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A Critical Evaluation of Gel Chromatography*,†

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Summary

Gel chromatography includes a number of related techniques for separating molecules by means of solvent-swollen gels. The gels consist of three-dimensional networks of cross-linked polymeric chains of controlled porosities which exclude molecules from the interior of the gel to a degree depending on relative dimensions of molecule and pore.

Current theories relating the elution volume to a partition coefficient or to molecular properties are discussed. A general equation has not yet been proposed. Such an equation would necessarily include the three important mechanisms: exclusion, restricted diffusion, and adsorption.

By the term "gel chromatography" we indicate a number of similar, if not identical, techniques for separating macromolecules on the basis of molecular dimensions through the use of solvent-swollen gels. In a more general sense, the method is not restricted to large molecules, nor is the separation based entirely upon geometric effects. As in most forms of chromatography, many people have developed the technique more-or-less independently, each group with their own materials, explanations, and terminology. By and large, biochemists and polymer chemists have done much the same thing under the names "gel filtration" and "gel permeation chromatography," respectively.

Gel filtration was first described by Porath and Flodin (*1*), who used a cross-linked hydrophilic dextran gel to separate dextran

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fractions and serum proteins. Biochemists generally have followed this lead and used dextran gels, spectrophotometric detectors, and have referred to the method as gel filtration, just as organic chemists retain the term "vapor-phase chromatography" rather than the generally accepted term "gas chromatography."

"Gel permeation chromatography" was first used as a name for a technique by Moore (*2*), who used a hydrophobic cross-linked polystyrene gel to separate polystyrene and polyglycol fractions; although essentially the same thing had been reported earlier by Vaughan (*3*), who called it "gel filtration." Polymer chemists, who are generally restricted to nonaqueous solvents, have used polystyrene gels, detection by refractive index, and have referred to the method as "gel permeation chromatography."

Other names, such as "exclusion chromatography," "molecular-sieve chromatography," and "restricted diffusion chromatography" have been suggested in an effort to identify the mechanism of separation. However, in most cases, a variety of mechanisms operate simultaneously and it would seem advantageous to extend the use of the term "gel chromatography," suggested by Determann (*4*), to all forms of separations using gels.

It is no longer obvious who was the first to use this technique. All the above work was preceded by earlier workers who used gels for much the same purpose; for example, Lathe and Ruthven (*5*) employed swollen starch gels to separate proteins and sugars in 1956, and Partridge (*6*) in 1952 used sulfonated polystyrene resins to separate peptides and proteins on the basis of size.

There are some unique features of gel chromatography which distinguish it from other forms of chromatography. If the separation is based primarily on total or partial exclusion from the interior of the gel, then:

1. The elution volume is normally less than the total solvent volume of the column.
2. There is very little dilution of the sample components.
3. Short columns can be used.
4. Large sample volumes can be used (to separate large molecules from small molecules, the sample volume can be as large as the internal volume in the gel).
5. The concentration in the stationary phase never exceeds that in the mobile phase.

In common with other forms of chromatography, the separation is based on a selective distribution between two phases, and the band spreading (nonideal behavior) must result from the same processes within the column.

NATURE OF THE GELS

All the materials used in gel chromatography consist of three-dimensional networks formed by cross-linking long polymeric chains. The matrices contain hydroxyl or other polar groups capable of absorbing water and similar polar solvents, or aromatic groups capable of absorbing less polar solvents. In either case, a large amount of solvent is imbibed, causing the gel structure to swell, providing interstices within the gel which are available to the solvent as well as solute molecules up to a critical size. The amount of cross-linking determines the extent of the swelling, which in turn determines the dimensions of the interstices or pores. Gels which are commonly used include:

Sephadex: dextran cross-linked with epichlorohydrin
Bio Gel: copolymer of acrylamide and methylenebisacrylamide
Styragel: polystyrene cross-linked with divinylbenzene
Agar: galactose residues with 1,3-glycosidic linkages
Starch granules: complex carbohydrate
Porous glass beads: silica
Rubber: chlorobutyl rubber–ZnO

The synthetic materials are available in many grades, by size of particles, amount of cross-linking, and degree of purity.

