CHROM. 6095

DETERMINATION OF PARAMETERS IN DEXTRAN GEL FILTRATION*

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(First received March 2nd, 1972; revised manuscript received April 17th, 1972)

SUMMARY

To calculate the height equivalent to a theoretical plate (HETP), the elution rate, the bed length of the gel, the particle size and the K_D values, the void volume, V_0, and the effective internal volume, $(V_i)_{eff}$, have to be determined. It can be seen from tests with low-molecular-weight organic molecules and inorganic salts that chloride ions (Sephadex G-25) and bromide ions (Sephadex G-10, G-15 and G-25) are suitable for the experimental determination of the effective internal volume of highly crosslinked dextran gels. F^-, I^-, SO_4^{2-}, SCN^-, Cu^{2+} and Fe^{3+} ions, dihydrogen orthophosphates, monohydrogen orthophosphates or tri-alkali orthophosphates, as well as glucose or neutral amino acids (α-alanine and leucine) should not be used for the determination of $(V_i)_{eff}$. The experimental data favour the introduction of an effective internal volume, $(V_i)_{eff}$, in place of the so far applied theoretical internal (imbibed) volume, V_i.

INTRODUCTION

To enable a comparison to be made of the K_D values of compounds separated by gel filtration[1], the experimental conditions and gel bed parameters have to be determined and calculated as carefully as possible. Data given by the manufacturer are often only approximate and are not sufficient for analytical calculations. The K_D values of peptides and nucleic acid components (pyrimidines, purines and nucleosides) we found[2,3] through analytical and preparative separations differ considerably from those determined by other workers[4–7], and similar considerations applied to the effective internal volume[4–7]. In this paper our investigations on the calculation of the effective internal volume and the HETP values for different organic molecules and inorganic cations and anions are described.

The total bed volume, V_t, of a column is composed of the gel matrix volume, V_m, the internal (imbibed) volume, V_i, within the gel grains, and the outer (void) volume, V_0, between the gel grains. To characterize the elution behaviour of large molecules[8–10], the relative elution volume, (V_e/V_0), the retention constant, $R = (V_0/V_e)$, or the relative elution volume, (V_e/V_t), which depends on the total volume, are usually applied. These parameters are dependent on the packing density[25]. The packing density-independent distribution coefficients, K_{av} and K_D, are

* In honour of the 50th birthday of Professor W. STICH.

suitable for use in describing the elution of low-molecular-weight substances. K_{av} is related[11] to the distribution of a component between the mobile phase (eluant) and the total gel phase according to eqn. 1. The K_D values[12] express the distribution between the mobile phase and the actually available internal volume of the stationary phase (gel grains); they are constants under constant conditions of gel filtration:

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

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

The K_D value acts as a distribution coefficient, which indicates the part of the internal volume that is available to the distribution solution $\rightleftharpoons$ gel of a substance during gel filtration. Molecules or ions, hindered from entering the interior of the grains by virtue of their molecular weight or configuration, use only the void volume in gel filtration ($V_e = V_0$; $K_D = 0$) and are preferred for the determination of the V_0 values of a gel bed. By using the parameters V_t, V_0, V_e, the imbibed volume (V_i), the dry weight of the bed material (a) and the partial specific volume ($V_g = 0.6$)[13], the K_D value of a substance can be calculated according to eqn. 3:

$$K_D = \frac{V_e - V_0}{V_i} = \frac{V_e - V_0}{V_t - V_0 - a \cdot V_g} \tag{3}$$

The imbibed volume, V_i, which depends on the degree of crosslinking, is calculated from the solvent regain, S_r (or water regain, W_r), the density of the eluant, ρ_E, and the dry weight of the bed material, a:

$$V_i = \frac{a \cdot S_r}{\rho_E} = a \cdot W_r = \frac{\rho_{gel} \cdot S_r}{\rho_E (S_r/\rho_E + 1)} \cdot (V_t - V_0) \tag{4}$$

As the dry weight of the bed material is not known, the density of the swollen gel, ρ_{gel}, has to be estimated.

All low-molecular-weight substances with no gel matrix interaction ($K_D > 1$) or restricted distribution ($K_D < 1$) are suitable[14,15] for use in an experimental determination of the internal volume, V_i. According to the investigations of GELOTTE[16], part of the water required for the swelling of the gel is needed for the hydration of its polysaccharide chains, and hence it follows that this part of the imbibed volume is not available for unrestricted distribution of the solutes. Thus these substances are eluted earlier than expected. According to GELOTTE[16], molecules or ions with a K_D value of 0.8 pass the gel bed without adsorption in unrestricted distribution.

We have designated this part of the imbibed volume that is available for unrestricted distribution the "effective internal volume", $(V_i)_{eff}$, and have calculated it according to eqn. 5 from the theoretical imbibed volume (see eqn. 4):

$$(V_i)_{eff} = 0.8 \cdot (V_i)_{th} \tag{5}$$

All freely diffusing compounds with a K_D value of 0.8 should show the

expected K_D value of 1.0 after the introduction of the hydration correction factor:

$$K_D = \frac{V_e - V_0}{(V_i)_{eff}} = 1 \text{ (unrestricted distribution)} \quad (6)$$

For these substances, the effective internal volume is determined directly in the filtration experiments as the difference of the elution volume and the void volume. From the elution diagrams, having the shape of Gaussian error curves, the number of theoretical plates, N, of a gel bed can be calculated easily by the formulae derived by GLUECKAUF (eqn. 7)[17] or FLODIN (eqn. 8)[18]:

$$N = 8 \cdot (V_e/\beta)^2 \quad (7)$$

$$N = (V_e/\sigma)^2 \quad (8)$$

where β is the width (measured in millilitres) of the elution diagram at the height $h/2.72$ over the base-line (Fig. 1) with S as the intersection of the tangents, and σ is the standard deviation of the stachyose* elution peak. The HETP corresponds

