PEAK BROADENING IN PAPER CHROMATOGRAPHY AND RELATED TECHNIQUES.

III. PEAK BROADENING IN THIN-LAYER CHROMATOGRAPHY ON CELLULOSE POWDER*

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SUMMARY

The mechanism of peak broadening in thin-layer chromatography on cellulose powder was investigated by comparing the peak widths obtained in chromatography with those caused only by diffusion in the cellulose powder, for a set of amino acids of widely differing R_F values and six kinds of cellulose powder.

The results are interpreted in terms of longitudinal diffusion in the mobile and the stationary phase and resistance to mass transfer in the mobile phase.

INTRODUCTION

The phenomenon of peak broadening has been amply studied for gas–liquid chromatography (GLC), but is still almost unexplored for other branches of chromatography. The main features of peak broadening in GLC can be described by the VAN DEEMTER equation[1,2]:

$$H = \frac{\sigma^2}{l} = A + \frac{B}{u} + C_M u + C_S u \qquad (1)$$

where:

H = height equivalent to a theoretical plate

σ = standard deviation of the solute distribution in the chromatography column

l = distance travelled by the solute

u = flow rate of the eluent

A, B, C_M, C_S = constants, accounting for peak broadening by eddy diffusion, molecular diffusion and resistance to mass transfer in the mobile and the stationary phases, respectively

However, in the course of years several shortcomings of this equation have become apparent, the most serious ones being:

* For parts I and II of this series, see refs. 6 and 7, respectively.

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(1) the A term is often negligeably small or even zero;

(2) the $C_M u$ term is about two orders of magnitude larger than its expected value, and (contrary to expectation) almost independent of the distribution of the solute between the mobile and the stationary phase.

GIDDINGS[3] explained the first point as the result of the influence of transversal molecular diffusion on peak broadening by eddy diffusion, yielding the expression:

$$\frac{A}{1 + E/u} \tag{2}$$

where E is a constant. In GLC, $E/u \gg 1$, so that eqn. (2) is approximately $(A/E)u$. Therefore, in GLC eddy diffusion does not give rise to a velocity-independent term, but contributes to the term proportional to u.

The second point is generally ascribed to the effect of unevenness of flow on a much larger scale than in the case of eddy diffusion—the scales being of the order of the column and the particle diameters respectively. This contribution to peak broadening was given by VAN BERGE, HAARHOFF, AND PRETORIUS[4] as Fu, but more correctly by SIE AND RIJNDERS[5] as:

$$\frac{F}{G + 1/u} \tag{3}$$

where F and G are constants. In GLC, $1/u \gg G$, so that eqn. (3) is approximately Fu.

Turning now to paper chromatography, we note the following points:

(1) Due to the low flow rates, the terms $A/(1 + E/u) \approx (A/E)u$, $C_M u$, $C_S u$ and $F/(G + 1/u) \approx Fu$ are likely to be small.

(2) The homogeneity of chromatography paper results in a remarkable evenness of flow—another reason for the expectation that the term Fu will be small.

(3) The diffusion coefficients in the mobile and the stationary phase being of the same order, peak broadening by longitudinal diffusion in the stationary phase cannot *a priori* be neglected (as was done by VAN DEEMTER *et al.* for GLC). It can be shown easily[6] that:

$$\sigma^2_{\text{diff}} = \{2\,\gamma_M D_M R_F + 2\,\gamma_S D_S\,(1 - R_F)\}t \tag{4}$$

where:

γ = tortuosity factor
D = diffusion coefficient
R_F = ratio of the distances covered by the solute and by the eluent
t = diffusion time

These points are fully borne out by experiment.

Using long elution times and, consequently, low mean flow rates ($\sim$ 0.0005 cm·sec^{-1}) DE LIGNY AND BAX[6] showed that under these conditions peak broadening is governed by longitudinal diffusion, both in the stationary and the mobile phase. They found (for Schleicher and Schüll 2043a paper):

$\gamma_M = 0.68 \pm 0.09$*
$\gamma_S = 0.06 \pm 0.01$
$A/E + C_M + C_S + F < 14$ sec

* Accuracy is given throughout this paper as the 90% probability interval.

Working at flow rates between 0.0003 and 0.002 cm.sec^{-1} DE LIGNY AND REMIJNSE[7] found (for Whatman No. 1 paper):

$\gamma_M = 0.65 \pm 0.26$
$\gamma_S = 0.13 \pm 0.04$
$A/E + C_M + C_S + F = 4$–10 sec

At flow rates which are obtained in practice ($\sim$ 0.001 cm·sec^{-1}) longitudinal diffusion appeared to cause about half of the total peak broadening.