The detailed structure of the swollen gels, including the size and shape of the interstices, determines the sieving action to a large extent. The indeterminate nature of the structure has given rise to a number of theories concerning the mechanism of separation. Freeman (7) has found that the structure of unsulfonated polystyrene gels is fairly regular, as contrasted with the random nature suggested for the dextran gels (8).

PROPOSED MECHANISMS OF THE SEPARATION

Each of the gels depends on a very strong adsorption of solvent molecules to cause them to swell, so it would seem that many types of solute molecules will be adsorbed as well. Adsorption is surely an important factor in many separations, yet it has not been considered in the theories suggested so far. Most authors have limited their treatment to nonadsorbed molecules of similar shapes and chemical properties. Several theories will be discussed in turn.

The first quantitative expression for the elution volume was developed by Porath and Flodin (*1*). The total volume within a column is

$$V_t = V_g + V_0 + V_i \tag{1}$$

where V_g is the volume occupied by the gel matrix, V_0 the void volume outside the gel particles, and V_i the volume occupied by the solvent within the gel. Solute molecules larger than a critical size are too large to enter the interior of the gel, and will be washed through the column in a volume equal to the void volume. Solute molecules which are small enough to penetrate all parts of the interior of the gel will require an elution volume equal to the sum of the void and interior volumes. Solute molecules which are intermediate in size are able to penetrate some fraction of the interior volume. The fraction depends on the relative size of the solute and the interstices. In general, the elution volume, V_e, equals

$$V_e = V_0 + K_D V_i \tag{2}$$

where K_D represents the fraction of V_i available to the solute. Equation (2) is analogous to the retention equation in gas-liquid chromatography. One might expect that K_D is a distribution coefficient, although it is here expressed as a function of volumes:

$$K_D = (V_e - V_0)/V_i \tag{3}$$

K_D can be thought of as the equilibrium ratio of the concentration of the solute within the gel (stationary phase) to that without (mobile phase), provided that one uses an "average" concentration within the gel. Presumably the concentration is zero in some parts of the interior and equal to the exterior concentration in other parts. K_D values describe the extent of penetration.

$K_D = 0$	complete exclusion
$0 < K_D < 1$	partial exclusion
$K_D = 1$	no exclusion
$K_D > 1$	adsorption

Typical K_D values are given in Table 1. Values for substances which should exhibit complete penetration are usually 0.8 to 0.9 rather than unity. The discrepancy is supposedly caused by a tightly adsorbed layer of water on the surface of the gel which is not available to solute molecules.

TABLE 1

Distribution Coefficients in Aqueous Dextran Gels

	Sephadex gel type					
	G-10[a]	G-25	G-50	G-75	G-100	G-200
p-Nitrophenol	23.0	—	—	—	—	—
Benzaldehyde	5.1	—	—	—	—	—
Benzyl alcohol	3.5	1.3	1.1	—	—	—
Benzoic acid	9.0	0.5 1.0[b]	—	—	—	—
Arginine HCl	—	>13 1.7[b]	—	—	—	—
Phenol	—	0.7	—	—	—	—
Ammonium sulfate	—	0.9	—	—	—	—
KCl	—	1.0	1.0	—	—	—
Tryptophan	—	~2	1.6	1.2	—	—
Tyrosine	—	~1.2	1.1	—	—	—
Phenyl alanine	—	~1.1	1.0	—	—	—
Glycine	—	0.9	—	1.0	—	—
Pepsin	—	0	0	0.3	—	—
Trypsin	—	0	0	0.3	0.5	0.7
Hemoglobin	—	0	0	0.1	0.3	0.5
Serum albumin	—	0	0	0	0.2	0.4
Fibrinogen	—	0	0	0	0	0

[a] Static experiments in this laboratory.

[b] 0.05 *N* NaCl solvent.