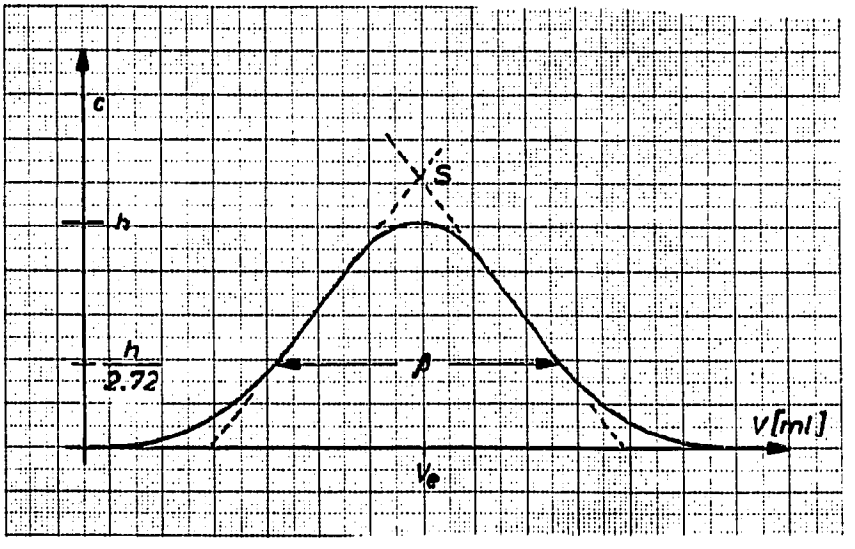

Fig. 1. Ideal Gaussian-shaped elution curve and scheme for calculation of the height equivalent to a theoretical plate (HETP).

to the gel bed length of one theoretical plate and can be calculated by dividing the column length, l_{gel}, by the number of theoretical plates (Table I):

$$\text{HETP} = l_{gel}/N \quad (9)$$

To estimate the number of theoretical plates required for a given degree of separation of two components, another mathematical relationship[3,17] exists between the number of theoretical plates and the ratio (eqn. 10) of the elution volumes of the two components:

$$\frac{V_{e,2}}{V_{e,1}} = \frac{(\alpha + K_{D,2})}{(\alpha + K_{D,1})} \quad (10)$$

* α-D-Galactosyl-α-D-galactosyl-α-D-glucosyl-β-D-fructose.

From the fraction $\alpha = (V_0/V_t)$ and the K_D values, the HETP values[19] and the number of theoretical plates, the particle size, the elution rate and the smallest gel bed length necessary for the separation of the components can be estimated. Owing to the zone spreading during gel filtration, the separation volume, V_{sep} (the difference between the fronts of the elution curves), has to be considered for effective preparative separations as well as for components with similar K_D values. It is apparent that a separation of two solutes is impossible owing to overlapping elution profiles if the volume of the sample is larger than the separation volume:

$$V_{sol} < V_{sep} = V_{e,2} - V_{e,1} = [V_0 + K_{D,2} \cdot (V_i)_{eff}] - [V_0 + K_{D,1} \cdot (V_i)_{eff}]$$
$$= (V_i)_{eff} \cdot [K_{D,2} - K_{D,1}] \qquad (11)$$

MATERIALS AND METHODS

Dextran gels

Sephadex G-10 (40–120 μm), Sephadex G-15 (40–120 μm), Sephadex G-25 fine (20–80 μm) and Blue Dextran 2000 (mol. wt. $2 \cdot 10^6$) were obtained from Deutsche Pharmacia GmbH, Frankfurt/Main (G.F.R.).

Gel bed dimensions. Sephadex G-10, gel bed length 200 cm (two LKB columns of dimensions 9 × 1000 mm, $V = 62.06$ ml and 12 × 1000 mm, $V = 109.88$ ml; total volume $V_t = 172.04$ ml); Sephadex G-25, gel bed length 100 cm (LKB column, 25 × 1000 mm, $V_t = 476.87$ ml).

Gel filtration

The dry gel was allowed to swell in twice-distilled water for 2 h in a boiling water-bath and after cooling it to room temperature it was carefully deaerated under reduced pressure. After sedimentation of the gel, the supernatant was removed by decantation. The sedimentation tube fitted to the top of the column was connected to a funnel with a diameter slightly smaller than the bore of the sedimentation tube and the connection was sealed with Parafilm. The system was then filled with water or buffer up to the funnel and the swollen gel added to the funnel quantitatively. After the sedimentation of the gel, the column was equilibrated and the compounds to be tested were put on to the column with a pump (LKB Perpex 10200).

The tubes from the pump and the top plug of the column, which usually became attached to each other, were disconnected easily by using strong alkaline solutions, and therefore we connected them with a capillary tubing connector (LKB 3055). The socket for the tube to the pump was bored open to correspond with the tube diameter (3 mm).

All filtrations were carried out at 10° in the LKB CombiCold Rac 5201 laboratory refrigerator with flow-rates of 14 and 18 ml/h. The effluent was collected in fractions of 3.5 ml (4.5 ml) in the LKB Ultrorac 7000 fraction collector.

To prevent microbial growth, the gel bed was rinsed occasionally with a 0.02% solution of NaN_3.

Elution buffers

(A) Twice-distilled water.

