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CALIBRATION OF TIGHTLY CROSS-LINKED GEL FILTRATION MEDIA FOR DETERMINATION OF THE SIZE OF LOW MOLECULAR WEIGHT, NON-INTERACTING SOLUTES*

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SUMMARY

A method for calibration of tightly cross-linked gel filtration chromatography media based on the elution characteristics of a series of selected sugars and alcohols is described. The elution position of all molecules is determined by the column effluent refractive index change and is correlated with both the molecular weight and the unhydrated radius estimated from molecular models.

INTRODUCTION

Gel chromatography has been employed for estimation of the size of high molecular weight compounds. Calibration is achieved by observing the elution position of macromolecules of known size[1]. In a similar manner, the size of small molecules may be determined by gel filtration chromatography on tightly cross-linked media. However, special consideration must be given to the choice of calibrating molecules and the method of determining their size.

EXPERIMENTAL

Materials and methods

The gels (Sephadex G-10, G-15*** and Bio-Rad P-2§) were prepared by swelling in distilled water for at least 24 h. Columns*** (1.5 × 90 cm) were packed by sedimentation taking care to exclude bubbles or discontinuities in the gel bed. The eluent, 0.15 *M* NaCl in 0.01 *N* acetic acid (pH 3.3), was allowed to flow at 10 ml/h for at least 24 h at room temperature prior to use. The gel bed was then adjusted to

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a height of 85 cm (total bed volume 150 cm^3) by removal of excess gel. All determinations were made at room temperature.

Hydrochloric acid-treated gels were prepared as follows: 150 cm^3 of expanded Sephadex G-15 or G-10 was mixed with 150 ml of 1 *N* or 6 *N* HCl and placed in a boiling water bath for 2 h with intermittent stirring. The gel was then allowed to settle and the supernatant decanted.

Hydrochloric acid was removed by repeated washes with distilled water followed by vacuum filtration. The gel was extracted with two 150 ml portions of chloroform–methanol (1:1) and one 150 ml portion of diethyl ether using vacuum filtrations between each extraction. The gel was dried in a hood for 24 h and heated in an oven at 100° for 2 h. The resulting xerogel was expanded in distilled water and packed in chromatographic columns as before.

Chromatography

The following calibrating molecules were employed: Blue Dextran 2000 (Pharmacia Fine Chemicals), stachyose, and raffinose (Sigma Chemical Co.), maltose, and propylene glycol (Eastman Organic Chemicals), glycerol (Baker Chemical Co.), ethylene glycol (Fisher Scientific Co.), methyl alcohol (Mallinckrodt Chemical Works), and 99.84 atom percent deuterium oxide (Volk Radiochemical Co.). The elution position of chromatographed molecules was determined from the effluent refractive index*. Fig. 1 indicates that the refractive index change of sugars and alcohols with