For thin-layer chromatography, points (2) and (3) mentioned for paper chromatography apply as well. However, due to the higher flow rates obtained in this technique ($\sim$ 0.003 cm·sec^{-1}) the contribution of longitudinal diffusion to peak broadening will be small. The remaining part can therefore be estimated quite accurately and it might be possible to estimate the relative importance of the various contributing terms. An important difference between these terms lies in their dependence on the distribution of the solute between the mobile and the stationary phase: the terms $A/(1 + E/u)$ and $F/(G + 1/u)$ are independent of this distribution, whereas C_M and C_S are equal to:

$$C_M = 0.01 \frac{k^2}{(1 + k)^2} \frac{d_p^2}{D_M} = 0.01\, (1 - R_F)^2 \frac{d_p^2}{D_M} \quad (5)$$

$$C_S = \frac{2}{3} \frac{k}{(1 + k)^2} \frac{d_f^2}{D_S} = \frac{2}{3} R_F\, (1 - R_F) \frac{d_f^2}{D_S} \quad (6)$$

where:

k = ratio of the amounts of solute in the stationary and the mobile phase, at equilibrium

d_p = diameter of the support particles

d_f = thickness of the layer of stationary fluid

Therefore, the relative importance of these four terms might be estimated by determining $(\sigma^2_{\mathrm{chrom}} - \sigma^2_{\mathrm{diff}})/l$ for a series of solutes having widely different R_F values and approximately equal diffusion coefficients.

EXPERIMENTAL

Chemicals

The following chemicals were used:

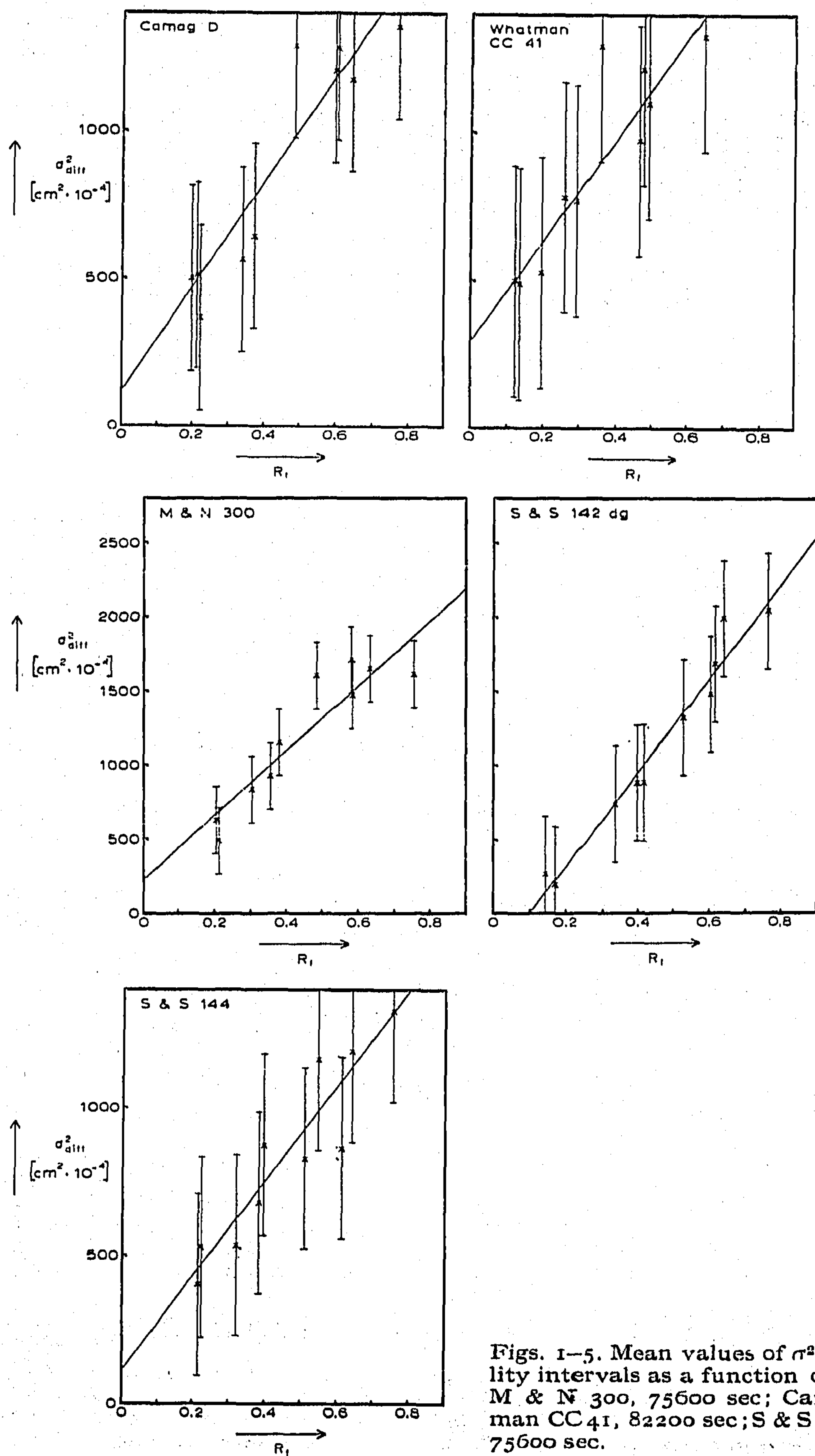
(1) L-α,γ-diaminobutyric acid; (2) L-ornithine; (3) L-aspartic acid; (4) L-glutamic acid; (5) L-threonine; (6) L-α-aminobutyric acid; (7) L-methionine; (8) L-valine; (9) L-norvaline; (10) L-leucine.