(B) 0.01 *M* citric acid/0.01 *M* Na_2HPO_4; pH 3.5.

(C) 0.1 *N* NH_3,H_2O/0.1 *N* NH_4Cl; pH 9.5.
(D) 0.1 *M* acetic acid/0.1 *M* sodium acetate; pH 4.3.
(E) 0.1 *M* NaCl and 0.01 *M* NaCl.
(F) 0.1 *N* NaOH; pH 13.

Outer (void) volume and internal volume

V_0. This was measured as the peak elution of 0.5 ml of a 3% solution of Blue Dextran 2000 (extinction at 256 nm). Eluants were buffers A, B (pH 3.5) and C (pH 9.5).

V_t. This was estimated by the elution of 1–3 ml of a 10% solution of the test samples (analytical-reagent grade). $FeCl_3$, NH_4SCN and the amino acids were used at a concentration of 2–5 mg/ml.

Amino acids

4 N sodium acetate buffer (pH 5.5). To 100 ml of water were added 135 g of sodium acetate trihydrate (analytical-reagent grade) and the mixture was stirred on a water-bath until dissolution was complete. After cooling to 20°, 25 ml of glacial acetic acid were added, and the volume was made up to 250 ml.

Ninhydrin reagent solution. Two grams of ninhydrin and 0.1 g of tin(II) chloride dihydrate were dissolved in a mixture of 75 ml of methyl cellosolve (ethylene glycol monomethyl ether) and 25 ml of sodium acetate buffer. During this procedure, a slow stream of pre-purified nitrogen was bubbled in.

Colour reaction. The effluent fractions (*ca.* 3 ml) were mixed with 0.5 ml of the reagent solution and the mixtures were heated for 20 min on a boiling water-bath. After dilution with 5 ml of ethanol–water (1:1), the tubes were cooled to 20° before being read at 570 nm.

Carbohydrates

Anthrone reaction. A 500-mg amount of anthrone (analytical-reagent grade) and 10 g of thiourea (analytical-reagent grade) were dissolved in 1000 ml of a hot (90°) 72% solution of H_2SO_4. The mixtures of the effluent fractions with 10 ml of the anthrone reagent solution were heated for 15 min on a boiling water-bath and after being allowed to cool to 20° they were read at 630 nm.

Phosphates

Alkali dihydrogen orthophosphates and di-alkali hydrogen orthophosphates were precipitated at pH 9 (with a few millilitres of a dilute ammonia solution, if necessary) with a 10% solution of $BaCl_2$ and estimated as $Ba_3(PO_4)_2$. $Ba(HPO_4)_2$ is soluble at pH < 7.

With tri-alkali orthophosphates, the effluent fractions were mixed with 3 ml of a 35% solution of NH_4NO_3 and 2 ml of 25% HNO_3. After heating on a boiling water-bath, 12 ml of a hot 3% solution of $(NH_4)_6Mo_7O_{24}$ were added to the test-tubes and the yellow precipitates of $(NH_4)_3(P^VMo_{12}O_{40})$ filtered off.

Sulphates

The effluent fractions were acidified with dilute HCl to pH 1 and mixed with 1–2 ml of a 10% solution of $BaCl_2$. The precipitated $BaSO_4$ was filtered off.

Fluorides

The 4-ml fractions of the effluent were acidified with 1 ml of 25% acetic acid,

then 1 ml of a 10% solution of $BaCl_2$ was added. The precipitated BaF_2 was isolated by centrifugation.

Chlorides, bromides and iodides

The effluent fractions were mixed with 1 ml of a 5% solution of K_2CrO_4 indicator and titrated to the end-point (precipitation of reddish brown Ag_2CrO_4) with 0.01 *N* $AgNO_3$ (Mohr titration).

RESULTS AND DISCUSSION

On examining the literature on gel filtration of low-molecular-weight substances, different inorganic cations and anions were found with K_D values of 1.0 but without experimental detail or an elution diagram. The derived V_t value of the gel bed was the basis of the calculations of the K_D values of the organic compounds

TABLE I

THE ELUTION VOLUMES AND K_D VALUES OF TEST SUBSTANCES FILTERED THROUGH A COLUMN (12 × 1000 mm AND 9 × 1000 mm) OF SEPHADEX G-10, 40–120 μm, AT DIFFERENT pH VALUES

N (Cl^-) ~ 2200; HETP (Cl^-) ~ 0.91 mm per plate; *N* (Br^-) ~ 2880; HETP (Br^-) ~ 0.69 mm per plate; *N* (I^-) ~ 2000; HETP (I^-) ~ 1.00 mm per plate.

Sephadex type; column length; $(V_t)_{eff}$	*Chemical compound*	*Elution buffer*	*pH*	*V_e (ml)*	*$K_D = \frac{V_e - V_0}{(V_t)_{eff}}$*
G-10; 200 cm; 57.8	Blue Dextran	B	3.5	69.8 (V_0)	0
	$CuSO_4$	B	3.5	83.4	0.23
	NaCl	B	3.5	110.2	0.70
	NaCl	B	3.5	114.3	0.77
G-10; 200 cm; 56.6 ml	Blue Dextran	H_2O		66.6 (V_0)	0
	Na_2SO_4	H_2O		89.4	0.40
	Na_2SO_4	H_2O		93.8	0.48
	NaF	H_2O		98.1	0.56
	KCl	H_2O		115.5	0.86
	KBr	H_2O		133.8	1.19
	KI	H_2O		173.9	1.90
	NaF	H_2O		101.6	0.62
	KCl	H_2O		119.6	0.94
	KBr	H_2O		133.1	1.18
	KI	H_2O		182.3	2.04
	NaH_2PO_4	D	4.3	93.7	0.48
	Na_2HPO_4	C	9.5	83.8	0.30
	Na_3PO_4	0.1 *N* NaOH	13	73.6	0.12
	NH_4SCN	H_2O		—	—
	$FeCl_3$	0.1 *N* NaCl/HCl	2.8	—	—
	Blue Dextran	H_2O		67.8	0
	α-L-Alanine	0.1 *N* NaOH	13	75.9	0.14
	α-L-Alanine	H_2O		91.2	0.41
	KBr	H_2O		132.1	1.14
	KCl	H_2O		115.1	0.84
	L-Leucine	H_2O		91.8	0.42
	D(+)-Glucose	0.1 *M* NaCl		97.8	0.55

