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The Mechanisms behind Peak Broadening in a Gel Chromatographic Column

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An equation for the elution profile in a linear chromatographic system derived in a previous paper is tested by recirculation experiments in a column packed with Sephadex G-25 Medium gel beads. As the elution velocity normally varied somewhat during the recirculation runs the details about the form of the elution curves could not be checked, but assuming that the elution profiles are approximately Gaussian it is possible to calculate apparent diffusion coefficients for different species at different elution velocities. Analysis shows that for substances excluded from the gel there exists a common eddy diffusion coefficient increasing in value with elution velocity. For the substances penetrating the gel beads the expression $D_{app}=D_{eddy}/(1+K)$, where K is the equilibrium distribution coefficient of the substance between the stationary and mobile phase, is a valid approximation at low elution velocities in accordance with simple quasi-equilibrium theory. At higher elution velocities there is an additional non-equilibrium contribution to D_{app} from which it is possible to estimate the relaxation time τ of equilibration between mobile and stationary phase. The order of magnitude of the calculated τ-values is controlled by comparison with a well known formula for the diffusion into or out of a sphere maintained in a bath of constant concentration.

In a preceding paper [1] it was shown that the asymptotic solution (large elution times) to the phenomenological equations governing a linear chromatographic system (linear or linearized "adsorption" and "desorption" kinetics) is given by the expression

$$c_{tot}(x',t')=c(o)+c(s)=$$
$$\frac{s}{\sqrt{2\pi}\,\sigma_x}\exp\left(\frac{-x'^2}{2\,\sigma_x^2}\right)\left[\left(1+\frac{A-2B^2}{\sigma_x^2}\right)+\frac{B}{\sigma_x^2}x'-\frac{A-2B^2}{\sigma_x^4}x'^2\right] \quad (1)$$

c_{tot} is the sum of the concentration in the mobile and the stationary phase based upon total column volume in a slice of column centered at the specific position x. The total number of moles in the chromatographic peak is s. The x'-variable is a Galilei-transformed position

$$x'=x-u_{cm}t' \quad (2)$$

The center of mass velocity is given by

$$u_{cm}=[1/(1+K)]u_{max} \quad (3)$$

where u_{max} is the mean velocity of the mobile phase and K is the "adsorption" equilibrium constant given by

$$K=k_1/k_2 \quad (4)$$

k_1 is the number of moles passing from the mobile to the stationary phase per unit of total column volume in the given slice and per unit of the number of moles in mobile phase per cm³ total column volume, *i.e.* per unit of $c(o)$. Analogously k_2 is the rate constant for "desorption". Finally in (1) the parameters A, B and σ_x are given by

$$A=-\tfrac{1}{2}D_{eddy}\frac{k_1-k_2}{(k_1+k_2)^2}+\frac{k_1k_2}{(k_1+k_2)^4}u_{max}^2 \quad (5)$$

$$B=\frac{k_1-k_2}{2(k_1+k_2)^2}u_{max} \quad (6)$$

$$\sigma_x=\sqrt{2\,D_{app}\cdot t'} \quad (7)$$

with the apparent diffusion coefficient

$$D_{app}=\frac{D_{eddy}}{1+K}+\frac{k_1k_2}{(k_1+k_2)^3}u_{max}^2 \quad (8)$$

The time t' corresponds to an initial condition of a Dirac's δ-function positioned at $x=0$

at $t'=0$. As the initial condition in an actual experiment is some irregular, though narrow, distribution we write

$$t'=t+t_0 \tag{9}$$

where the additive constant t_0 relating actual time to "Gaussian" time t' is to be found in each experiment. The parameter D_{eddy} in (8) is an eddy diffusion coefficient describing the spreading out of the zone due to the many possible routes by which the molecules can pass through the mobile phase. It is assumed to be concentration independent, but it may be a function of u_{max}, and if so it is anticipated that it will increase with u_{max}.

Eqn. (8) has previously been derived by Bak [2] in a study primarily dealing with the electrodiffusion phenomena. It can be shown too that the random walk theory of Giddings and Eyring [3,4] yields a solution with the same center of mass velocity and a D_{app} equal to the second term in (8) when the solution is power expanded to the second degree in the argument of the exponential function to yield a Gaussian distribution.[1,5] However, their calculations of k_1 and k_2 from experimental elution curves are in error, because of their neglect of eddy diffusion.[1] Several authors have used integral transformations with respect to *time* [eqn. (1) is obtained by complex Fourier transformation with respect to position] to obtain solutions to the chromatographic transport equations, *e.g.* Lapidus and Amundson,[6] Kučera,[7] and Kubin [8] (Laplace-Carson transform). The solutions given by these authors are very complicated, however, and not immediately suited for practical applications. Van Deemter *et al.*[9] have made a Gaussian approximation to the solution of Lapidus and Amundson, and their formulae may be translated to our equations (3), (7), and (8) after a rather long series of tedious transformations. The latter authors have checked the formulae on experimental elution curves from ion exclusion and gas-liquid partition chromatographic columns. The arguments in the present article are strengthened by the use of recirculation chromatography. Also, it seems to be the first time that peak broadening has been investigated in gel chromatographic columns by appropriate methods. In this connection it should be mentioned that the formulae derived by Ackers [10] are not sufficient to describe gel chromatography at higher flow rates, because the transport equations used by him are based upon quasi-equilibrium and do not take into account the additional contribution of peak broadening from the finite rate of mass transfer between the stationary and the mobile phase. Vink [11] has solved the chromatographic transport equations proposed by himself by the method of moments. His expression for u_{cm} is identical to eqn. (3), and for the second moment he gets a result similar – but not quite identical – to eqn. (7) and (8). However, his transport equation contains a rather unrealistic assumption on longitudinal diffusion in the stationary phase besides the exchange out of and into the gel beads. The most serious mistake in his equations is that he puts $D_{eddy}/(1+K)$ in eqn. (8) equal to a constant (D_1) which is independent of the partition coefficient K. Thus, he does not correct for the fact that molecules in the stationary phase do not contribute to eddy diffusion. Of course, this assumption has most serious consequences for his theory of determination of molecular weight distributions by gel chromatography.[12] The theoretical conclusions of the previous article [1] and the experimental results in the present are not in accordance with Vink's assumption.

