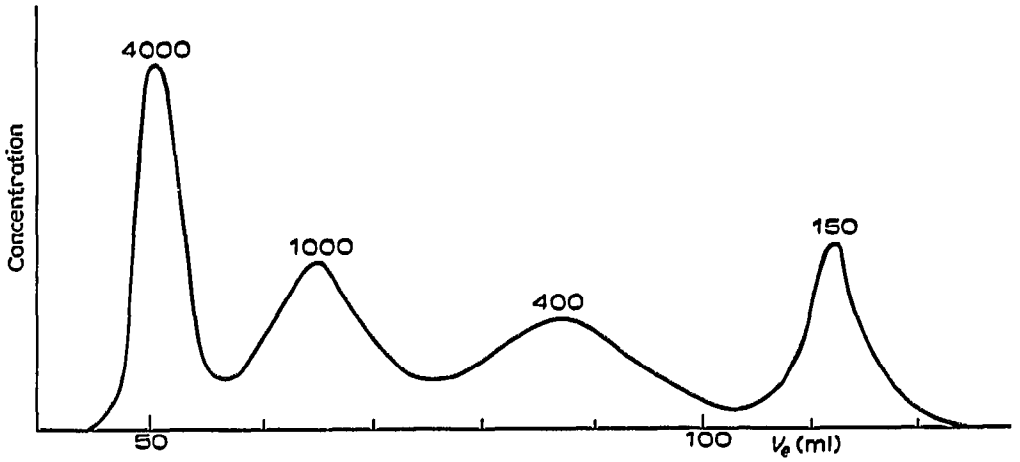

Fig. 1. Elution volumes of polyethyleneglycols of different sizes from Sephadex LH with ethyl alcohol.

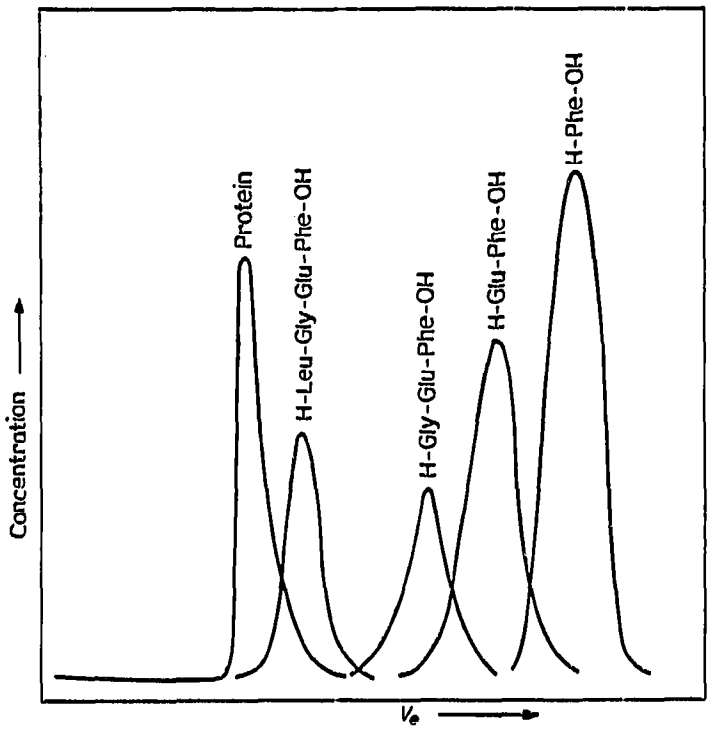

Fig. 2. Chromatographic behaviour of an amino acid, three peptides and a protein on Sephadex G-10 in water.