All amino acids were purchased from Fluka and were "chromatographically pure".

Cellulose powders: Macherey and Nagel 300, Camag D, Whatman CC 41, Schleicher and Schüll 144, 140 dg and 142 dg.

Procedure

Values for l, R_F and $\sigma^2_{\mathrm{chrom}}$ were taken from previous work[8]. Values of σ^2_{diff}



Figs. 1–5. Mean values of σ^2_{diff} and their 90% probability intervals as a function of R_F. Diffusion times are: M & N 300, 75600 sec; Camag D, 74400 sec; Whatman CC41, 82200 sec; S & S 144, 75600 sec; S & S 142 dg, 75600 sec.

TABLE I

PEAK BROADENING IN THIN-LAYER CHROMATOGRAPHY

Amino acid No.	M & N 300 ($t_{elution}$ = 6600 sec)					Camag D ($t_{elution}$ = 6000 sec)					Whatman CC 41 ($t_{elution}$ = 3840 sec)				
	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)
1	2.05	0.212	0.014	0.004	0.0046	2.11	0.213	0.011	0.004	0.0035	1.24	0.131	0.018	0.002	0.0122
2	1.99	0.207	0.012	0.006	0.0032	2.16	0.221	0.012	0.003	0.0043	1.12	0.119	0.026	0.002	0.0215
3	2.94	0.303	0.018	0.007	0.0036	1.96	0.200	0.012	0.004	0.0043	1.79	0.195	0.023	0.002	0.0112
4	3.67	0.378	0.016	0.010	0.0016	3.35	0.338	0.012	0.005	0.0019	2.77	0.291	0.032	0.004	0.0101
5	3.43	0.357	0.017	0.008	0.0026	3.62	0.370	0.016	0.006	0.0028	2.44	0.259	0.038	0.004	0.0140
6	4.69	0.484	0.014	0.014	−0.0001	4.74	0.484	0.017	0.010	0.0015	3.24	0.354	0.028	0.006	0.0069
7	5.63	0.580	0.012	0.013	−0.0001	5.98	0.603	0.013	0.010	0.0004	4.52	0.475	0.030	0.006	0.0053
8	5.56	0.577	0.018	0.015	0.0005	5.84	0.597	0.014	0.010	0.0008	4.36	0.464	0.045	0.004	0.0093
9	6.11	0.631	0.018	0.014	0.0006	6.29	0.641	0.014	0.010	0.0007	4.51	0.491	0.063	0.005	0.0129

Amino acid No.	S & S 144 ($t_{elution}$ = 3000 sec)					S & S 142 dg ($t_{elution}$ = 2820 sec)					S & S 140 dg ($t_{elution}$ = 1500 sec)*				
	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)	l (cm)	R_F	σ^2_{chrom} (cm²)	σ^2_{diff} (cm²)	$(\sigma^2_{chrom} - \sigma^2_{diff})/l$ (cm)
1	1.99	0.222	0.051	0.002	0.0245	1.62	0.171	0.040	0.001	0.0245	2.28	0.242	0.066	0.001	0.0285
2	1.92	0.214	0.046	0.002	0.0233	1.34	0.144	0.037	0.001	0.0266	2.22	0.236	0.070	0.001	0.0309
3	2.84	0.318	0.024	0.002	0.0077	3.16	0.337	0.022	0.003	0.0060	3.86	0.410	0.056	0.002	0.0140
4	3.56	0.394	0.033	0.004	0.0083	3.91	0.415	0.023	0.003	0.0051	4.69	0.497	0.076	0.002	0.0156
5	3.47	0.383	0.032	0.003	0.0084	3.70	0.397	0.019	0.003	0.0042	4.46	0.475	0.091	0.002	0.0199
6	4.52	0.507	0.033	0.003	0.0067	4.94	0.527	0.018	0.005	0.0025	5.68	0.604	0.064	0.003	0.0107
7	5.50	0.611	0.032	0.003	0.0051	5.76	0.611	0.020	0.006	0.0024	6.57	0.696	0.047	0.003	0.0067
8	5.40	0.596	0.032	0.005	0.0052	5.59	0.601	0.015	0.006	0.0017	6.40	0.682	0.074	0.003	0.0112
9	5.70	0.639	0.037	0.005	0.0056	5.97	0.636	0.019	0.007	0.0019	6.73	0.717	0.104	0.003	0.0149