Later, Porath (9) treated the interstices as a collection of hollow cone-like shapes which, on the average, could be treated as identical regular cones. The size of cone depends on the degree of cross-linking. If the average cone has a diameter A and a total depth H, its total volume V is

$$V = \pi H A^2/12 \tag{4}$$

A molecule of effective radius R can reach a maximal depth h so that the center of the molecule can move freely within the partial volume v:

$$v = \pi h(A - 2R)^2/12 \tag{5}$$

The distribution coefficient K_D is proportional to the fractional volume v/V; hence

$$K_D = kv/V = k\,\frac{h}{H}\left(\frac{A - 2R}{A}\right)^2 \tag{6}$$

from which it follows that

$$K_D = k\left(1 - \frac{2R}{A}\right)^3 \tag{7}$$

where k is a proportionality constant. Porath then assumes that R is proportional to $M^{1/2}$ (for flexible polymers consisting of identical segments), and that A^3 is proportional to the volume of solvent in the gel, which can be expressed in terms of "solvent regain" S_r and a correction term α for the part of the solvent which is tightly adsorbed and unavailable to the solutes. This leads to

$$K_D = k\left[1 - k_1\,\frac{M^{1/2}}{(S_r - \alpha)^{1/3}}\right]^3 \tag{8}$$

where $k = 1.64$, $k_1 = 0.012$, and $\alpha = 0.8$ for Sephadex (values obtained empirically). Porath has plotted the data given by Granath and Flodin (*10*) as $K_D{}^{1/3}$ vs. $M^{1/2}$ and obtained the linear relation predicted by Eq. (8) (see Fig. 1).

Squire (*11*) independently developed a theory similar to Porath's, but included other shapes for the interior spaces—cones, cylinders, and crevices. The distribution of these shapes was arbitrarily prorated to obtain the best fit to the data available. This variation of the geometry gives a relation in terms of relative elution volume:

$$\frac{V_e}{V_0} = \left[1 + g\left(1 - \frac{2R}{A}\right)\right]^3 \tag{9}$$

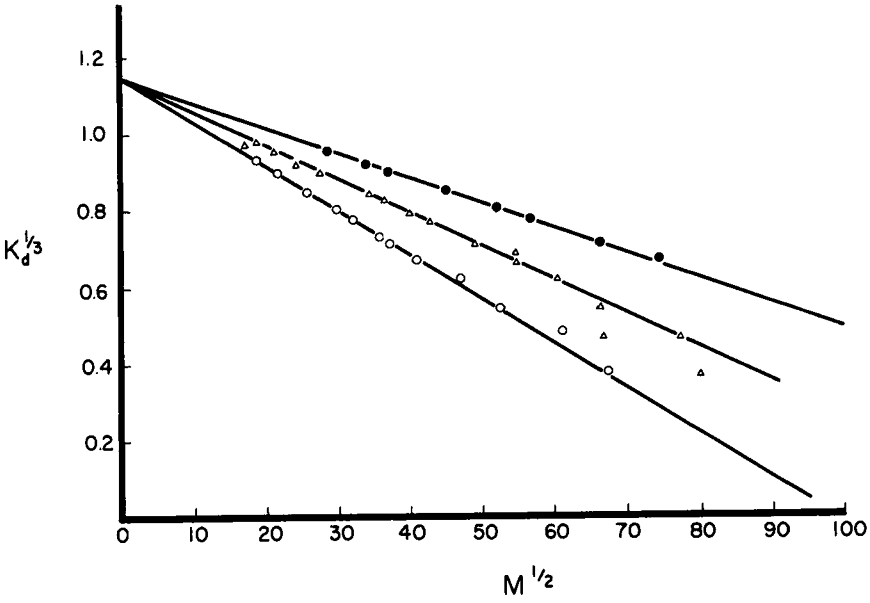

FIG. 1. Linear relationship between $K_D^{1/3}$ and $M^{1/2}$ proposed by Porath (9). Dots, circles, and triangles are data of Granath and Flodin (*10*).

where g is an arbitrary constant introduced to define the distribution of shapes. As an additional assumption, he treated proteins as spheres, so that R is proportional to $M^{1/3}$ rather than $M^{1/2}$ (Porath). Thus Squire's working equation is

$$\frac{V_e}{V_0} = \left[1 + g\left(1 - \frac{M^{1/3}}{C^{1/3}}\right)\right]^3 \tag{10}$$

where C corresponds to the molecular weight of the smallest protein that cannot enter the gel. The constants g and c are evaluated empirically by plotting $(V_e/V_0)^{1/3} - 1$ vs. $M^{1/3}$. Squire used data from several authors to determine best values of g and C for each Sephadex and found that molecular weights of most substances could be predicted from Eq. (10) to within 10% of the known value.