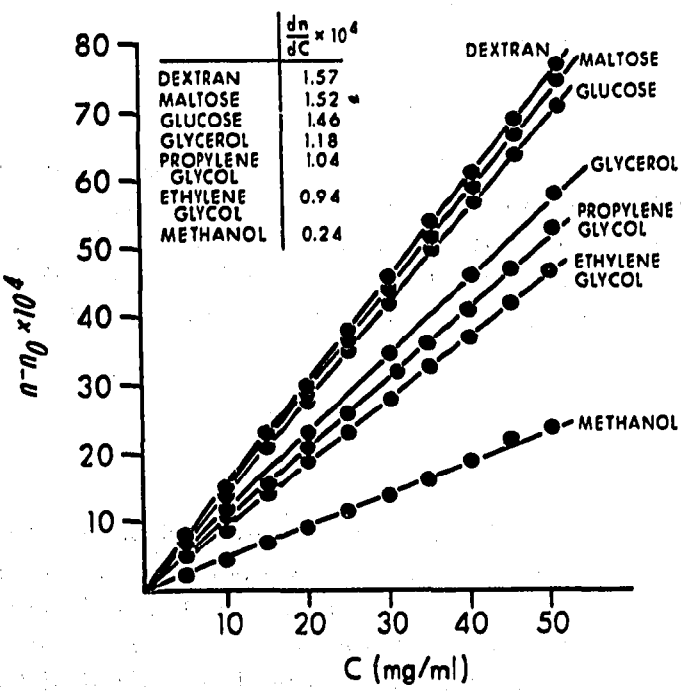

Fig. 1. Refractive index characteristics of sugars and alcohols. The refractive index change from that of the column eluent ($n-n_0$) for sugars and alcohols of various concentrations (C(mg/ml)) is linear over the concentration range $C \leq 50$ mg/ml. The characteristic refractive index change (dn/dC) varies with the molecular size. The data were taken from a compilation by WOLF AND BROWN[2].

* E-C Apparatus Corp., Philadelphia, Pa. Refractive index column monitor Model EC 211.

concentration (dn/dc) was relatively linear over the range $C \leq 50$ mg/ml. The refractive index monitor response was adjusted for a full-scale deflection of 35×10^{-4} refractive index units.

Elution data are presented in terms of the distribution coefficient (K_D) defined by the equation:

$$K_D = \frac{V_e - V_0}{V_t} \tag{1}$$

where V_e is the observed elution volume of the chromatographed substance, V_0 is the void volume or volume external to the gel mesh network and V_t is the accessible volume within the gel mesh.

On Sephadex gels, the void volume (V_0) was measured by the elution volume of Blue Dextran*, a high molecular weight (2×10^6 daltons) polysaccharide which is completely excluded from the gel pores. Since polyacrylamide gels bind Blue Dextran, void volume determinations were made with bovine serum albumin**.

The internal volume (V_t) was determined from the elution position of deuterium oxide. The lower refractive index[3] of deuterium oxide ($^2H_2{}^{16}O$) compared with protium oxide ($^1H_2{}^{16}O$) resulted in a negative refractive index peak which co-chromatographed with tritium oxide ($^3H_2{}^{16}O$). Internal volume (V_t) was computed as the difference between the elution volume of deuterium oxide and the void volume indicator. Resolution in terms of zone broadening was determined by measuring the standard deviation of the elution peak (ref. 1, p. 70).

Unhydrated radii of probe molecules

The molecular sieving characteristics of tightly cross-linked gels were determined from the elution volume of stachyose, raffinose, maltose, glucose, glycerol, propylene glycol, ethylene glycol and methanol. Unhydrated radii were measured from CPK*** molecular models by the method of GOLDSTEIN AND SOLOMON[4]. Geometric mean radii in Å were computed from three mutually perpendicular diameters (d_1, d_2, d_3 in cm) on molecular models in their most extended and collapsed configurations as:

$$r_g = \frac{(d_1 d_2 d_3)^{\frac{1}{3}}}{(2)(1.25)} \tag{2}$$

The unhydrated radius of a given molecule is taken as the arithmetic mean of the largest and smallest geometric mean radii determined by this method.

$$r = \frac{r_{g(\max.)} + r_{g(\min.)}}{2} \tag{3}$$

The unhydrated radius (r) was related to the molecular weight (M_W) by a function of the form[6,7]:

$$r = K\,(M_W)^n \tag{4}$$

where n and K are constants to be evaluated.

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** Bovine serum albumin, Fraction V, Pentex, Inc., Kankakee, Ill.

*** Corey-Pauling-Koltun space-filling models; Schwarz Bioresearch, Inc., Orangeburg, N.Y. Scale factor = 1.25 cm/Å.

TABLE I

MOLECULAR WEIGHTS AND UNHYDRATED RADII OF SELECTED SUGARS AND ALCOHOLS USED FOR COLUMN CALIBRATION

The maximum radii ($r_{g(max.)}$) and minimum radii ($r_{g(min.)}$) computed by eqn. 2 from measurements on molecular models were used to determine the unhydrated radii (r) by eqn. 3.