The purpose of the present is to test eqs. (1), (3), (7), and (8) as far as possible for a gel chromatographic column. Seven substances with K-values from 0 to 5.1 were subjected to recirculation chromatography in a column packed with beads of cross-linked dextran (Sephadex). Values of u_{max} ranging from 0.75 to 4.7 cm/min were applied. The spread in parameters is sufficient to sort out approximately the u_{max} dependence of D_{eddy} from the contribution from the second term in (8). Values for the relaxation time of equilibration between stationary and mobile phase

$$\tau=(k_1+k_2)^{-1} \tag{10}$$

can therefore be calculated and because the separating principle in gel-chromatography is diffusion into and out of spherical beads with known upper and lower limits for the radius, it is even possible to check the order of magnitude of these relaxation times.

However, the nomenclature used in the literature concerning gel chromatography appears to be somewhat confusing. Therefore it was found appropriate to start by covering the definitions and symbols used here in order to make the present paper self-consistent.

DEFINITIONS AND INTERRELATIONS IN GEL CHROMATOGRAPHY

In Fig. 1 a schematic subdivision of the inner space of a gelchromatographic column is shown. The primed concentrations refer to that section of the volume in which the molecule is positioned. Thus $c_k'(i)_{x,t}$ is the concentration of substance k at a position x and time t in mol/cm³ of solvent imbibed in the gel. The primed concentrations should be carefully distinguished from the unprimed concentrations based upon total column volume. We define a "molecular sieving" equilibrium constant by

$$K_{ms} = c'(i)/c'(o) \tag{11}$$

In the pure molecular sieve case (without any differences in activity coefficients in V_i and V_0) K_{ms} ranges from 0 for big molecules which cannot enter the network of crosslinked polymer chains to 1 for small molecules which can move freely everywhere in V_i. Many substances show a specific adsorption to the polymer chains in the gel, and we therefore define an adsorption coefficient

$$K_{ads} = c'(g)/c'(i) \tag{12}$$

The equilibrium constant K defined in eqn. (4) can also be expressed as

$$K = \frac{c(i)+c(g)}{c(o)} = \frac{V_i + V_g K_{ads}}{V_0} K_{ms} \tag{13}$$

It is still possible to maintain the picture of linear "adsorption" and "desorption" kinetic given in the introduction, if the diffusion process in the gel beads can be properly linearized and the adsorption process is rapid in comparison to the diffusion process. It should be carefully noted that the concentrations used in (11), (12), and (13) are the *equilibrium concentrations* and not the concentrations at the same x and t in an actual chromatographic experiment. The value of u_{max} may be small enough to make that difference insignificant. In this "quasi-equilibrium" solution to the chromatographic problem, D_{app} is given by the first term in (8) only. The second term, however, reflects the deviation from quasi-equilibrium. In any case it can be shown that in a linear chromatographic system the center of mass velocity is given by (3), whether or not quasi-equilibrium can be used as an approximation. For a detailed discussion of these subjects the reader is refered to the preceding theoretical paper.[1] Combination of (3) and (13) yields

$$u_{max}/u_{cm} = 1 + K_d V_i/V_0 \tag{14}$$

with the *definition*

$$K_d = K_{ms}(1 + V_g K_{ads}/V_i) \tag{15}$$

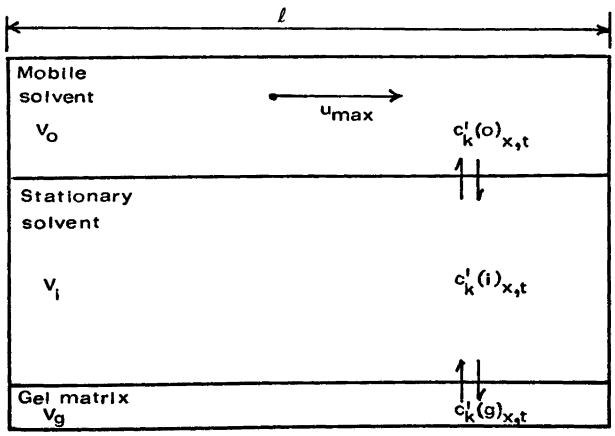

Fig. 1. Schematic subdivision of a gelchromatographic column. Total volume $V_t = V_0 + V_i + V_g$. Cross-sectional area A.