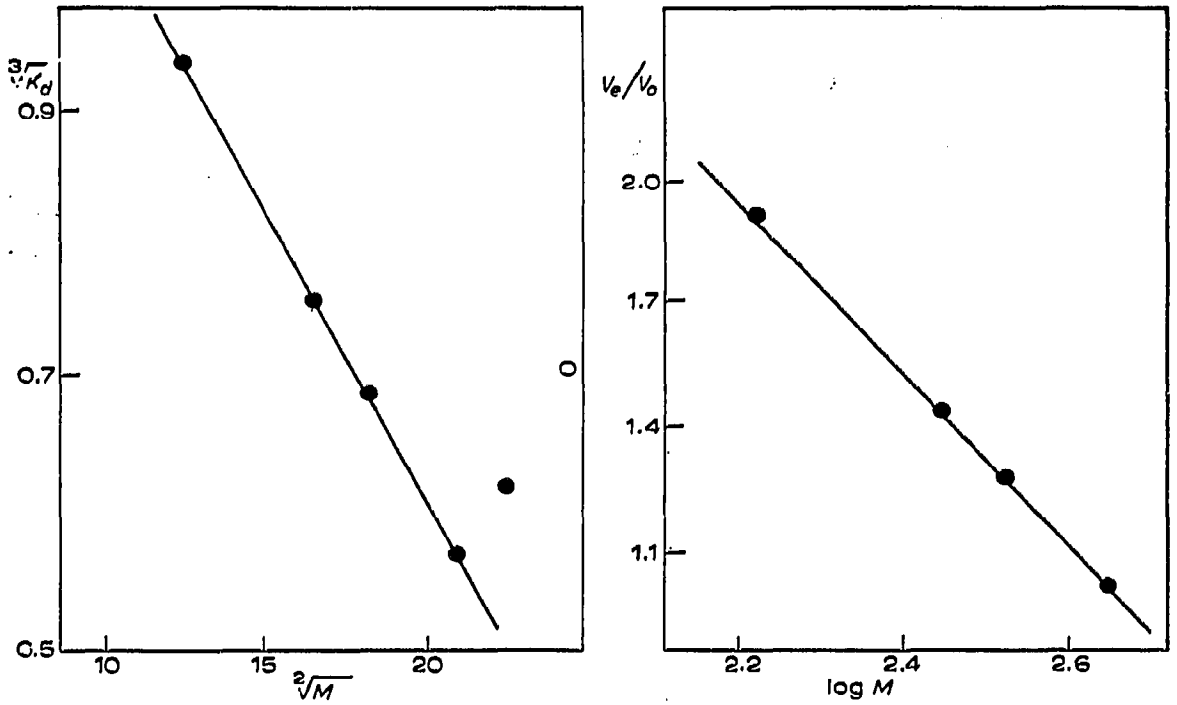

Fig. 3. Plot of *M versus* elution volumes in two different expressions.

The numerical expressions are shown in Table I. To determine the molecular weight of a protein, it is therefore only necessary to establish its V_e/V_0 and to insert its value into the appropriate equation.

With enzymes, it is not necessary to isolate the pure substances before chromatography as their appearance in effluent can be traced by virtue of their catalytic action. Elution volumes were found to be independent of accompanying proteins, as *e.g.* AURICCHIO AND BRUNI[4] have shown. A further application in the enzyme field is that of examining the reversible binding of several substances to proteins. Thus, as an example, the affinity of the hydrogenated pyridinium nucleotide (NADH) to lactic acid dehydrogenase (LDH) could be measured according to PFLEIDERER AND AURICCHIO[5]. The protein was run in a NADH containing buffer on Sephadex G-50. The LDH–NADH complex was visible in the eluent as a positive peak followed by a negative peak corresponding to the deficient, protein-bound NADH (see Fig. 4).

TABLE I

EQUATIONS OF THE STANDARD CURVES OF FIG. 3

G-200	$\log M = 6.698 - 0.987\ (V_e/V_0)$
G-100	$\log M = 5.941 - 0.847\ (V_e/V_0)$
G-75	$\log M = 5.624 - 0.752\ (V_e/V_0)$
G-50	$\log M = 5.415 - 0.864\ (V_e/V_0)$

THIN-LAYER TECHNIQUES

By 1962, it had been found that a suspension of particles of Sephadex G-25 in water could be spread over clean glass plates to form solid layers which could be

used in thin-layer chromatography (TLC) of the ascending type[6]. Later on, Sephadex was manufactured in bead form. These particles do not adhere and cohere sufficiently for vertical layers. They can, however, be used on inclined plates in a descending technique like that described below for proteins. One of the first applications of TLC was in peptide chemistry in the investigation of the so-called plastein reaction.

Plasteins are formed by the action of pepsin at pH 4 on concentrated solutions of proper oligopeptides[7]. Tyr–Leu–Gly–Glu–Phe being a particularly reactive "monomer"[8]. The enzyme catalyses the condensation by splitting off water between the constituents. After TLC on Sephadex G-25, the plastein appeared as a mixture of several polymers, whose resolution was possible by column chromatography on Sephadex G-25 (Fig. 5). The component with highest molecular weight was the pentamer which contained 25 amino acids[9].