TABLE II

THE ELUTION VOLUMES AND K_D VALUES OF TEST SUBSTANCES FILTERED THROUGH A COLUMN (25 × 1000 mm) OF SEPHADEX G-25 FINE, 20-80 μm

N (Br^-) ~ 4200; HETP (Br^-) ~ 0.24 mm per plate.

Sephadex type; column length; $(V_t)_{eff}$	*Chemical compound*	*Elution buffer*	*pH*	V_e *(ml)*	$K_D = \frac{V_e - V_0}{(V_t)_{eff}}$
G-25; 100 cm; 190.6 ml	Blue Dextran	H_2O		211.3 (V_0)	0
	D(+)-Glucose	H_2O		374.5	0.86
	Na_2SO_4	H_2O		375.3	0.86
	NaH_2PO_4	D	4.36	382.1	0.90
	NaH_2PO_4	D	4.36	388.2	0.93
	Na_2HPO_4	C	9.5	384.1	0.91
	NaF	H_2O		386.6	0.92
	KCl	H_2O		407.4	1.03
	KBr	H_2O		432.7	1.16
	KI	H_2O		455.3	1.28
	NaF	H_2O		391.7	0.95
	KCl	H_2O		419.9	1.09
	KBr	H_2O		438.9	1.19
	KI	H_2O		467.4	1.34

investigated. The cations and anions differ not only in the ionic radii and ionic charge, but also to a great extent in their degree of hydration. The differences in the elution behaviour[20,21] in filtration with highly crosslinked dextran gels due to the different mobilities of the ions in the solvent should disappear only with a smaller degree of crosslinking (from Sephadex G-75 to G-200) or a larger porosity.

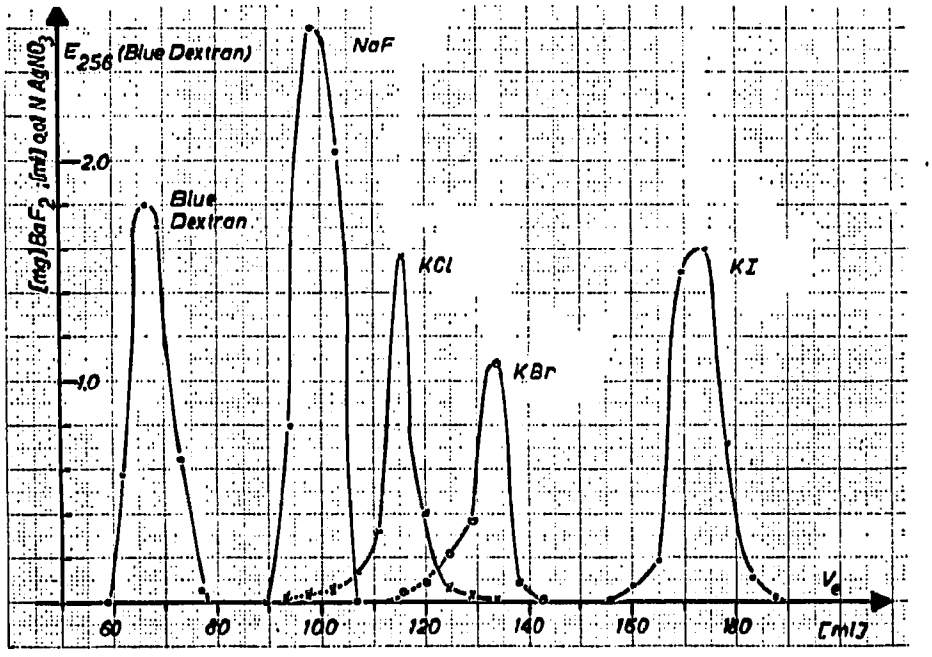

Fig. 2. Elution curves for Blue Dextran 2000 and alkali halides at an elution rate of 14 ml/h. Bed material: Sephadex G-10. Eluant: H_2O.