In many gelchromatographic experiments u_{max} and u_{cm} will not be constant in time because of fluctuations or systematic variations. In such cases it is much more practical to measure *elution volumes* than velocities. To prove this we note that the differential increment of the elution volume when the peak advances the distance dx_{cm} in the column is

$$dV_e/dx_{cm} = (dV_e/dt)u_{cm}^{-1} = (V_0/V_t)A(u_{max}/u_{cm})$$

wherein A is the area of column cross section. The instantaneous value of the ratio u_{max}/u_{cm} is still given by (14) and is consequently time independent. By integration through the column

$$V_e = \int_0^l (dV_e/dx_{cm})dx_{cm} = V_0 + K_d V_i \qquad (16)$$

In practice it is indeed found that the elution volumes are independent or nearly independent of elution velocity showing that a gelchromatographic system in this respect behaves as a linear chromatographic system. This fact has been of immense value for the practical utilisation of the technique, and equations based upon elution volumes such as (16) first stated by Wheaten and Baumann [13] pervade the literature about the subject. The present work will make it clear, however, that if the phenomena of peak broadening shall be properly described in order to assess the resolution power of the column, it is *not* sufficient with an elution volume description.

Still another distribution coefficient has been widely used in the literature. It is defined as the ratio between the equilibrium concentration in the gel based upon the volume $V_i + V_g$ and the equilibrium concentration in the mobile phase based upon the volume V_0:

$$K_{av} = \frac{c(i)(V_t/V_i + V_g) + c(g)(V_t/V_i + V_g)}{c(o)(V_t/V_0)} = \frac{V_0}{V_i + V_g} K \qquad (17)$$

The importance of that equilibrium constant rests upon the possibility of a direct theoretical calculation within the framework of statistical mechanics as a ratio between two molecular configuration integrals as has been done in a number of cases, *e.g.* by Giddings *et al.*[14] Explicitly

$$K_{av} = \frac{\int dr \int d\psi \int d\lambda \exp(-\varepsilon_{i+g}/kT)}{\int dr \int d\psi \int d\lambda \exp(-\varepsilon_0/kT)} \qquad (18)$$

wherein r denotes the position of the center of mass of the molecule, ψ the rotation coordinates and λ the possible internal conformation coordinates. The energies ε_{i+g} and ε_0 are the molecular energies of a given configuration in $V_i + V_g$ and V_0, respectively, and the r integration is taken over the same volumes. Typical molecular sieving problems focus upon "on-off" potentials, *i.e.* $\varepsilon_{i+g} = 0$ or ∞, For example Giddings *et al.* were able to derive as a special case the Ogston expression [15] for spherical molecules in a network of randomly oriented fibers

$$K_{av} = \exp[-\pi L(r + r_0)^2] \qquad (19)$$

where L is the total length of fibers per cm^3, r is the molecular radius and r_0 is the radius of the gel-fibers. As well known this equation has been successfully applicated by Laurent and Killander [16] to describe the elution of proteins from Sephadex gels using Stokes radii (from diffusion coefficients) as r.

Also the distribution coefficients K and K_{av} can, of course, be calculated as simple functions of the elution volume as well as K_d. Comparison of eqns. (13) and (15) yields

$$K = (V_i/V_0)K_d \qquad (20)$$

Insertion of (16) in this equation yields

$$K = (V_e - V_0)/V_0 \qquad (21)$$

Application of (21) to (17) yields finally

$$K_{av} = (V_e - V_0)/(V_i + V_g) = (V_e - V_0)/(V_t - V_0) \qquad (22)$$

Eqn. (16) and derived equations are based upon the assumption that the imbibed solvent in the gel does not move, *i.e.* no solvent is forced through the gel beads by the pressure drop in the column. If this assumption were not correct it would show up in variations in the elution volume of excluded molecules with the rate of elution.

EXPERIMENTAL

Materials. The substances used were: DL-tryptophan, L-tyrosine (BDH Chemicals, chromatographically homogeneous), *N*-acetyl-L-tryptophan methyl ester (Mann Res. Lab., chrom. hom.), myoglobin (Seravac, Horse skeletal muscle, 1 × cryst), ovalbumin (Sigma Chem. Comp., cryst. and lyophil., salt free,

electrophoretic purity 99 %), methylene blue (3,9-bisdimethylamino-phenazothionium chloride, Merck) and "Blue Dextran 2000" (Pharmacia). The last substance is dextran with an average molecular weight $M_n \sim 2 \times 10^6$ bound to a polycyclic chromophor. The gel used was Sephadex G-25 Medium (Pharmacia) swollen in 0.1 M KCl. This gel allows for relatively wide variations in elution rate without crack-formation in the gel bed at the high velocities. The eluent was 0.1 M KCl throughout to reduce ionic association of the substances (especially the amino acids and methylene blue) with the gel.