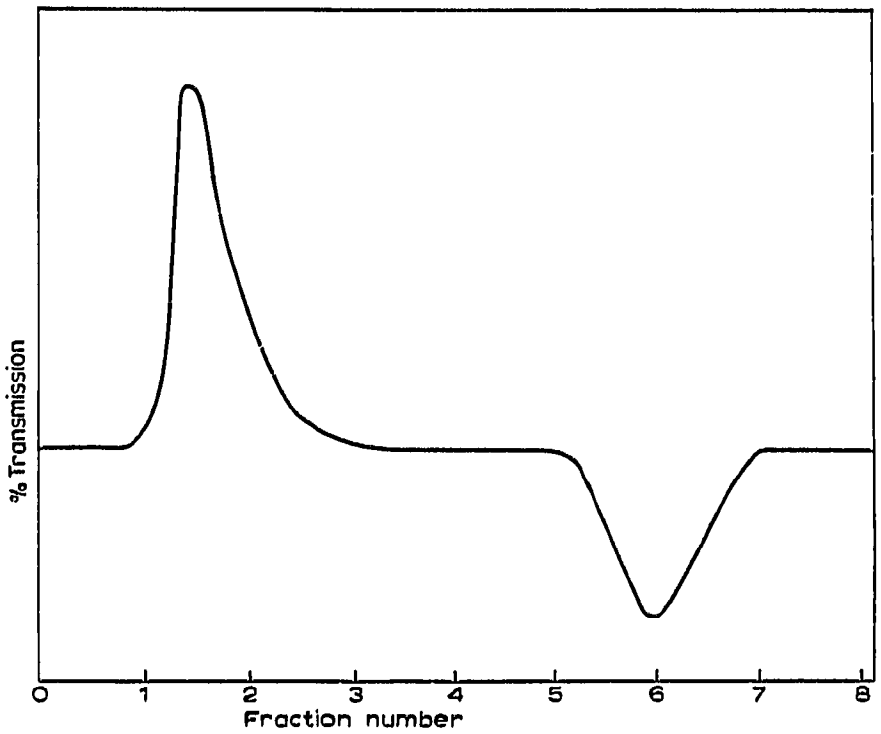

Fig. 4. Elution profile (253 mμ) of LDH after passage through a Sephadex gel column being equilibrated with 0.7 mM NADH.

Thin layers of the large-pore types G-100 and G-200, suitable for separations of proteins, have been employed later by different workers[10–12]. They can only be used in a descending technique. In these cases the flow rate can be regulated by inclining the plate at different angles. The chromatograms of Fig. 6 show a series of proteins which have been run at different angles.

Localization of the protein spots has been made by printing onto a filter paper and staining the prints with the usual dyestuffs or, better, coupling with diazotized sulfanilic acid (Pauly reaction). In TLC, a linear correlation also holds between R-values (which may be referred to cytochrome c) and log M, but there are considerable differences between various gel batches. By using reference proteins it is, however, easily possible to determine molecular weights by the TLC method. In the case of

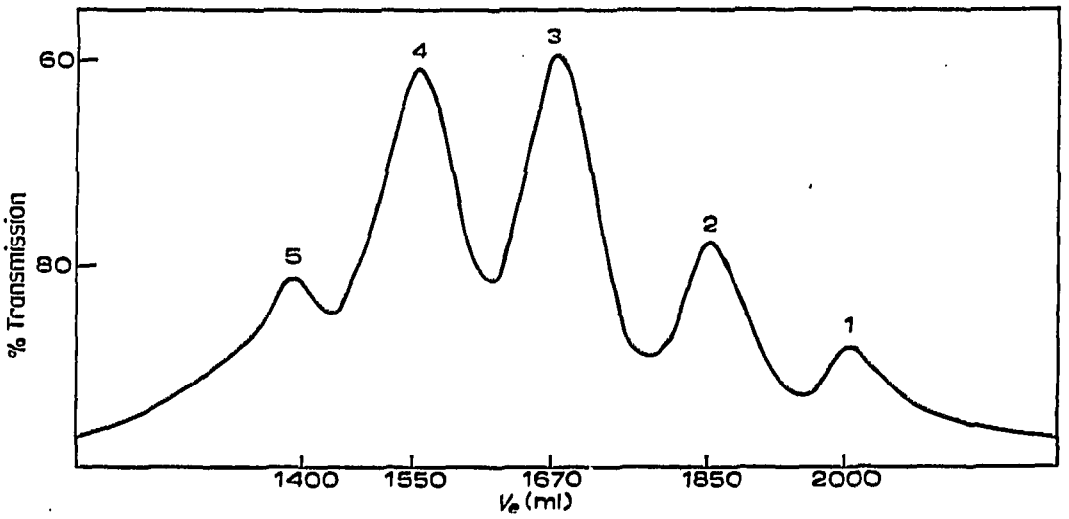

Fig. 5. Resolution of a "plastein" mixture by chromatography on Sephadex G-25. 1 = Pentapeptide; 2 = decapeptide, etc.