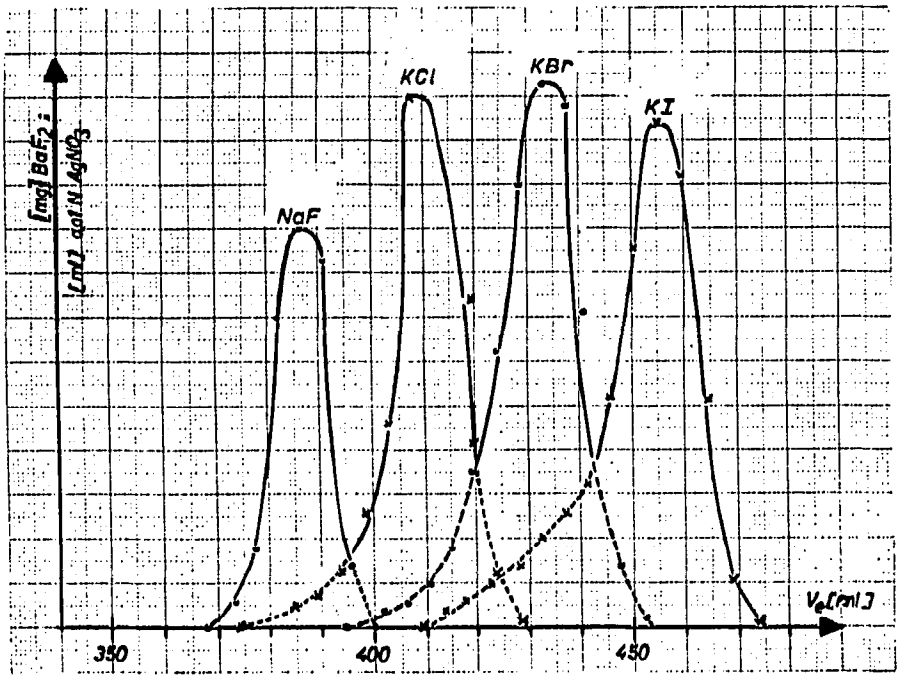

Fig. 3. Elution curves for alkali halides at an elution rate of 18 ml/h. Bed material: Sephadex G-25 fine. Eluant: H_2O.

It is known that the increasing radius of the central ion of halogen anions, which are less hydrated than alkali cations, is compensated to a certain extent by the decreasing hydration. These ionic hydrates should demonstrate similar elution behaviour if there were no other effects. It can be seen that the halides were eluted in our filtration experiments in the sequence F^-, Cl^-, Br^-, I^- (Tables I and II; Figs. 2 and 3). It can be concluded from our investigations that only chloride and bromide ions are able to diffuse completely into the effective internal volume of the highly crosslinked Sephadex gels, but not F^-, I^- or the other ions examined (Figs. 6 and 7). Even the influence of the degree of crosslinking was clearly detectable for all ions. Whereas the F^- ions were diffusing into the available internal volume of Sephadex G-10 at 62 %, in Sephadex G-25 the F^- ions participated in the distribution at about 90% (Tables I and II; Figs. 2 and 3). The I^- ions were unusually retarded (Sephadex G-10, $K_D = 2.04$; Sephadex G-25, $K_D = 1.34$). The untimely elution of F^- and the retardation of I^- cannot be explained without the supposition of a considerable difference in the degree of hydration and an additional interaction of I^- with the gel matrix. The differences in the elution behaviour[20–24] between the alkali ions or the alkaline earth metal ions are a result of the marked decrease in the hydration.

We are of the opinion that the different degrees of hydration of the three anions of orthophosphoric acid: PO_4^{3-} (pH 14.5) $\rightleftharpoons$ HPO_4^{2-} (pH 9.5) $\leftrightharpoons$ $H_2PO_4^-$ (pH 4.5) appears to be the cause of the experimentally found elution sequence at various pH values (Table I; Fig. 4). Whereas the triply charged PO_4^{3-} anion is excluded from the inner phase of Sephadex G-10 at 88% ($K_D = 0.12$) the $H_2PO_4^-$ anion[20,21] of lower valency participated in the distribution into the effective internal volume at about 50% ($K_D = 0.48$). With Sephadex G-25, these differences became blurred and the orthophosphates behaved in a similar manner to the F^- ion

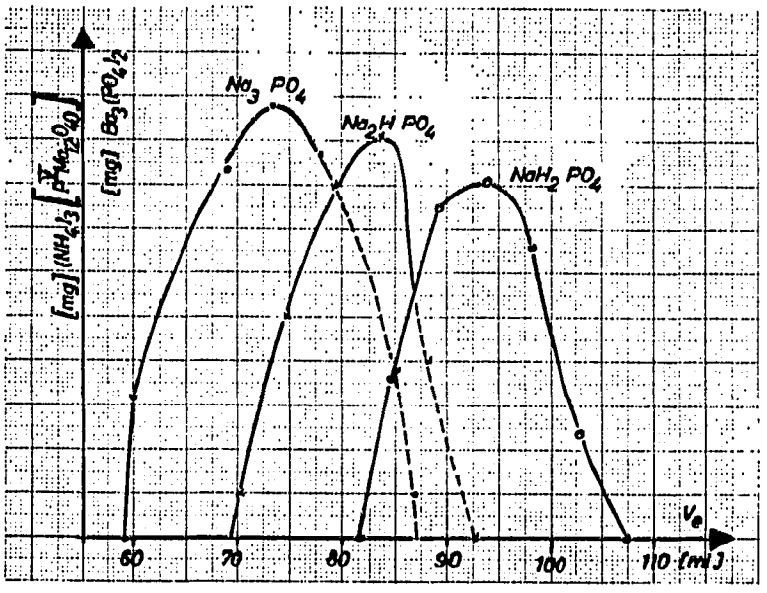

Fig. 4. Elution curves for alkali orthophosphates at different pH values and at an elution rate of 18 ml/h. Bed material: Sephadex G-10. Eluants: 0.1 *M* sodium acetate buffer (pH 4.3); 0.1 *M* ammonium chloride buffer (pH 9.5); 0.1 *N* NaOH (pH 13).