Apparatus. The column used was manufactured in the laboratory workshop of a perspex tube with two adjustable perspex plungers with filter caps for the inlet and outlet of eluent introduced into each column end and tightened against the inside surface of the tube by two O-rings each. The construction follows very nearly the one used in commercial available columns, *e.g.* the type supplied by LKB-Produkter AB (Stockholm-Bromma, Sweden). The bed was packed by decanting small portions of a well-stirred slurry of Sephadex G-25 in 0.1 M KCl into the column. After the total amount of gel had been transferred to the column the gel bed was slightly compressed by the upper plunger to prevent crack formation at the high elution velocities. The final data for the gel bed are the following: length = 30.5 ± 0.2 cm, diameter = 3.19 ± 0.01 cm, volume $V_t = 243.6 \pm 1.8$ cm^3, amount of dry gel = 55.0 ± 0.1 g.

The metering pump was a peristaltic pump "Minimet metering pump, type B" (Protech). By means of a "Recychrom, selector valve, type 4911 B" from LKB the chromatographic system was able to function in two ways. In the "open" state the sample was sucked into a polythene tube leading to the metering pump and from there to the bottom of the column. From the top of the column the eluent was let out of the system in the same rate as sample is introduced. In state "closed" the eluent was lead back to the pump and once more to the bottom of the column so that the sample might be recirculated as many times as desired. The external tubing was made as short as possible. The volume in the external circuit was estimated to be less than 2 % of the column volume. Most of the external volume was located in the tube in the peristaltic pump.

To make possible a registration of the elution profile a spectrophotometer "Uvicord" from LKB was introduced between the top of the column and the selector valve. The light path is 0.3 mm and the wavelength used 280 nm. The supplied signal in percent transmission was converted to absorbance in the Servogor recorder.

Recirculation experiments. The room temperature was 25°C within 1 – 2 °C. The elution rates were varied between 3 ml/min to 11 ml/min. The sample volumes from 1.2 ml to 7.8 ml. The samples were 1 wt % in 0.1 M KCl in the case of ovalbumin, myoglobin, and methylene blue (exp. 7), 10 wt % in the case of exp. 8 (methylene blue) and saturated solutions in the other cases. The gel bed was tested for homogeneity by the direct observation of a zone of blue dextran. During the first passage of the column the zone was irregular, but at the end of the second passage it had become a regular horizontal ring. The center of mass of the zone was moving upwards in the column with a uniform velocity.

RESULTS AND DISCUSSION

Treatment of elution peaks. Fig. 2 shows the outcome of a recirculation experiment with DL-tryptophan. It is noticed that the time difference between the maxima is not quite constant (*cf.* Table 1). It was not possible with the equipment used to maintain a strictly constant flow in the experiments of long duration. Therefore we cannot expect to test eqn. 1 in the most rigorous way in all details and we have to do some approximations. It is seen that even if we assume the instantaneous profile to be Gaussian ($A = B = 0$), the elution curve – *i.e.* c_{tot} as a function of t at constant x given also by (1) – is not a Gaussian curve

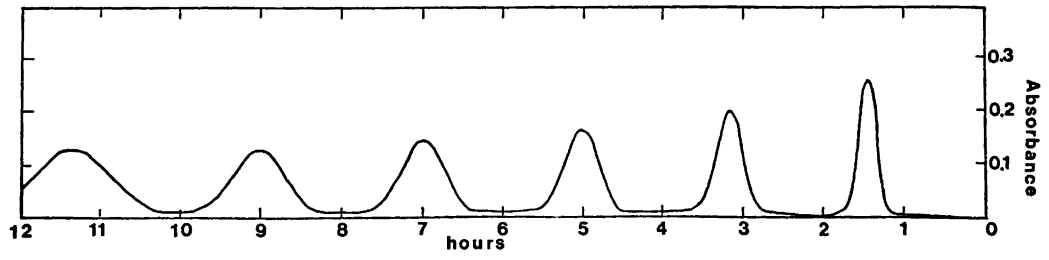

Fig. 2. Absorbance *vs.* time in a recirculation experiment.

Table 1. Analysis of a recirculation experiment. Exp. 18. D,L-Tryptophan. 3.00 ml sat. sol.

Peak No.	t_{max} min	Δt_{max} min	A_{max} abs	h abs	σ_t min	σ_t(corr) min	s abs min	Elution flux ml/min
1	86	86	0.255	0.319	6.50	8.16	5.38	4.03
2	190	104	0.200	0.227	11.0	11.5	5.38	
3	300	110	0.162	0.192	13.5	13.3	5.27	
4	420	120	0.142	0.170	16.5	14.8	5.20	
5	540	120	0.127	0.147	20.8	18.7	5.70	
Mean		108						

Regressions:
$\sigma_t^2(\text{corr}) = (0.578 \pm 0.076) t_{max} + 11.32$
$t_{max} = (1.65 \pm 0.21) \sigma_t^2(\text{corr}) - 3.69$