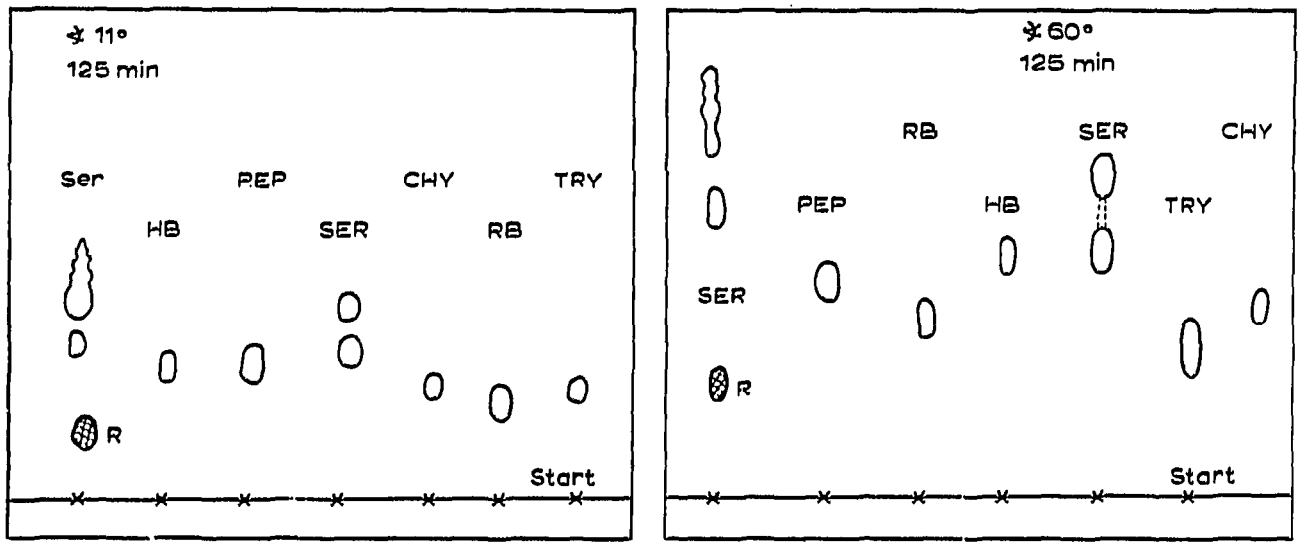

Fig. 6. R_F values of several proteins in descending thin-layer chromatography at two different inclination angles. SER = Serum proteins, HB = hemoglobin, PEP = pepsin, CHY = chymotrypsin, RB = ribonuclease, TRY = trypsin; R = reference compound (cytochrome c).

enzymes, visualization is much more sensitive when an appropriate optical method is used. Thus, LDH can be detected in minimal amounts by spraying a print with NADH containing pyruvate buffer, whose fluorescence is quenched in the presence of traces of enzyme. In studies with isozymes of LDH it was found that the heart muscle type (H_4) travels distinctly faster than the skeletal muscle type (M_4) although both of them have the same molecular weight in the ultracentrifuge. Perhaps dissociation into protomers (subunits), which occurs more readily in M_4, plays a role in this phenomenon.

LIPOPHILIC GELS

We tried some years ago[13] to transfer simply our experience gained with hydrophilic gels to a system of water-insoluble monomer (methyl methacrylate) and cross-linker (glycol bismethacrylate). The gels obtained in such experiments were able to separate partially very inhomogenous mixtures of low molecular weight polystyrenes. The resolving efficiency clearly depended on the degree of cross-linking, the weaker gel (0.25 % cross-linked) having more accessibility for bigger molecules, as compared with the strong gel (1 % cross-linked), a smaller part of the separation mixture being excluded. The applicability, however, was only for resolving molecules up to molecular weight 4000. We, therefore, tried to reduce the amount of cross-linking agent to get bigger "pores", but gels made up in this way proved too soft for handling in a chromatographic column. The experiments of MOORE[14] were then published, in which he made use of the fact that gel formation of polystyrene/divinylbenzene in suitable solvent systems gives a rather rigid, although large-pore gel. In our system pentanol was added in various amounts to the toluene solution of monomers before polymerization. Thus we also obtained relatively solid gels with better accessibility for larger molecules. Fig. 7 shows the fractionation of polystyrenes in tetrahydrofuran up to molecular weights of about 20,000.