(Table II; Fig. 5). The matrix of the dextran gels, containing some carboxyl groups (0.1–10 μval* per gram of dry gel)[16,25], acts insignificantly as a cation exchanger in ion-free solvents, but this effect disappears completely with eluants with an ionic strength of $\mu > 0.01$. The cations we have examined, which are easily analysed

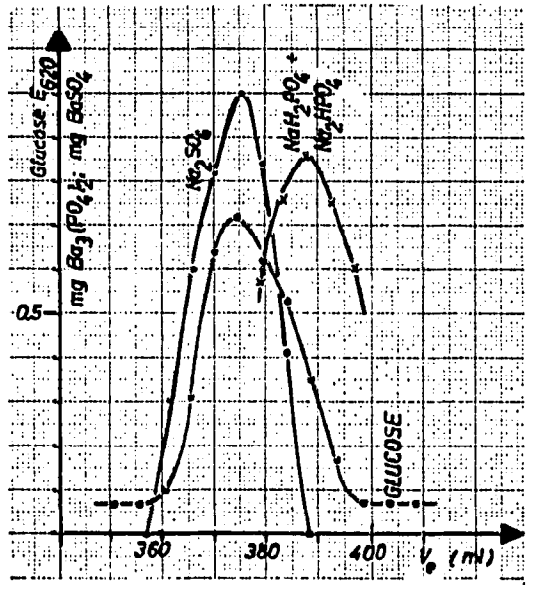

Fig. 5. Elution curves for glucose, sodium sulphate, sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate at an elution rate of 18 ml/h. Bed material: Sephadex G-25 fine. Eluants: twice-distilled water; 0.1 *M* sodium acetate buffer (pH 4.3); 0.1 *M* ammonium chloride buffer (pH 9.5).

by the light absorbance of strongly coloured complexes [$Cu^{2+} \rightarrow [Cu(NH_3)_4]^{2+}$; $Fe^{3+} \rightarrow Fe(SCN)_3$], were not suitable for the determination of $(V_t)_{eff}$, owing to the

* 1 val = 1 equivalent.

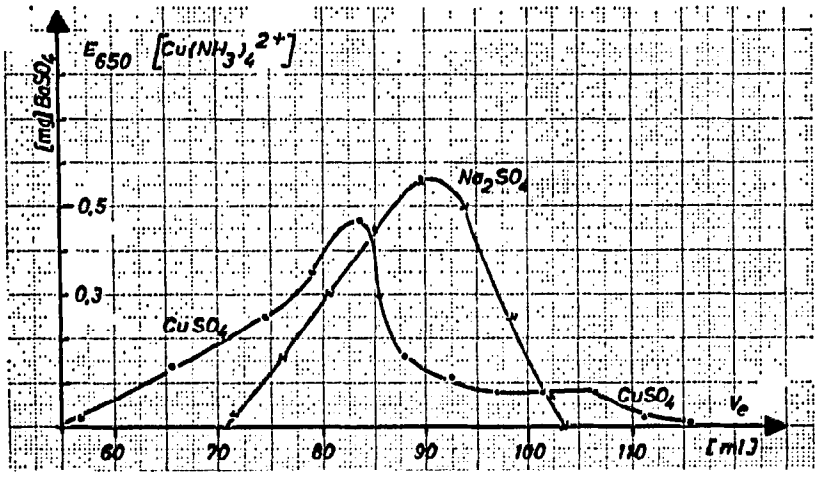

Fig. 6. Elution curves for copper sulphate and sodium sulphate at an elution rate of 18 ml/h. Bed material: Sephadex G-10. Eluants: H_2O; 0.01 *M* citrate buffer (pH 3.5).

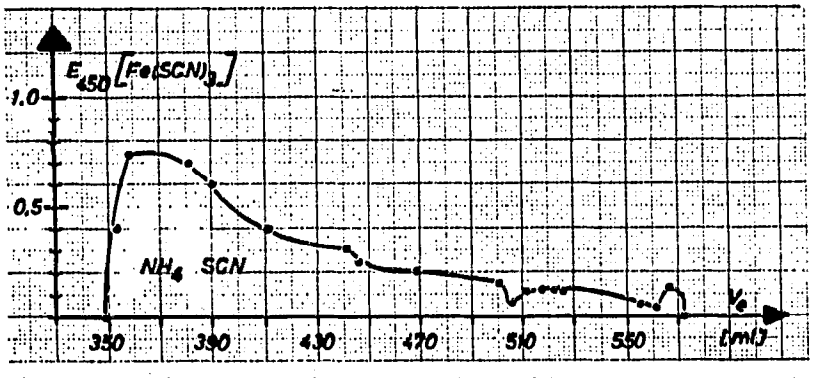

Fig. 7. Elution curve for ammonium thiocyanate at an elution rate of 14 ml/h. Bed material: Sephadex G-10. Eluant: twice-distilled water.

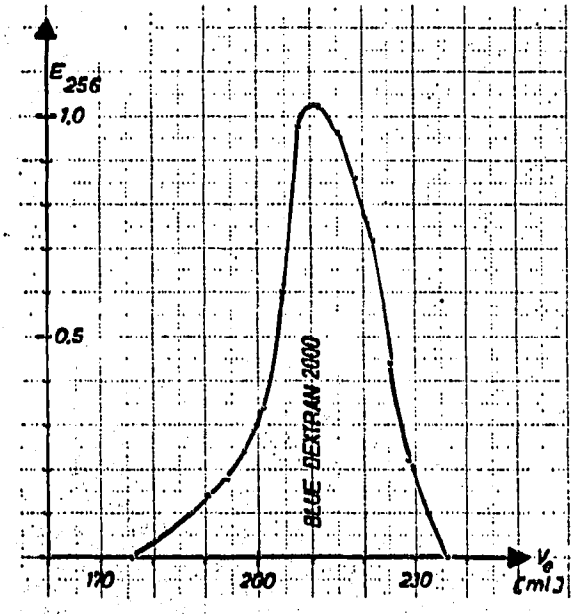

Fig. 8. Elution curve for Blue Dextran 2000 at an elution rate of 14 ml/h. Bed material: Sephadex G-25 fine. Eluant: twice-distilled water.