If the chains in 1 ml of an average dextran gel were stretched in one long chain, there would be a total length of some 60 million miles. The distance of closest approach that a sphere can make to a rod is a function of the sum of the radii of the sphere r_s and the rod r_r. Thus there is a cylindrical space around the rod which is unavailable to the spheres. With a chain length of the order quoted above, this excluded space can be an appreciable fraction of the total space. Laurent and Killander (*8*) have used this approach to compute K_{av}, which they define as

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} = K_D \frac{V_i}{V_i + V_g} \tag{11}$$

Using an expression derived earlier by Ogston (*12*), they give

$$K_{av} = \exp\left[-\pi L(r_s - r_r)^2\right] \tag{12}$$

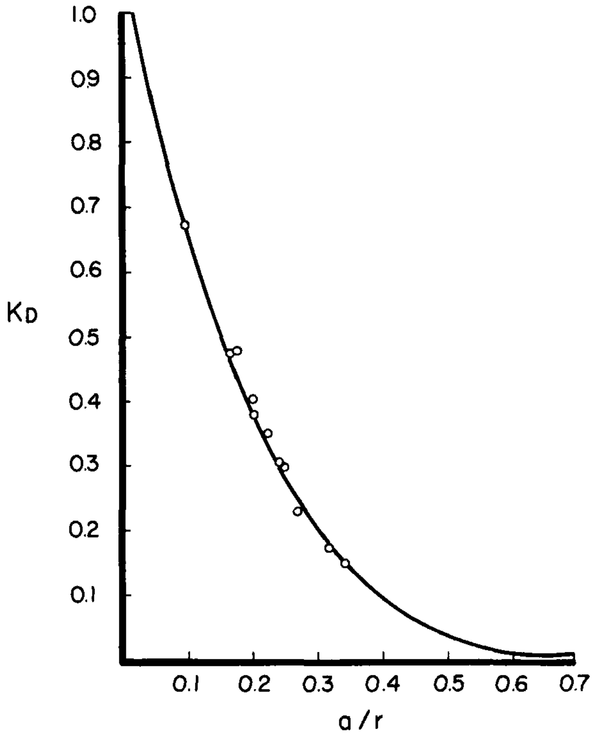

FIG. 2. Test of Eq. (13) according to Ackers (*13*). Solid line is the theoretical curve with $r = 18.76$ mμ. Circles are experimental values for proteins.

where L is the concentration of rods in cm per cm^3 of gel. The radius of the equivalent sphere r_s was calculated from the diffusion constant, using Stoke's formula and data in the literature. The radius of the chains, or rods, r_r, was estimated to be 7×10^{-8} cm. A value for L for each type of Sephadex was chosen for closest fit to the experimental K_{av} values: for G-75, $L = 4.6 \times 10^{12}$; G-100, $L = 2.9 \times 10^{12}$; and G- 200, $L = 1.6 \times 10^{12}$. Thus with fixed values for r_r and L, they plotted K_{av} [calculated from Eq. (12) and experimental from (11)] and obtained good agreement using the results of several independent groups.

Ackers (*13*) compared values of K_D measured through Eq. (3) with those measured in static equilibrium measurements. For Sephadex G-75 and G-100 the two sets gave close agreement, but for the more loosely cross-linked G-200, the static values were considerably larger. He explained this on the basis of restricted diffusion based on a model in which the gel pores are represented as uniform cylindrical channels. A molecule of radius a, which is smaller than the pore radius r, can penetrate the gel only if its center passes within a virtual pore of radius $r - a$. If the molecule does enter the pore channel, it encounters increased frictional resistance and therefore has a lower diffusion coefficient than it had in free solution. Such a diffusional resistance is greater for large molecules than for small. Ackers used the Renkin equation (*14*), which he had previously (*15*) applied to the diffusional restriction encountered by macromolecules during migration through thin agar gel membranes. For gel chromatography the relation can be written

$$K_D = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\,\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^2 - 0.95\left(\frac{a}{r}\right)^3\right] \qquad (13)$$