V_e (directly measured between the two last recorded peaks $= 338.5 \pm 5$ ml
$u_{cm} = 0.282$ cm/min
$K = (V_e - V_0)/V_0 = 2.47 \pm 0.06$
($V_0 = 97.5 \pm 0.8$ ml)
$u_{max} = (1+K) u_{cm} = 0.979$ cm/min

because of the time-dependence of σ_x (*cf.* eqn. 7). As the instantaneous profile broadens and becomes more flat during elution, the ascending limb of the elution curve is steeper than the descending limb. This is observed in Fig. 2 and was indeed observed for all the elution curves observed in this work. If we want to approximate the elution curve by a Gaussian distribution, however, we can do this by substituting for t' in σ_x in the Gaussian x-distribution a suitable mean time, *e.g.* the time of elution maximum. The t-distribution is now given by the Gaussian distribution

$$c_{tot}(l,t') \cong \frac{s}{\sqrt{2\pi}\sigma_t} \exp(-\{t' - t_{max}'\}^2/2\sigma_t^2) \qquad (23)$$

with the root mean square of time of arrival given by

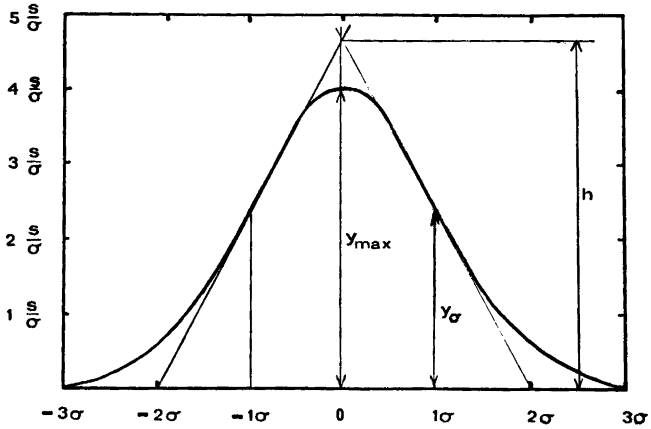

Fig. 3. Geometrical relations in a Gaussian peak with areas s and standard deviation σ.

$h = (2/\pi e)^{\frac{1}{2}}(s/\sigma) \simeq 0.484 s/\sigma$
$y_{max} = [1/(2\pi)^{\frac{1}{2}}](s/\sigma) \simeq 0.399 s/\sigma$
$y_\sigma = [1/(2\pi e)^{\frac{1}{2}}](s/\sigma) \simeq 0.242 s/\sigma$
Area of triangle $= (8/\pi e)^{\frac{1}{2}} s \cong 0.968 s$

$$\sigma_t \cong \sqrt{2D_{app}t_{max}}\,\frac{1+K}{u_{max}} \qquad (24)$$

In Fig. 3 some simple geometry of the Gaussian distribution is shown. It is seen that the turning tangents are well determined and the distance between their intersection with the base-line is simply 4σ. We determine σ_t by this method from the elution peaks and it is seen that this method corrects somewhat for the asymmetry. Eqn. (24) may be written

$$\sigma_t^2 = 2D_{app}u_{cm}^{-2}(t_{max}+t_0) \qquad (24a)$$

so that t_0 and D_{app} can be determined by linear regression of σ_t^2 upon t_{max}. The following factors are responsible for the uncertainty in the determination of these parameters:

(1) The elution curves are not Gaussian. The applied geometrical method corrects roughly for the asymmetry.

(2) Beer's law is not exactly fulfilled. It should be mentioned that the band of the "Uvicord" at 280 nm is rather wide ($\pm$ 10 nm).

(3) The elution velocity is not constant; especially not within experiments of long duration (small and binding molecules). A suitable mean value for u_{cm} is found as the ratio between column length and the mean value of time difference between subsequent elution maxima, in a single recirculation experiment. By means of the K-values determined from the elution volumes (eqn. 21) we find simultaneously a mean value for u_{max} in the recirculation experiment.

(4) The first peak(s) may still be somewhat irregular, and the maximal absorbances may fall outside the domain of Beer's law. The last peaks show an increasing amount of overlap and the background concentration in the column will eventually become too high for the geometrical determination according to Fig. 3 to be permissible. Furthermore the uncertainty in the determination of $4\sigma_t$ in all cases becomes very great when the peak is very flat.

A total of 109 peaks distributed over 21 experiments has been investigated. Table 1 shows the analysis of the experiment shown in Fig. 2. The values of σ_t are corrected for systematic variation in elution velociy by multiplying with $(\Delta t)_{mean}/(\Delta t)_{preceding}$. The area of the peaks is calculated as $2.066h\sigma_t(corr)$ according to Fig. 3 as a control. Preliminary plots were made to determine the number of peaks which should be included in the regression. The number of subsequent peaks showing good linearity was mostly between 5 and 7. In experiments with less than 5 peaks (3 and 4 peaks) all peaks are included. Fig. 4 shows the

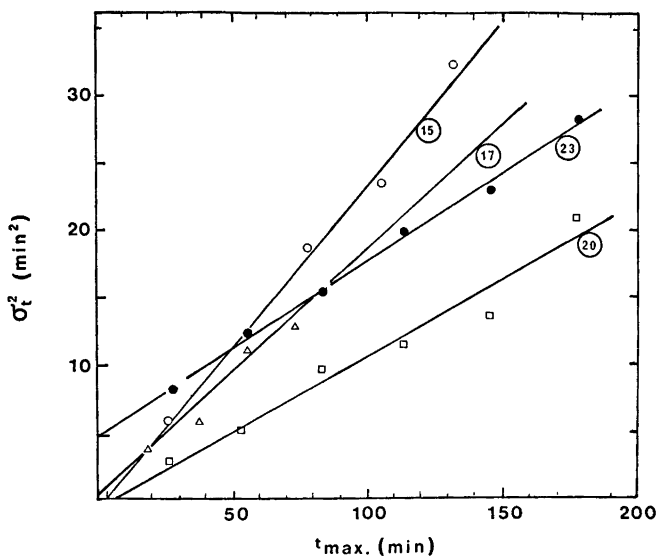

Fig. 4. Plots for determination D_{app}. ○ exp. 15; △ exp. 17; □ exp. 20; ● exp. 23.