Higher molecular weight polymers were also excluded from this gel. Further improvement in pore size was achieved by a method which we have called "inclusion polymerization"[15]. Here the cross-linking polymerization is conducted in the presence of large amounts (ratio to monomer 1:1) of a solid powder. The size of the granules is variable; the filler is removed afterwards by dissolving. In Fig. 8 the resolving power of a gel which has been prepared from the former monomers in the presence of sodium carbonate particles of 250 μ diameter is demonstrated. Here distinct differences exist in elution volumes of high polymer polystyrenes up to molecular weights of about half a million. The properties of several of these macroporous gels are summarized in Table II.

The principle of inclusion polymerization was also applied to the preparation of hydrophilic gels[16]. A gel prepared from acrylamide plus methylene bis(acrylamide) in the presence of powdered $CaCO_3$ (the inorganic substance was extracted by treating with 50 % acetic acid) allowed the resolution of the several components of "dextran blue" (mol.wt. 2×10^6) with the result demonstrated in Fig. 9.

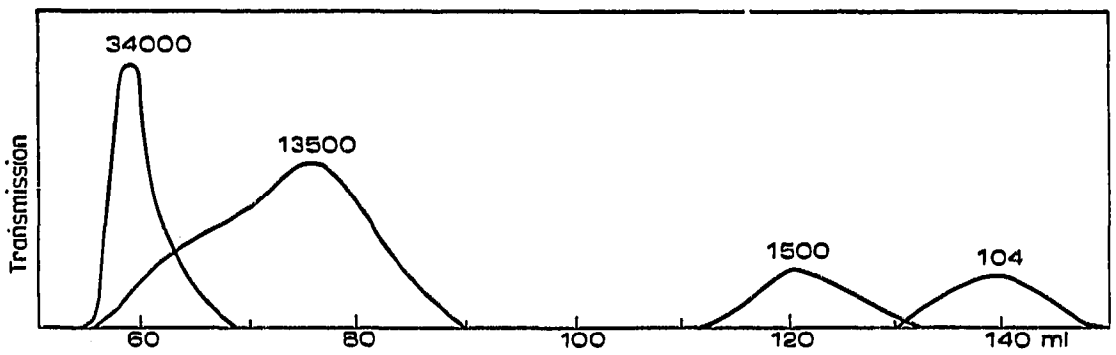

Fig. 7. Elution volumes of polystyrenes of different molecular weight from a methacrylate gel.

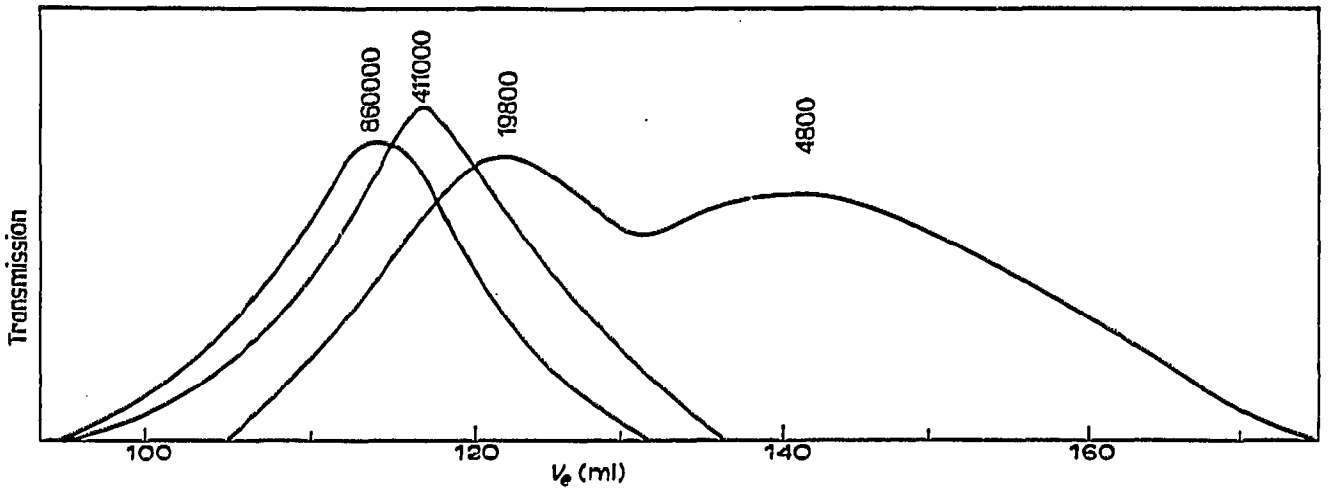

Fig. 8. Chromatography of high molecular weight polystyrenes on a gel obtained by inclusion polymerization.