The value of the pore radius r for a given column is obtained by using compounds of known Stoke's radius a and the elution data for K_D. The value of r for Sephadex G-200 was 20.4 mμ, with a mean deviation of 1.8%. This small deviation supports the validity of Eq. (13) and allows one to compute Stoke's radii for unknown compounds from column data, or vice versa. The excellent agreement between experiment and theory is shown in Fig. 2. Several workers (*1,8,16–18*) have maintained that if a restricted diffusional mechanism is operative, the elution volume should be dependent on the flow rate of the eluent. No such dependence has been reported. Ackers, himself, did not observe a flow-rate dependence, but limited his study to flow rates between 1.5 and 6.0 ml/hr. This does not agree with his statement that at zero flow rate there is a large difference in K_D from that observed with the above flow rates. Ackers also suggests that a stagnant layer of liquid surrounding the gel particles offers a resistance to diffusion into and out of the gel and that the thickness of this layer decreases with increasing flow rate. This feature tends to produce extreme insensitivity to flow rate. Ackers concludes that "It is likely that both the molecular-exclusion effect and the diffusional restriction effect are operative to some extent in all types of molecular sieve columns."

Still another mechanism has been proposed by Brewer (*18*), who worked with columns filled with rubber swollen with cyclohexane. Whereas polystyrene gels are supposed to consist of a rigid matrix, swollen rubber is presumed to be elastic. Brewer assumes that the solvent within the elastic network must be under a pressure which must equal the osmotic pressure. With this model he shows that the difference in pressure (inside and outside the gel) can partly explain the decrease in solubility of macromolecules in the gel and also the fact that K_D decreases as molecular weight increases. Using the Boltzmann distribution in terms of the work required to move a solute from the outside to the inside, and the Flory-Rehner theory, he derives an expression for K_D:

$$\ln K_D = (V_s/V_0)[\ln (1 - \varphi_p) + \varphi_p + \chi\varphi_p^2] \tag{14}$$

where V_s and V_0 are the molar volumes of the solute and solvent, respectively, φ_p is the volume fraction of rubber in the swollen rubber, and χ is the rubber-solvent interaction parameter. Thus log K_D is proportional to the ratio of molar volumes rather than to the molecular weight of the solute. Experimental values were used for φ_p, 0.227 for natural rubber and 0.135 for butyl rubber. The values of χ were taken as 0.36 for natural rubber–toluene and 0.44 for butyl rubber–cyclohexane. Values of K_D calculated from Eq. (14) were considerably higher than the experimental values. Although there is some doubt about the values used for φ_p and χ, there is still a discrepancy which Brewer attributes to another mechanism operating—presumably molecular sieving.

A structured gel is not really necessary at all for chromatographic separations based on molecular size. Pedersen (*19*) observed that blood corpuscles in capillaries move faster than does the plasma—

presumably because the larger corpuscles stay in the center of the narrow tubes. He finds a similar separation of large from small molecules by passing the mixture through a glass tube filled with micro-glass beads (diameter 20 to 35 μ). Such separations should be possible on any rough surface having irregularities of the same order as the sizes of the solutes. Small molecules would travel close to the surface and would therefore follow a longer path than large molecules.

In addition, Laurent and Killander (*8*) report that the available volumes of moderately large proteins, calculated from the decrease in solubility in non-cross-linked dextran solution, correspond reasonably well with those in gels.

In a recent review, Anderson and Stoddart (*20*) have replotted all Ackers' computed values of K_D vs. the logarithm of (a/r) as shown in curve A of Fig. 3. They also show that in terms of Acker's symbols, Porath's exclusion theory reduces to

$$K_D = k(1 - a/r)^3 \tag{15}$$

where $k = 1.64$ for dextran fractions on Sephadex gels. Using known values of a/r, they calculated values of K_D and plotted K_D vs. log (a/r), as shown in curve B in Fig. 3.