σ_t^2(corr) *vs.* t_{max} plots in a number of experiments. It is difficult to judge *a priori* whether the conditions for the correctness of a regression of σ_t^2 upon t_{max} are fulfilled or not. Of course the uncertainty of the measured t_{max} relative to the span in t_{max}-values is much smaller than the corresponding relative uncertainty in σ_t^2, but the "effective" uncertainty in t_{max} must be greater because u_{cm} is not a constant throughout the experiments. Therefore, the regression of t_{max} upon σ_t^2 was also calculated. The straight lines shown in Fig. 4 are the mean of these two regressions. The estimate of the standard deviation of the slope calculated from the regression of σ_t^2 upon t_{max} showed up to be greater than the difference between the slope and reciprocal slope of the two regressions, however, so that the former standard deviation can be used as uncertainty.

Calculation of apparent diffusion coefficients. Table 2 shows the outcome of the least squares treatment of the recirculation experiments. The numbering refers to the chronological sequence of experiments. The calculated K-values and mean values of u_{max} are also tabulated. The minimum elution volume V_0 of the three excluded substances (Blue Dextran, myoglobin and ovalbumin) determined at various velocities is 97.5 ± 0.8 ml with an estimate of the standard error of a single determination ± 2 ml. Therefore, no eluent is forced through the gel beads by the pressure drop. The following conclusion can be drawn from an inspection of Table 2:

(1) The K-values of a single substance do not vary significantly with the elution velocity. Thus, in this respect the system behaves as a linear chromatographic system.

(2) There is no significant difference between

Table 2. Summary of recirculation experiments. BD = blue dextran, Tryp = DL-tryptophan, Ov = ovalbumin, Myo = myoglobin, Me = methylene blue, Tyr = L-tyrosine, and ATM = *N*-acetyl-L-tryptophan methyl ester.

Subst.	Exp.	u_{max}	K	$2D_{app}/u_{cm}^2$	t_0	$10^3 D_{app}$	$10^3 D_{app}(1+K)$	$10^3 D_{term}$
		cm/min		min	min	cm²/min	cm²/min	cm²/min
Elution velocity: medium								
BD	2	1.72	0	0.040±.006	−8 ±2	5.9 ±0.9	5.9±0.9	
Tryp	3	1.58	(2.54±.07)	0.35±.04	−22 ±2	3.5 ±0.4	12.3±1.4	0.0395 [a]
Tryp	4	1.32	(2.54±.07)	0.41 ±.04	−2 ±2	2.8 ±0.3	10.1±1.0	0.0395
Ov	5	1.85	0	0.037±.004	+5 ±2	6.3 ±0.7	6.3±0.7	0.00468 [b]
Myo	6	2.03	0	0.031±.003	−5 ±2	6.4 ±0.6	6.4±0.6	0.00678 [b]
Me	7	2.31	(5.11±.06)	0.61 ±0.4	+43.8±.5	4.4 ±0.3	26.6±1.8	
Me	8	2.46	5.11±.06	0.80 ±.12	+30 ±5	6.5 ±1.0	39.5±6.0	
Tyr	9	2.26	1.72±.09	0.32 ±.07	−9 ±2	11.0 ±2.4	30.0±6.5	
ATM	10	2.30	2.70±.03	0.36 ±.02	−0.9 ±.8	7.0 ±0.4	25.8±1.5	
Elution velocity: high								
Myo	11	4.55	0	0.0247±.0014	−3.2 ±.1	25.5 ±1.5	25.5±1.5	0.00678
Ov	13	4.48	0	0.0244±.0012	+4.8±.1	24.5 ±1	24.5±1	0.00468
Myo	14	4.42	0	0.0268±.0014	+4.2 ±.1	26.0 ±1.5	26.0±1.5	0.00678
Tryp	15	4.15	2.61±.06	0.244±.012	−3.3 ±.3	16.1 ±0.8	58 ±3	0.0395
ATM	16	4.20	2.53±.06	0.26 ±.08	−3 ±6	18 ±6	65 ±20	
Tyr	17	4.66	1.81±.05	0.184±.016	+1.5 ±2.3	25.5 ±2	71.5±6.5	
Elution velocity: low								
Tryp	18	0.979	2.47±.06	0.59 ±.08	+12 ±8	2.3 ±.3	8.1±1.1	0.0395
ATM	19	0.841	2.50±.06	0.62 ±.21	+86.6±.7	1.8 ±.6	6.3±2.1	
Myo	20	1.03	0	0.114±.011	−6 ±2	6.0 ±.6	6.0±0.6	0.00678
Tyr	21	0.750	1.63±.05	0.48 ±.09	+53 ±11	1.95±.35	5.1±1.0	
Ov	22	1.05	0	0.138±.009	−19.5±1	7.6 ±.5	7.6±0.5	0.00468
BD	23	1.03	0	0.130±.004	+36.4±.4	6.9 ±.2	6.9±0.2	

[a] 25 °C Ref. 17. [b] 20 °C Ref. 18.

the K-values of DL-tryptophan and N-acetyl-L-tryptophan methyl ester.