	M_w	$r_{g(max.)}$ (Å)	$r_{g(min.)}$ (Å)	r (Å)
Stachyose	667	7.30	5.69	5.78[a]
Raffinose	505	5.73	4.83	5.28
Maltose	342	4.28	5.01	4.64
Glucose	180	3.67	3.46	3.56
Glycerol	92	3.05	2.72	2.89
Propylene glycol	76	2.99	2.70	2.85
Ethylene glycol	62	2.76	2.24	2.50
Methanol	32	2.12	2.14	2.13
Deuterium oxide	20	—	—	1.53

[a] Determined by the method of Robinson and Stokes (ref. 7, p. 124).

Consideration of gel filtration models

Four mathematical models were tested for their ability to predict distribution coefficients (K_D) from molecular size (see DISCUSSION). Of these, the equations

$$K_D = a - b\,[\log_{10} r] \quad \text{and;} \tag{5}$$

$$K_D = A - B\,[\log_{10} M_W] \tag{6}$$

were used to represent the elution characteristics of test molecules.

RESULTS

Unhydrated radii of sugars and alcohols selected for column calibration

The molecular models constructed for measurement of unhydrated radii are illustrated in Fig. 2. Radii computed from eqns. 2 and 3 are listed in Table I. The radius determination for stachyose was less precise due to the flexibility of the molecular model and is indicated in Table I by the relatively large difference between the measured maximum ($r_{g(max.)} = 7.30$) and minimum ($r_{g(min.)} = 5.69$) radii. For this oligosaccharide, a radius approximation was made by extrapolation (ROBINSON AND STOKES (ref. 7, p. 124)). The unhydrated radius estimate thus obtained was 5.78 Å.

Correspondence of these molecular radii to eqn. 4 was tested by a least-squares fit of the data from Table I. The resulting equation, $r = 0.65\ (M_W)^{0.336}$ predicted the radii of this series from their molecular weights with an average error of 2.2% (Fig. 3).

Elution properties of selected calibration molecules

Elution profiles of the selected sugars and alcohols on four different columns are illustrated in Fig. 4. Concentrations of the test molecules were selected to give

Fig. 2. CPK molecular models used to determine the unhydrated radius of calibrating molecules. In the order of their increasing size, the models represent water, methanol, ethylene glycol, propylene glycol, glycerol, and glucose (bottom row); maltose, raffinose, and stachyose (top row).

All sugars were constructed in the D form with the following configurations. Glucose: β-glucopyranose; maltose: β-glucopyranose-(1-4)-α-glucopyranose; raffinose: β-fructofuranose-(1-6)-α-galactopyranose-(1-6)-α-glucopyranose; stachyose: α-galactopyranose-(1-6)-α-galactopyranose--(1-4)-α-glucopyranose-(1-2)-fructufuranose.

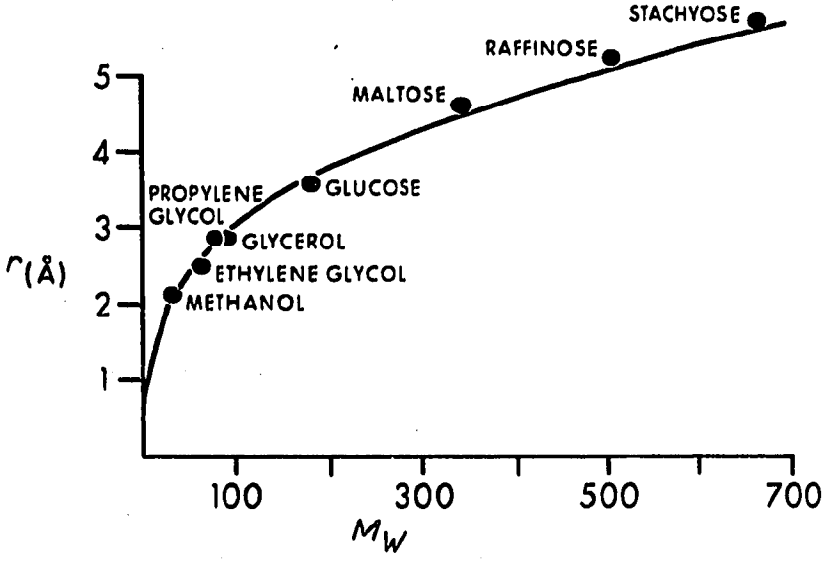

Fig. 3. Molecular weights (M_w) and unhydrated radii (r) of molecules selected for column calibration. The solid line is the function $r = 0.65\ (M_w)^{0.336}$, a least-squares fit equation from the data of Table I.