For either curve a central portion is nearly linear and is of the general form

$$K_D = -\, k_1 \log(a/r) - k_2 \tag{16}$$

where k_1 and k_2 are empirical constants. Since a is proportional to some fractional power of the molecular weight M, they express Eq. (16) as

$$K_D = -\, b \log M + c \tag{17}$$

which suggests that a plot of K_D vs. log M should be linear over a limited range dependent on the nature of the solute molecules and the nature of the gel. Further, since V_0 and V_i are constants for a particular column, Eq. (17) can be rewritten

$$V_e = -b' \log M + c' \tag{18}$$

Equation (18) is of the form commonly used to determine molecular weights from gel-chromatographic measurements. Andrews (*21*) found linear plots of V_e vs. log M over molecular weight ranges of 3000 to 35,000 for Sephadex G-75 and 5000 to 60,000 for G-100, and

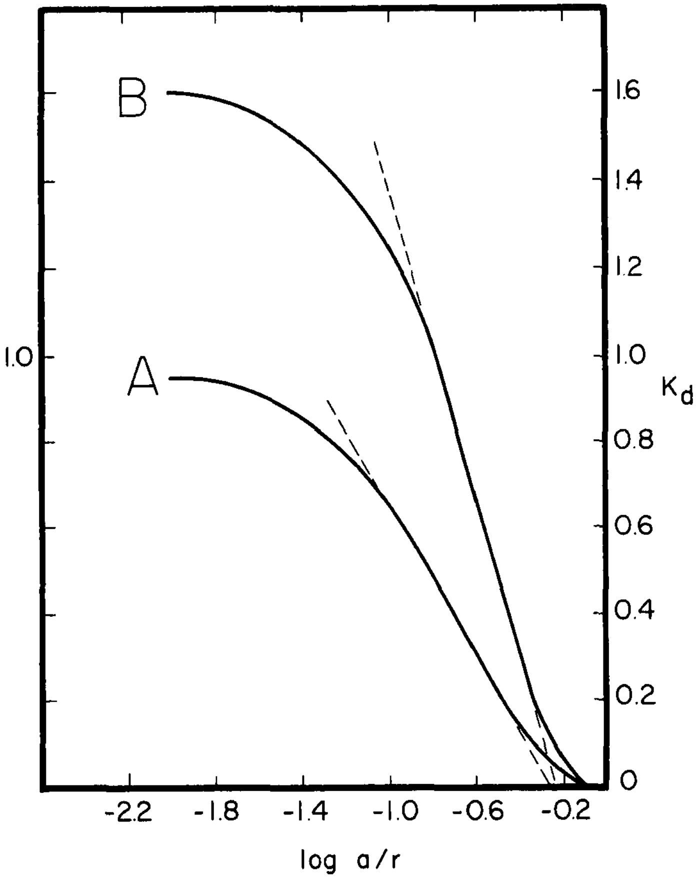

FIG. 3. Relationship between K_D and log (a/r) shown by Anderson and Stoddart (*20*). Curve A, Ackers' theory (*13*); Curve B, Porath's theory (*9*).

in addition found excellent correlation of his data with Eq. (8) (Porath). Likewise Whitaker (22) used a plot of V_e/V_0 vs. log M in the range of molecular weights of 10,000 to 100,000. Moore (23) and co-workers, and others who have followed his lead, have used plots of log M vs. V_e for characterizing the molecular-weight distribution of polystyrene and polyglycol mixtures, with good results.

There have been a number of variations involving different functions of the molecular weight as a basis for a better fit to experimental data. Hendrickson and Moore (24) used 130 compounds to establish a scale of "effective carbon numbers" for the elements usually found in organic molecules. With these "building blocks" they obtained an effective chain length in an additive fashion. When the log of effective number of carbon atoms in a molecule is plotted vs. V_e, they obtained a nearly perfect linear fit over a range of carbon numbers from 2.5 to about 30, as shown in Fig. 4.