(3) As $V_0 = 97.5$ ml and $V_i = W_r \times$ (gram dry gel) $= 2.5 \times 55 = 137$ ml, wherein W_r is the water regain (ml water taken up per g dry gel) given, *e.g.*, in Ref. 19, p. 28, K-values greater than $V_i/V_0 = 1.41$ correspond to K_d-values greater than 1 according to eqn. (20). All the small molecules investigated here are therefore adsorbed to the dextran chains. If the molecular sieving distribution coefficient is assumed to be 1, we can calculate K_{ads} from eqn. (15). For example we have for DL-tryptophan $K_{ads} V_g/V_i = 0.80$ and with a dry density of the gel 1.64 g/ml [19] we find $K_{ads} = 3.28$.

(4) The apparent diffusion coefficients bear no relation to the ordinary coefficients of diffusion. They are 10^2 to 10^3 times as great.

(5) There is no significant difference between the values of D_{app} for the experiments 2, 5, 6, 20, 22, and 23 corresponding to substances with $K = 0$ at low and medium elution velocities (approximately 1 – 2 cm/min). Thus, at these velocities we have a constant eddy diffusion coefficient independent of molecular size and flexibility (Blue Dextran, myoglobin, ovalbumin). This eddy diffusion apparently is caused by "trajectory splittings" in V_0 of macroscopic dimensions compared with the dimensions of the macromolecules. The mean value of D_{eddy} is 6.5×10^{-2} cm²/min with a standard deviation of the single values ± 0.8 cm²/min. Furthermore it is seen that there is no significant difference between the D_{app} values for the experiments 11, 13, and 14, *i.e.* $K = 0$ and high elution velocities. But here the common D_{eddy} has increased to a value of 25.3×10^{-2} cm²/min with an uncertainty $\pm 0.8 \times 10^{-2}$ for a single determination.

(6) By comparison of the columns D_{app} and $D_{app}(1+K)$ for the group "low elution velocity", it appears that the variation within the latter (relative to the errors) are considerable smaller than within the former. All the values of $D_{app}(1+K)$ fall within the interval determined by the found mean value of D_{eddy} 6.5×10^{-2} cm²/min $\pm$ two times the estimate of the standard deviation of a single determination of D_{eddy} (0.8 cm²/min). Thus, quasi-equilibrium is a reasonable approximation in the estimation of peak broadening at low elution velocities.

(7) In the groups "medium" and "high elution velocity" it is apparent that the values of $D_{app}(1+K)$ for the species entering the gel are significantly *higher* than for the species with $K = 0$. Furthermore it is seen that the values for *different* species deviate significantly from each other and that the values for the *same* species at *different velocities* differ significantly too in such a way that the deviation from quasi-equilibrium is larger for larger velocities.

Calculation of relaxation times. The last observation makes natural the assumption that the deviation from quasi-equilibrium is due to the second term in eqn. (8). For the purpose of analysis we rewrite this expression in the following way:

$$(1+K)D_{app} = D_{eddy} + \Delta \qquad (8a)$$

Table 3. Calculation of relaxation times of diffusion equilibration.

Exp.	Substance	$D_{eddy} \times 10^2$ cm²/min	$\Delta \times 10^2$ cm²/min	$(1+K/K)u_{max}^{-2}$ (min/cm)²	$\tau \times 10^2$ min
3	DL-Tryptophan		5.8 ± 1.6	0.558	3.2 ± 0.9
4	DL-Tryptophan		3.6 ± 1.3	0.800	2.9 ± 1.0
7	Methylene blue		20 ± 2	0.224	4.5 ± 0.5
8	Methylene blue	6.5 ± 0.8	33 ± 6	0.1976	6.5 ± 1.2
9	L-Tyrosine		23.5 ± 6.5	0.310	7.5 ± 2
10	*N*-Ac.-L-tryp. methyl ester		19.3 ± 1.7	0.260	5.0 ± 0.4
15	DL-Tryptophan		32.7 ± 3	0.0803	2.6 ± 0.2
16	*N*-Ac.-L-tryp. methyl ester	25.3 ± 0.8	40 ± 20	0.0791	3.2 ± 1.6
17	L-Tyrosine		46 ± 6.5	0.0715	3.3 ± 0.5

$$\Delta = K\tau u_{max}^2/(1+K) \quad (8b)$$

with the relaxation time of equilibration between stationary and mobile phase given by (10).