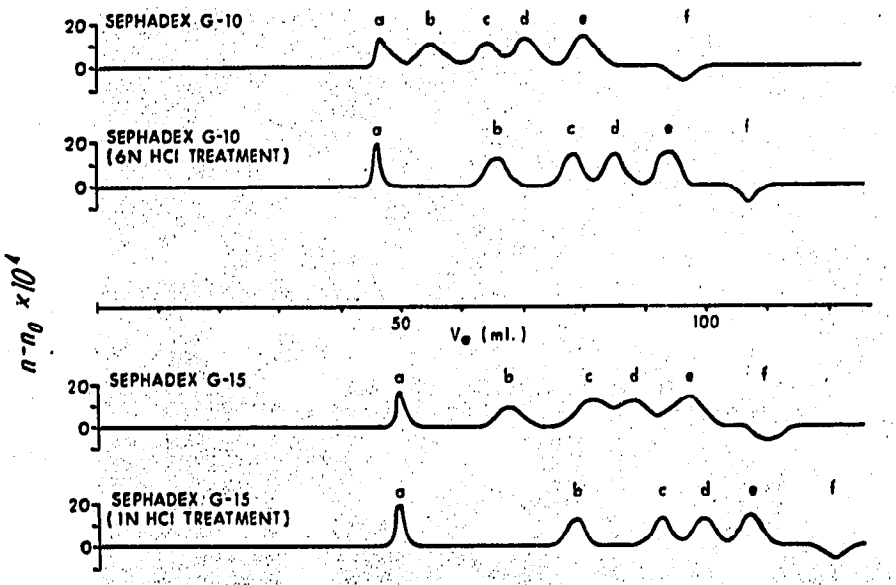

Fig. 4. Characteristic elution patterns of a mixture of test molecules on 1.5 × 85 cm Sephadex G-10 and G-15 columns. The elution volume (V_e) of each molecule was determined from the maximum refractive index change (n-n_0) of the column effluent. The samples contained: (a) Blue Dextran, 1 mg; (b) stachyose, 50 mg; (c) maltose, 50 mg; (d) glucose, 50 mg; (e) ethylene glycol, 100 μl; and (f) deuterium oxide, 900 μl; and NaCl 9 mg (eluent concentration). Of the other calibrating compounds the amounts used were: raffinose, 50 mg; glycerol, 50 μl; propylene glycol, 50 μl and methanol, 200 μl.

approximately equal maximal refractive index deflections. The results from multiple chromatographic runs utilizing the series of test molecules on each of the gel filtration media are shown in Table II. These K_D values were fitted by least squares to eqns. 5 and 6 utilizing the radii and molecular weights in Table I. The coefficients a, b, A and B of Table III define equations which describe the elution properties of the test molecules on each of the gel filtration media studied. Extrapolation of the functions to $K_D = 0$ gives an estimate of the largest molecular size which will just diffuse into the gel pores. Similarly, extrapolation to $K_D = 1$ provides an estimate of the largest molecular size that will diffuse completely into the internal volume of the gel.

DISCUSSION

The elution properties of a series of sugars and alcohols were studied on three commercially available, tightly cross-linked gel filtration media and three acid-treated dextran gels. The acid-treated Sephadex G-10 and Sephadex G-15 showed a significant improvement in chromatographic resolution. This improvement is illustrated by increased internal volumes (V_i) and smaller elution peak standard deviations ($\bar{\sigma}$). As previously reported[17], acid treatment resulted in an enlargement of the external pore aperture.