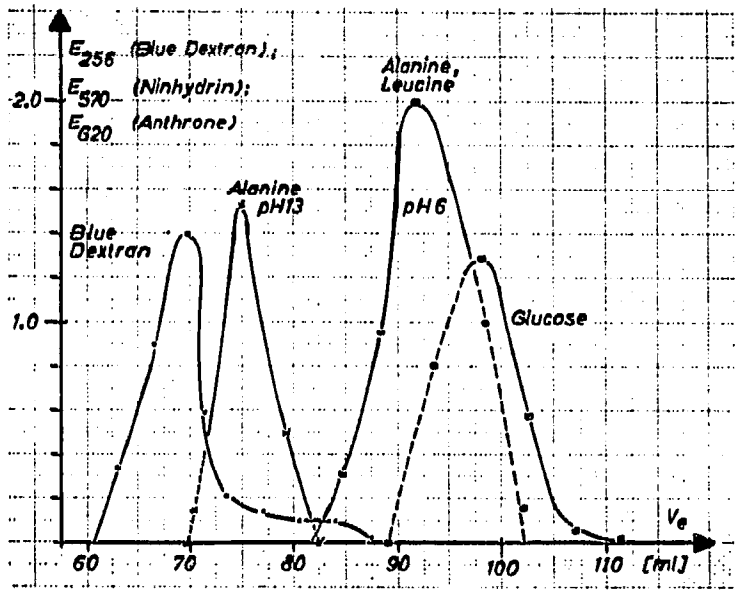

Fig. 9. Elution curves for Blue Dextran 2000, glucose, L-α-alanine and L-leucine at various pH values and at an elution rate of 18 ml/h. Bed material: Sephadex G-10. Eluants: twice-distilled water and 0.1 *N* NaOH.

restricted distribution of copper ions ($K_D = 0.23$) and the almost irreversible adsorption of the Fe^{3+} ions, in spite of eluants having a pH of 2.8 and an ionic strength of $\mu = 0.1$. Even the reversed method, gel filtration of thiocyanate ions[26] and their estimation as $Fe(SCN)_3$, were not successful as a consequence of the interaction of the salt with the gel matrix (Figs. 6 and 7). The estimations of the void volume with Dextran Blue 2000 were not influenced by the different eluants at various pH values (Tables I and II; Figs. 2, 8 and 9).

It can be deduced from our investigations on the determination of the effective internal volume, that the theoretical imbibed volume, $(V_i)_{th}$, is not completely available for the distribution of low-molecular-weight substances. Calculating (see eqn. 3) the effective internal volume from the expression $(V_i)_{eff} = V_t - V_0 - a \cdot V_g$

TABLE III

CALCULATED AND DETERMINED PARAMETERS FOR SEPHADEX G-10 AND SEPHADEX G-25 FINE

Sephadex type; column length	*Amount of dry gel, a (g)*	*Water regain, W_r (ml H_2O/g)*	$(V_i)_{th}$ *(ml)*	$(V_i)_{eff} = 0.8 \cdot (V_i)_{th}$ *(ml)*	V_0 *(ml)*	$(V_i)_{eff} + V_0$ *(ml)*
G-10, 200 cm	72.2	1.0 ± 0.1	72.2 ± 7.2	57.8 (calc. 58.9)	69.8	127.6 (calc. 128.7)
	1.0		1		0.97	
G-10, 200 cm	70.7	1.0 ± 0.1	70.7 ± 7.1	56.6 (calc. 63.0)	66.6	123.2 (calc. 129.6)
	1.0		1		0.94	
G-25, 100 cm	95.3	2.5 ± 0.2	238.2 ± 19.1	190.6 (calc. 208.4)	211.3	401.9 (calc. 419.7)
	1.0		2.5		2.2	

from the results obtained in the experiments (Table III), the following values were obtained with $V_0 = 0.6$ (ref. 13):

Sephadex G-10, gel bed length 200 cm (9 × 1000 mm and 12 × 1000 mm):

(1) $(V_i)_{eff} = 172.0 - 69.8 - 43.3 = 58.9$ ml.
$(V_i)_{eff} + V_0 = 128.7$ ml.
$V_m = 43.3$ ml.

(2) $(V_i)_{eff} = 172.0 - 66.6 - 42.4 = 63.0$ ml.
$(V_i)_{eff} + V_0 = 129.6$ ml.
$V_m = 42.4$ ml.

Sephadex G-25, gel bed length 100 cm (25 × 1000 mm):

$(V_i)_{eff} = 476.9 - 211.3 - 57.2 = 208.4$ ml.
$(V_i)_{eff} + V_0 = 419.7$ ml.
$V_m = 57.2$ ml.

These effective internal volumes, calculated from the total volume, V_t (see MATERIALS AND METHODS), the void volume and the weight of the dry gel were completely in agreement (see Table III) with the calculated effective internal volumes according to eqn. 5. The estimation of the gel matrix volume, $V_m = a \cdot V_g = V_t - V_0 - (V_i)_{th}$, by means of the theoretical imbibed volume, $(V_i)_{th}$, according to eqn. 3 also favours the effective internal volume instead of the theoretical imbibed volume, as V_m is to be calculated with the dry weight of the gel. The following values of the gel matrix volumes would result from the calculations with $(V_i)_{th}$:

Sephadex G-10, gel bed length 200 cm:

(1) $V_m = 172.0 - 69.8 - 72.2 = 30.0$ ml.
(2) $V_m = 172.0 - 66.6 - 70.7 = 34.7$ ml.