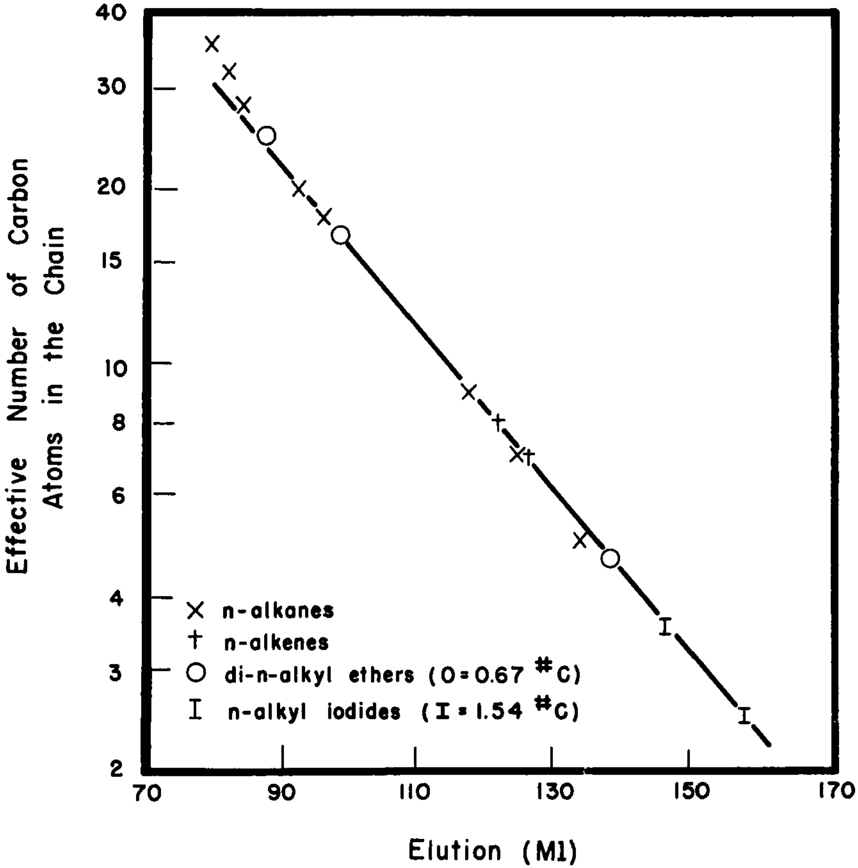

FIG. 4. Elution volume of nonbranched compounds as a function of "effective" number of carbon atoms according to Hendrickson and Moore (24).

Cazes (25) suggests that the chain length ought to be replaced by the radius of gyration of the coiled polymer. Benoit et al. (26) noted that the elution volumes for branched polystyrenes of known structure were far higher than for linear polystyrenes of the same molecular weight. They therefore assumed that the determining factor is the hydrodynamic volume of the solvated molecule, which they computed as the product of the molecular weight and the intrinsic viscosity $[\eta]$. A plot of log $M[\eta]$ vs. V_e was used. Meyerhoff (27) found that the hydrodynamic volume was not a satisfactory parameter when tested with polymethacrylate and cellulose nitrate. He obtained a better fit for all compounds tested with log $M^{1/2}[\eta]^{1/3}$.

The use of chain length has also been criticized by Smith and Kollmansberger (28), who tried both log (chain length) and log (molar volume) plots. The average deviation for the former was 12.2%, while that for the latter was only 3.7%. From this they conclude that the molar volume is the more fundamental yardstick relating elution volume to molecular structure.

All the semiempirical approaches just described give satisfactory results over limited ranges of molecular weights, provided the column is precalibrated with known compounds of a similar type. The ionic strength is often critical, and the temperature is critical if a macromolecule can be uncoiled. In addition, a small but significant concentration effect has been observed (*21,38*). No one has attempted a general equation to predict the elution volume from the characteristic properties of the molecules. A general equation would necessarily be cumbersome and of little use, because of the many mechanisms involved in the separation: (1) the sieving effect, (2) the restricted diffusion effect, and (3) the adsorption effect. To this combination of effects we must add the uncertain nature of the structure of the swollen gels, and the possibility of liquid-liquid partitioning.

Although each author is able to produce data to substantiate his equation or method of plotting, there is essentially no independent data available for rigorous testing of the several approaches. Reliable data, of course, require reasonably sharp elution peaks, and this is another area in which there is a great paucity of useful data.

Several polymer chemists (*17,29–34*) have developed various methods which utilize the peak shapes of gel chromatograms for the estimation of molecular-weight distributions. Chevron Research Company has a computer program available for this purpose (*35*). These methods must correct for natural band spreading which occurs during the chromatographic process. The processes leading to zone spreading have been described and theoretically treated by Giddings (*36,37*). As yet polydispersity in biological materials has not been examined.

Within the framework of the limitations described above, gel chromatography compares quite favorably, in terms of time, cost, and quantity of material, with all other methods utilized for molecular-weight estimations and distributions. However, before it can be fully exploited, a better understanding of processes involved in separation must be attained.

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