Table 3 features the calculation of τ from the deviation term Δ. The correspondence of τ's for the experiments 3, 4, and 15 is remarkable in view of the widely differing values of Δ. Also the experiments 7, 8; and 9, 17 have τ-values which are (in relation to the uncertainty of the difference) a little closer to each other than the Δ-values. The uncertainty in Δ in exp. 16 is too large for a reasonable comparison between exp. 10 and 16, but τ in exp. 10 is significantly greater than τ in exp. 3, 4, and 15 in accordance with the lower diffusion coefficient of *N*-acetyl-L-tryptophan methyl ester compared to tryptophan.

Check of the order of magnitude of relaxation times. The large uncertainties of these calculations make it desirable to judge in some other way the correctness of the values found for the relaxation times. Jost cites in his monograph on diffusion [20] the following formula for the time dependence of the mean concentration c in a sphere which at time $t=0$ is surrounded by a reservoir with a constant concentration

$$\frac{c(t)-c_\infty}{c_0-c_\infty} = \frac{6}{\pi^2}\sum_{\nu=1}^{\infty}\frac{1}{\nu^2}\exp\{-\nu^2\pi^2 Dt/r^2\} \quad (25)$$

wherein c_0 is the position independent initial concentration in the sphere, c_∞ is the final concentration in the sphere and D is the diffusion coefficient in the sphere. Apart from a short transient period the solution (25) can be approximated by the first term in the sum

$$\frac{c(t)-c_\infty}{c_0-c_\infty} \cong \frac{6}{\pi^2}\exp\{-t/\tau\} \quad (26)$$

so that the kinetics are linear with a relaxation time of equilibration

$$\tau = r^2/\pi^2 D \quad (27)$$

If this relaxation time is taken as a reasonable approximation to the one defined in (10) it is possible from the values in Table 3 to calculate the diffusion coefficients in the gel beads. Because the distribution of radii in Sephadex G-25 Medium is rather broad (dry radii from 25 to 75 μ [19]) and the relaxation time according to (27) is very sensitive to variations in r, we shall prefer in a single case to make as good an estimation on the diffusion coefficient in the gel beads as possible. Then from (27) and the determined values of τ we calculate an "effective" radius which should have a value within the "wet radius" interval $45-130\mu$ ($W_r=2.5$ g H_2O/g dry gel and density 1.64 g/ml dry gel [19]). For tryptophan Longsworth [17] has determined the diffusion coefficient in crosslinked dextran gel beads ($W_r=3$, 4″) swelled in water for a number of substances (monovalent alifatic alcohols, acetamid, urea, thiourea, glycerol, and tritiated water). They used (for an isolated bead) the formula (26) in the limit $t \to \infty$. At 25 °C the ratio between these diffusion coefficients and the free diffusion coefficients was found to lie in the interval 0.59 to 0.73. Assuming that none of the above substances are absorbed significantly to the dextran chains we may put this ratio equal to about 2/3 and ascribe it to the combined effect of the reduced cross sectional area and a tortuosity factor both due to the presence of the gel chains. The latter is an expression of the mean value of $\cos^2\phi$ with ϕ being the angle between the mean direction of movement in a small (but macroscopic) volume of V_i and the overall driving force (for pure diffusion the negative overall concentration gradient). The investigated gel does not differ very much in degree of crosslinking from Sephadex G-25, and we assume a factor 2/3 in G-25 too. We have seen, however, that the aromatic small molecules used here have a specific adsorption to the gel matrix. If the adsorption-desorption exchange is rapid compared to the diffusion process, the diffusion coefficient will simply be lowered by a factor $(1+K_{ads}')$ where the modified adsorption constant $K_{ads}' = K_{ads}(V_g/V_i)$ is the ratio between the concentrations of bound and free component on the basis of the volume V_i+V_g. For tryptophan we have found in the present case (*cf.* point 3 above) $K_{ads}'=0.80$, and we have the final estimation of the diffusion coefficient in a G-25 gel bead

$$D=(2/3)\times 3.95\times 10^{-4}/1.80 = 1.46\times 10^{-4}\ \mathrm{cm^2/min}$$

From Table 3 it appears that τ is approximately 3×10^{-2} min, and we find from eqn. (27)

$$r_{eff} = 66\mu$$

a value well within the wet radius interval of Sephadex G-25.

The resolution power of a gelchromatographic column. By following the preceding train of arguments in reverse direction it is now possible to describe a procedure for estimation of the separation power of some given substances in a given column:

(1) Determine the *eddy diffusion characteristic* of the column, *i.e.* the eddy diffusion coefficients of excluded substances as a function of u_{max}. The best thing is to make recirculation experiments as done here, but if this is not feasible the initial zone should be made as possible and the correction time t_0 put equal to zero as a crude approximation.

(2) Estimate the K-values from literature calibration curves, Ogston-Laurent-Killander theory or another adequate theory.

(3) Calculate τ from (27) with the mean radius of the gel beads and some estimate of the diffusion coefficient in the gel inserted.

(4) Calculate the apparent diffusion coefficient at a given u_{max} from eqns. (8a – b).

(5) Estimate the time corresponding to elution maximum by $t_{max} \sim l(1+K)/u_{max}$.

(6) Calculate the root mean square time of arrival to the column end σ_t from eqn. (24) with $t_0 \sim 0$.

(7) Comparison of the difference between t_{max} with $4\sigma_t$ for two substances indicates whether the column will separate these or not.

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