TABLE II

SOME PROPERTIES OF SEVERAL MACROPOROUS GELS

Pore sizes have been adjusted by inclusion polymerization of methyl methacrylate and glycol bismethacrylate (cross-linking agent, CA) in the presence of various substances which are eluted afterwards by appropriate solvents. I.S. = included substance.

Gel No.	*Included substance*	*Particle size* (μ)	*Ratio I.S.: monomer*	*% CA in polymer*	*Swelling factor*	*Minimal molecular weight of polystyrene excluded* ($\times 10^3$)
O	None	—	—	1	7	5
D1	Dextrose	<50	1:1	1	6.5	20
P1	Polystyrene	mean molecular weight $\sim 3 \cdot 10^5$	1:2	1	7	130
C1	$CaCO_3$	10–20	1:1	1	7	145
C2	same	10–20	1:1	5	3	42
C4	same	1–5	1:1	2	5	28
C5	same	10–20	2.5:1	1	7	150
S1	Na_2CO_3	100–125	1:1	1	7	300
S2	same	200–250	1:1	1	7	*ca.* 500

SEPARATIONS DUE TO ADDITIONAL INTERACTIONS

In addition to the graduated accessibility of substances to the liquid part of a gel, the classical principles of chromatography can also be developed to maximal resolving power by the nature of the gels. It is well known that Sephadex has a definite adsorbing strength for aromatic systems. In Fig. 10 the differences in elution volumes, of several amino acids, in salt-free aqueous solution, are shown.

Glutamic acid moves even faster than the neutral glycine; repulsion of the two negatively charged carboxylated groups by equally charged centers of the gel seems to prevent diffusion into the pores here. Of the three aromatic amino acids the indole system is most strongly adsorbed. This is also the reason for the widespread applica-

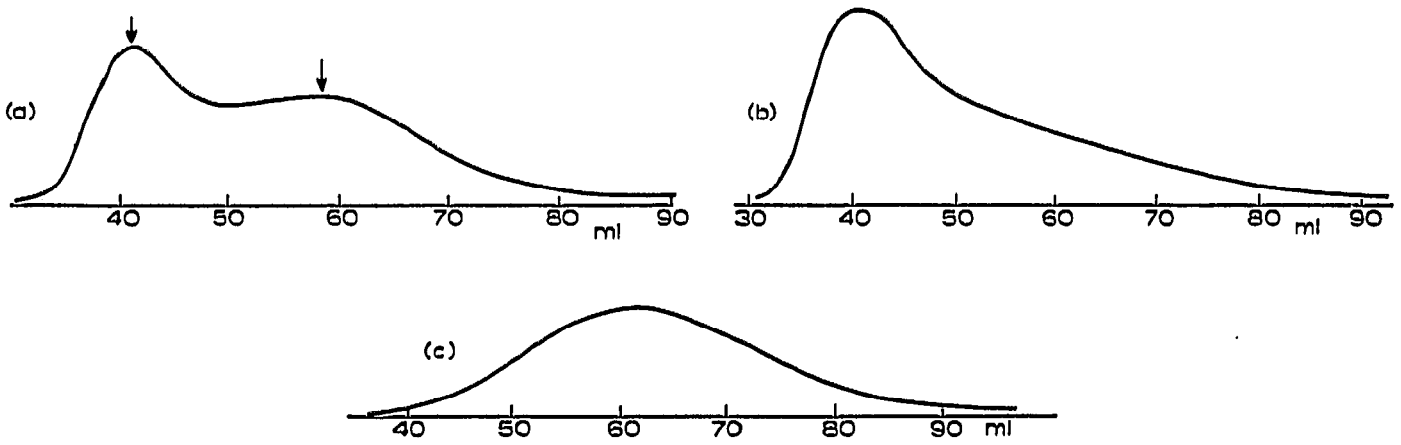

Fig. 9. Chromatographic behaviour of dextran blue on a column of macroporous polyacrylamide gel. (a) Dextran blue; (b) rechromatography of fractions of 30–40 ml; (c) fraction of 60–80 ml.

bility of Sephadex in the chromatographic resolution of the numerous constituents of the green toxic mushroom *Amanita phalloides*. The water-soluble fraction is divided into rather homogeneous fractions by chromatography on Sephadex G-50 in water as a solvent[17]. Fig. 11 shows clearly the very effective separation *e.g.* of phalloidin from α-amanitin, the two peaks before and after the 18.3 liter mark.