Bio-Gel P-2, the most tightly cross-linked acrylamide gel commercially available, exhibited a much larger pore size than the dextrans, Sephadex G-10 and G-15. Thus the acrylamide gel was found to be less suitable for the separation of small molecules and to possess molecular sieving characteristics similar to those of Sephadex G-25.

TABLE II

CHROMATOGRAPHIC PARAMETERS DESCRIBING THE CHARACTERISTIC ELUTION OF TEST MOLECULES ON TIGHTLY CROSS-LINKED GEL PREPARATIONS

The void volume (V_0) was taken as the elution volume of Blue Dextran and internal volume (V_i) was computed from the elution volume of deuterium oxide (N determinations). The distribution coefficients (K_D) of test molecules were computed from eqn. 1. Multiple determinations of K_D for the same molecule indicated an average standard deviation of 0.027. The mean standard deviation, $\bar{\sigma}$, of the elution peak determined from the peak width is a measure of zone broadening.

	V_0 (ml) ± S.D.	V_i (ml) ± S.D.	N	K_D								$\bar{\sigma}$ ± S.D. (N)
				Stachyose	*Raffinose*	*Maltose*	*Glucose*	*Glycerol*	*Propylene glycol*	*Ethylene glycol*	*Methanol*	
Sephadex G-10	46.6 ± 0.8	51.0 ± 1.6	3	0.17	0.24	0.35	0.48	0.60	0.67	0.67	0.80	2.17±0.23(14)
Sephadex G-10 1*N* HCl	45.5 ± 0.9	50.1 ± 0.6	3	0.13	0.21	0.32	0.53	0.58	0.67	0.67	0.80	1.83±0.16 (12)
Sephadex G-10 6*N* HCl	46.4 ± 2.3	61.4 ± 3.5	3	0.31	0.43	0.52	0.63	0.76	0.78	0.77	0.96	1.53±0.27 (11)
Sephadex G-15	48.6 ± 0.8	63.0 ± 1.9	5	0.31	0.39	0.52	0.63	0.72	0.76	0.75	0.87	2.42±0.37 (13)
Sephadex G-15 1*N* HCl	48.8 ± 1.1	72.7 ± 1.7	3	0.41	0.49	0.60	0.70	0.77	0.79	0.80	0.87	1.89±0.31 (14)
Bio-Gel P-2	46.8 ± 0.9	69.5 ± 11.0	6	0.48	0.63	0.65	0.82	0.89	0.85	0.88	0.86	2.23±0.80 (14)

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TABLE III

EQUATION COEFFICIENTS, STATISTICAL MEASURES OF VARIATION AND EXTRAPOLATED VALUES DESCRIBING THE RELATIONSHIP BETWEEN ELUTION POSITION AND MOLECULAR SIZE OF TEST COMPOUNDS CHROMATOGRAPHED ON TIGHTLY CROSS-LINKED GEL MEDIA

The coefficients a and b relate to eqn. 5 and the coefficients A and B relate to eqn. 6 where K_D is the distribution coefficient, r is the average geometric mean radius, and M_W is the molecular weight of the chromatographed test molecules. σK_D is the standard error of observed diffusion constant values from the least-squares fitted equation. $\%E_r$ and $\%E_{M_W}$ are the average percent error in radius and molecular weight, respectively, for predicted K_D values from those observed experimentally.

Evaluations of each model at $K_D = 0$ gives the radius ($r(K_D = 0)$) and molecular weight ($M_W(K_D = 0)$) of a hypothetical molecule which is marginally excluded. Evaluation of each model at $K_D = 1$ gives the radius ($r(K_D = 1)$) and molecular weight ($M_W(K_D = 1)$) of the smallest molecule participating in chromatographic process.