Sephadex G-25, gel bed length 100 cm:

$V_m = 476.9 - 211.3 - 238.2 = 27.4$ ml.

These values, however, are about 40% and 100%, respectively, too low!

Glucose and neutral amino acids are not suitable for use in the determination of the effective internal volume of highly crosslinked dextran gels, as shown by our experiments. The K_D values of α-alanine, leucine and glucose were below 0.6 (Sephadex G-10), and glucose with Sephadex G-25 showed the elution behaviour of species such as sulphate anions (Table II and Fig. 5). In strongly alkaline solutions (pH 13), α-alanine was nearly excluded from the available internal volume (Table I and Fig. 9).

Finally, it may be mentioned that the sample concentration, the sample volume[20,21], the charge and the solvation of inorganic ions[24,27–33] influence their elution behaviour and for that reason only a few ions are suitable for use in the estimation of the effective internal volume. In addition to the coincidence of the calculated and the experimental data for the effective internal volumes with Br^- or Cl^-, their easy titrimetric determination with $AgNO_3$ is of great advantage.

ACKNOWLEDGEMENTS

This study was supported by the Assoziation mit Euratom and the Gesellschaft für Strahlen- und Umweltforschung, No. 031-64-1 BIAD.

REFERENCES

1 J. PORATH, *Nature*, 218 (1968) 834.
2 H. J. ZEITLER AND E. STADLER, *Hoppe-Seyler's Z. Physiol. Chem.*, in press.
3 H. J. ZEITLER AND E. STADLER, *J. Chromatogr.*, in preparation.
4 R. BRAUN, *Biochim. Biophys. Acta*, 142 (1967) 267.
5 R. BRAUN, *Biochim. Biophys. Acta*, 149 (1967) 601.
6 TH. HOHN AND W. POLLMANN, *Z. Naturforsch.*, 18b (1963) 919.
7 J. DE BERSACQUES, *J. Chromatogr.*, 31 (1967) 222.
8 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
9 L. W. NICHOL, W. H. SAWYER AND D. J. WINZOR, *Biochem. J.*, 112 (1969) 259.
10 A. POLSON AND W. KATZ, *Biochem. J.*, 112 (1969) 387.
11 T. C. LAURENT AND J. KILLANDER, *J. Chromatogr.*, 14 (1964) 317.
12 R. M. WHEATON AND W. C. BAUMANN, *Ann. N.Y. Acad. Sci.*, 57 (1953) 159.
13 K. H. GRANATH, *J. Colloid. Sci.*, 13 (1958) 308.
14 N. V. B. MARSDEN, *J. Chromatogr.*, 58 (1971) 304.
15 J. M. GOODSON, V. DISTEFANO AND J. C. SMITH, *J. Chromatogr.*, 54 (1971) 43.
16 B. GELOTTE, *J. Chromatogr.*, 3 (1960) 330.
17 E. GLUECKAUF, *Ion Exchange and Its Applications*, presented at *The Conference in William Beveridge Hall, London University, April 5th–7th, 1954*, Society of Chemical Industry, London, 155, pp. 34–48.
18 P. FLODIN, *J. Chromatogr.*, 5 (1961) 103; also in P. FLODIN (Editor), *Dextran Gels and Their Applications in Gel Filtration*' Pharmacia AB, Uppsala, 1962 pp. 39–41.
19 J. L. MULDER AND F. A. BUYTENHUYS, *J. Chromatogr.*, 51 (1970) 459.
20 P. A. NEDDERMEYER AND L. B. ROGERS, *Anal. Chem.*, 40 (1968) 755.
21 P. A. NEDDERMEYER AND L. B. ROGERS, *Anal. Chem.*, 41 (1969) 94.
22 H. ORTNER AND H. SPITZY, *Fresenius' Z. Anal. Chem.*, 238 (1968) 167.
23 H. ORTNER AND H. SPITZY, *Fresenius' Z. Anal. Chem.*, 238 (1968) 251.
24 N. YOZA AND S. OHASHI, *J. Chromatogr.*, 41 (1969) 429.
25 H. DETERMANN, in H. DETERMANN (Editor), *Gelchromatographie*, Springer Verlag, Berlin Heidelberg and New York, 1967, p. 29.
26 H. DETERMANN AND I. WALTER, *Nature*, 219 (1968) 604.
27 J. G. MCKELVEY, JR., K. S. SPIEGLER AND M. R. J. WYLLIE, *Chem. Eng. Prog. Symp. Ser.*, 55 (1959) 199.
28 W. B. SMITH AND A. KOLLMANNSBERGER, *J. Phys. Chem.*, 69 (1965) 4157.
29 L. M. SIEGEL AND K. J. MONTY, *Biochim. Biophys. Acta*, 112 (1966) 346.
30 E. F. CASASSA, *J. Polym. Sci.*, B 5 (1967) 773.
31 Z. GRUBISIC, P. REMPP AND H. BENOIT, *J. Polym. Sci.*, B 5 (1967) 753.
32 B. Z. EGAN, *J. Chromatogr.*, 34 (1968) 382.
33 N. YOZA, T. OGATA, Y. UENO AND S. OHASHI, *Bull. Chem. Soc. Jap.*, 41 (1968) 2550.