Adsorption chromatography is also very effective on lipophilic gels in apolar solvents. The pattern of peaks in Fig. 12 shows, impressively, the resolving ability of a methacrylate gel for nearly all pigments of spinach eluted with benzene[18].

Finally the usefulness of gels as supporting media in partition chromatography is mentioned. By adding an organic solvent to the gel–water system a change will take place whereby the hydrophilic gel phase will contain more water than the mobile phase. The same holds for partition chromatography in general, but with gels, particularly in the large-pore bead form, the interface between both the phases is much wider than in previously used materials. Thus, not only the resolving power, but also

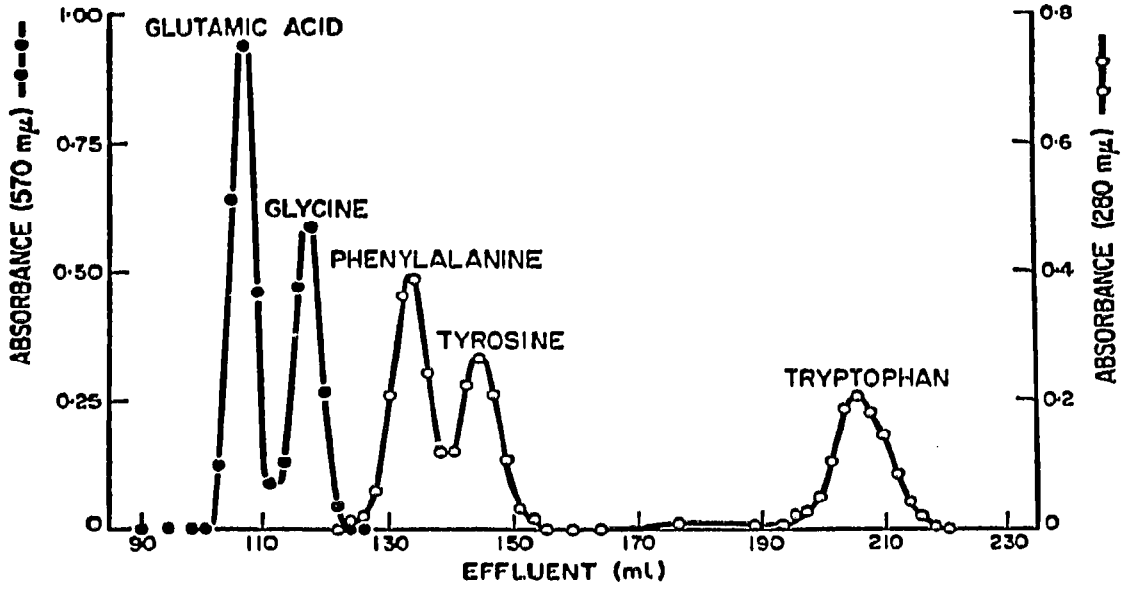

Fig. 10. Adsorption chromatography of amino acids on Sephadex G-25 in salt-free water. (After J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.)