	Equation coefficients				*Statistical measures of variation*				*Extrapolated radius and molecular weight*			
	(5)		*(6)*		*(5)*		*(6)*		*(5, in Å)*		*(6, in Daltons)*	
	a	*b*	*A*	*B*	σK_D	$\%E_r$	σK_D	$\%E_{M_W}$	$r(K_D=0)$	$r(K_D=1)$	$M_W(K_D=0)$	$M_W(K_D=1)$
Sephadex G-10	1.274	1.43	1.545	0.480	0.027	3.6	0.022	8.5	7.8	1.6	1,660	14
Sephadex G-10 1*N* HCl	1.322	1.53	1.618	0.517	0.040	4.5	0.041	11.3	7.3	1.6	1,340	16
Sephadex G-10 6*N* HCl	1.387	1.37	1.645	0.458	0.043	4.8	0.033	12.8	10.4	1.9	3,900	26
Sephadex G-15	1.298	1.25	1.537	0.421	0.037	5.1	0.037	15.6	10.9	1.7	4,500	19
Sephadex G-15 1*N* HCl	1.247	1.05	1.451	0.354	0.035	5.8	0.040	18.3	15.6	1.7	12,600	19
Bio-Gel P-2	1.320	1.03	1.532	0.355	0.066	11.3	0.080	39.8	18.9	2.0	20,800	32

The sugars and alcohols used in this study as calibration molecules were selected for their availability in high purity and their lack of demonstrable gel interaction. Certain molecular species were categorically excluded as test molecules because of their chemical similarity to molecules which have been demonstrated to exhibit weak gel interactions. Thus, low molecular weight aldehydes, such as glyceraldehyde and glycolaldehyde, were excluded because of an observed interaction of acetaldehyde with dextran gels. Similarly, high molecular weight aliphatic alcohols were excluded on the basis of gel interaction with *n*-butanol[10]. Inorganic electrolytes were not used because of their extensive hydration[11,12].

The use of molecular radius in this study depends on the validity of the measurement procedure described. This procedure has been used with success by GOLDSTEIN AND SOLOMON[4] to estimate the pore size of erythrocyte membranes. The measurement procedure assumes that the "true" molecular configuration lies between the two extreme configurations: $r_{g(\max.)}$ and $r_{g(\min.)}$. The CPK models are designed to approximate the space-filling characteristics of molecules from accepted values of bond angles and Van der Waal's radii of constituent atoms[5]. A principal disadvantage of the estimation of molecular size from CPK models is the degree of subjective variability possible in a single measurement which is contingent on the molecular configuration and measurement axes chosen. However, the algebraic mean between the maximum and minimum radius values observed from multiple determinations decreases subjective variability and establishes limits within which the true radius must lie.

Other methods have been used to estimate the radii of small molecules[6,7]. The radius of a sphere of equal weight and density[8] is a commonly used measure of unhydrated radius. However, the use of solid or liquid densities of pure substances in this computation is questionable. The solid or liquid density of a pure substance reflects the efficiency of packing and the intermolecular forces existing in pure substances rather than the effective density of a substance dissolved in a relatively large amount of water. Molecular radii calculated by this method exhibited poorer correlations with both molecular weight and column chromatographic elution volumes. Determination of Stokes' radii[7] from the diffusion coefficient or electrophoretic mobility requires an empirical correction for molecules with radii of less than 5 Å. The exact form of the correction is uncertain and its application to small neutral molecules is questionable[11].

Radius measurements from molecular models constitute a simple and direct method which circumvents the problem of density while incorporating the effect of molecular configuration. It has been demonstrated, here, that this measurement produces an internally consistent set of radius values which behave in the manner expected for approximately spherical molecules.

An evaluation of the correspondence of elution data to theoretical models described by eqns. 5 and 6 indicated (Table III) that under the conditions described they could be used to predict the observed diffusion constant values (K_D) within a standard deviation of approximately 0.04 from either the radius measured on molecular models or the known molecular weight. Conversely, eqn. 5 could be used to predict the measured molecular radius within an average error of about 5% and eqn. 6 could be used to predict the molecular weight within an average error of about 10%. The inferior correspondence of Bio-Gel P-2 data to these models arises largely

from the small fraction of the total gel sieving range covered by the test molecules.