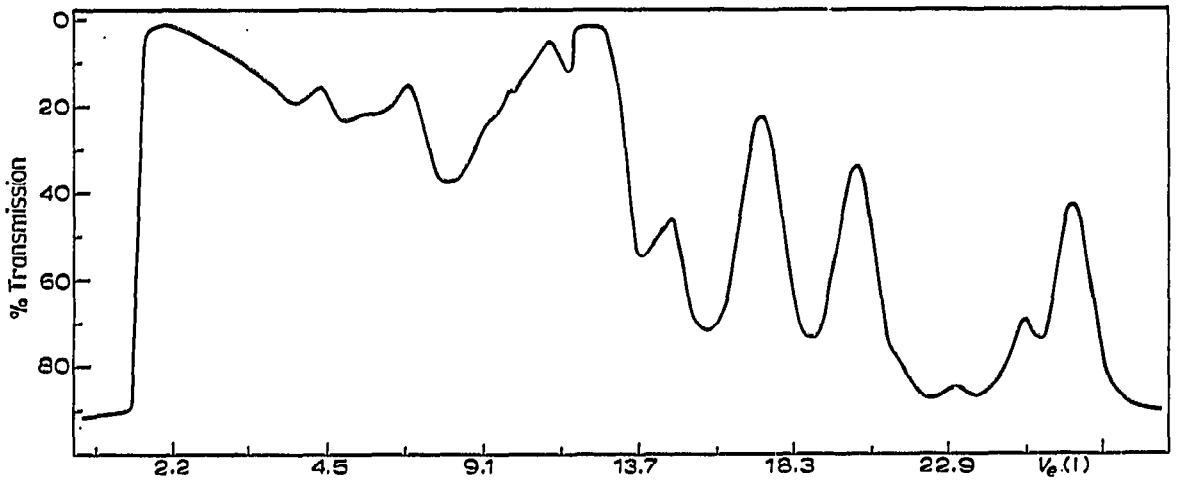

Fig. 11. Adsorption chromatography of hydrophilic components of *Amanita phalloides* on Sephadex G-50 in aqueous solution.

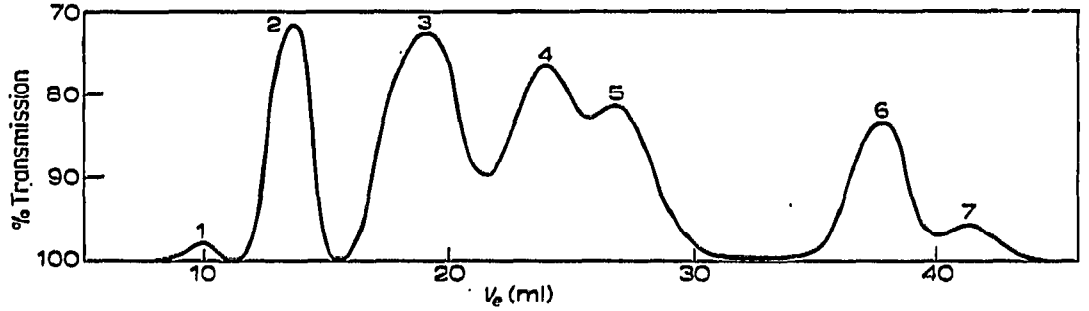

Fig. 12. Adsorption chromatography of leaf pigments on a methacrylate column in benzene (according to ref. 18).

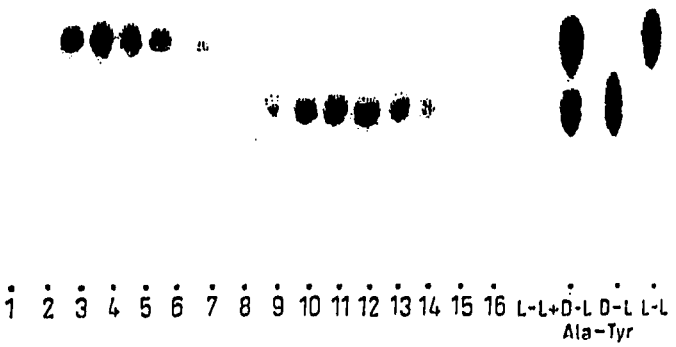

Fig. 13. Separation of L- and D-alanyl-L-tyrosine on a column of Sephadex G-50 with pyridine–water (1 : 1 moles) as an eluent. Left hand side, ninhydrin reaction of eluted fractions; right hand side, reference substances as analyzed by thin-layer chromatography on cellulose powder in the same solvent.

the capacity of Sephadex columns is surprisingly high. Fig. 13 shows the separation of two dipeptides, which differ only in the configuration of one amino acid, *viz.*, L-alanyl-L-tyrosine (left) and D-alanyl-L-tyrosine (right). In this case, 0.2 g of a 1:1 mixture could be resolved quantitatively, 0.5 g almost totally, on Sephadex G-50 in pyridine–water (1:1 moles) on a column of 1 m length and only 1.3 cm width, *i.e.* only 25 g of supporting medium[19].

Partition chromatography on Sephadex in butanone–water mixtures also plays an inportant role in the isolation of the lipophilic constituents of *A. phalloides* in the authors' laboratory[17].

CONCLUSION

It is realized that we are offending against the settled nomenclature by gathering together all these chromatographic principles under the term gel chromatography. At least in the range of low molecular substances, however, a strict discrimination between the factors governing the mechanisms of separation is impossible. Therefore it seemed plausible to maintain the term until a better name for the subject under consideration has been found.

SUMMARY

A review is given on some applications of different gels in chromatographic procedures: separations of amino acids, peptides, proteins—here especially on thin layers of Sephadex. Lipophilic gels are useful in separating polystyrenes; large-porous gels have been obtained by inclusion of different particles during polymerization and eluting the particles out of the gel. Sephadex and methacrylate gels are also suitable carriers for adsorption or partition chromatography.

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