Other theoretical models tested included that of ACKERS[8]:

$$K_D = \left(1 - \frac{r}{K_1}\right)^2 \left[1 - 2.104\left(\frac{r}{K_1}\right) + 2.09\left(\frac{r}{K_1}\right)^3 - 0.95\left(\frac{r}{K_1}\right)^5\right] \quad (7)$$

and that of PORATH[9]:

$$K_D = K_2\left(1 - \frac{2r}{K_3}\right)^3 \quad (8)$$

where K_1, K_2 and K_3 are fitted parameters. The model of ACKERS, based on the assumption of restricted molecular diffusion in a cylindrical pore gave poor correspondence to the data of Table II. The basic assumption of elution dependency on diffusion rate has been criticized[1], since the elution volume of a molecule is independent of the column flow rate. In addition, the poor correlation found in this study indicated a lack of general applicability of this model to predict elution values on dextran media with high cross-linking ratios. The Porath model, though accurately predicting elution positions, was found to give large values for the marginally excluded molecule ($K_D = 0$) and failed to predict the observed exclusion of inulin on Sephadex G-15. For these reasons, eqns. 5 and 6 were found to be superior to the other mathematical models tested.

Extrapolation to unity distribution coefficient gave the smallest molecular size which could participate in the chromatographic process. The diffusion constant of deuterium oxide was defined as unity, and therefore was eliminated in the curve fitting procedure so that extrapolation constituted an unbiased estimate of the internal chromatographic limit. Table III indicated that the chromatographic process on both Sephadex G-10 and G-15 extended to a limiting value imposed by the dimensions of water. With the Sephadex gels, extrapolation to unity distribution coefficient ($r(K_D = 1)$, $M_W(K_D = 1)$, Table III) gave molecular weight values close to that of monomeric water and radius values close to that measured on molecular models of monomeric water (1.53 Å). This result has an important corollary. Under the conditions of the experiment, deuterium oxide and tritiated water do not exhibit statistically significant anomolous behavior on Sephadex G-10 and G-15 in relation to the sugars and alcohols used as test molecules, indicating that these labeled water species behave as monomers.

The use of isotopically labeled water for the determination of internal volume has been criticized by some authors[10,12]. MORSI AND STERLING[13] have demonstrated that purified starch completely exchanges hydroxyl hydrogen with deuterium oxide after one hour of equilibration. MARSDEN[10] has suggested that a correction for the distribution constant determined by tritiated water should be used to account for this phenomenon. A value of 5.9% was given for Sephadex G-25 based on its epichlorhydrin cross-linking ratio. Cross-linking ratios for Sephadex G-10 and G-15 have not been determined[14]. However, the close correspondence of the observed elution values of deuterium oxide and tritiated water with that expected theoretically indicates that if this exchange occurs the effect does not significantly alter the elution properties of isotopically labeled water in this chromatographic system.

The question of the form in which water exists within dextran gel pores has been raised by YOZA AND OHASHI[15]. There is a large literature concerned with the

existence of polymeric aggregates of water in certain physical and biological systems[16]. The large size of water aggregates within the gel pores would result in an earlier apparent elution. Close correspondence of the internal chromatographic limit to the dimensions of monomeric water is consistent with the hypothesis that water exists in a completely dissociated form within the gel pore.

Since the processes of hydrogen exchange and water aggregation have opposing effects, it cannot be ruled out that both phenomena occur simultaneously, resulting in a cancellation of their chromatographic results. Neither can the possibility be ruled out that all test molecules are significantly hydrated in solution resulting in an uniform additive increase in radius. However, if hydration of these molecules occurs, the degree of hydration must be small. ROBINSON AND STOKES (ref. 7, p. 306) have suggested that glycerol may move with one molecule of water. GOLDSTEIN AND SOLOMON[4] has commented that the glycols move through solution more slowly than expected, although he was unable to differentiate between chemical hydration effects and hydrodynamic drag. In the absence of evidence for significant hydration of sugars and alcohols in solution, it may be concluded that the radii measured for the calibrating molecules are internally consistent and reasonable estimates of their effective molecular sizes.

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