* Values of σ^2_{diff} were not determined experimentally, but calculated (see Discussion).

were determined by applying bands of each of the ten amino acids to a thin-layer plate[8]. The plate was placed, coated side down, on two glass rods around which filter paper had been wrapped, and equilibrated with the vapour of the lower layer of a 4:1:5 butanol–acetic acid–water mixture for 18.5 h at 21.5°. Then an amount of the upper layer (the eluent used in the chromatographic experiments) was poured into the trough containing the plate and glass rods, so that the liquid ran up into the paper and cellulose powder from both ends. When, after a few minutes, both liquid fronts had reached each other, the liquid stayed at rest for the remainder of *ca.* 21 h during which time the amino acids were allowed to diffuse in the cellulose powder, at 21.5°. Then the plate was dried and stained, and the densitogram obtained. From the peak widths at half height, the peak variances were obtained, and corrected for the variances, originating during the equilibration period[8]. The experiments were performed in duplicate.

RESULTS

The mean values of σ^2_{diff} and their 90% probability intervals are shown as a function of R_F in Figs. 1–5. The mean values of l, R_F, σ^2_{chrom}, σ^2_{diff} (recalculated for a diffusion time equal to the elution time in the chromatography experiments) and $(\sigma^2_{chrom}-\sigma^2_{diff})/l$ are given in Table I.

DISCUSSION

The values of σ^2_{diff} and σ^2_{chrom} are mean values of two and three determinations, respectively, and therefore have an approximately normal distribution, so that the usual statistical methods can be applied.

(1) σ^2_{diff}

In the first place it was ascertained, by means of BARTLETT's tests, that the variances of σ^2_{diff} for a particular cellulose powder are homogeneous.

According to eqn. (4) there is a linear relationship between σ^2_{diff} and R_F, if the diffusion coefficients of the solutes are approximately equal. It follows from the equation of WILKE AND PIN CHANG[9] that in our case the individual diffusion coefficients do not deviate more than 10% from their mean value (which is about the accuracy of the values, calculated by this equation). The relationship between σ^2_{diff} and R_F was calculated by the method of least squares, allocating equal statistical weights to

TABLE II

VALUES OF γ_M AND γ_S IN THIN-LAYER CHROMATOGRAPHY

Cellulose powder	γ_M	γ_S
M & N 300	0.52 ± 0.33	0.03 ± 0.09
Camag D	0.42 ± 0.29	0.02 ± 0.08
Whatman CC 41	0.40 ± 0.08	0.04 ± 0.02
S & S 144	0.37 ± 0.10	0.02 ± 0.03
S & S 142dg	0.62 ± 0.09	−0.04 ± 0.03

the values of σ^2_{diff}. The corresponding straight lines are shown in Figs. 1–5. From their slopes and intercepts, together with the calculated values of $\bar{D}_M$ and $\bar{D}_S$[6] and the known diffusion times, γ_M and γ_S can be calculated. These values are given in Table II.

We note the following points:

(a) The calculated lines represent the measurements very well, passing through 48 out of the 50 90% probability intervals (this would still hold if in the case of S & S 142 dg the line was constrained through the origin).

(b) 4 out of the 5 γ_S values are positive. The chance of obtaining this result if, in reality, γ_S is zero is only 9%[10]. Therefore, it is also very probable that in thin-layer chromatography longitudinal diffusion in the stationary phase contributes to peak broadening.

(c) The spread of the γ values is somewhat larger than can be accounted for on the basis of the estimated accuracies. However, if the data for S & S 142 dg are omitted (the negative γ_S value being impossible and the γ_M value being abnormally high because of the abnormally low γ_S value) the remaining data are not significantly different. Hence, it is probably justified to state that peak broadening by longitudinal diffusion in thin-layer chromatography is governed by the weighted mean values $\bar{\gamma}_M = 0.39 \pm 0.06$ and $\bar{\gamma}_S = 0.03 \pm 0.02$. Both are appreciably smaller than the values obtained for paper chromatography.

We are now also able to calculate σ^2_{diff} for S & S 140 dg, a powder that was taken from the market since the chromatographic data were obtained.

(2) $(\sigma^2_{chrom}-\sigma^2_{diff})/l$

In the first place it was investigated, by means of BARTLETT's tests, whether the variances of σ^2_{chrom} for a particular cellulose powder are homogeneous. 2 out of the 6 tests gave significant results on the 10% probability level. This suggests strongly that the variances are not homogeneous (the chance of obtaining the above mentioned result if the variances are, in reality, homogeneous is only 3%).

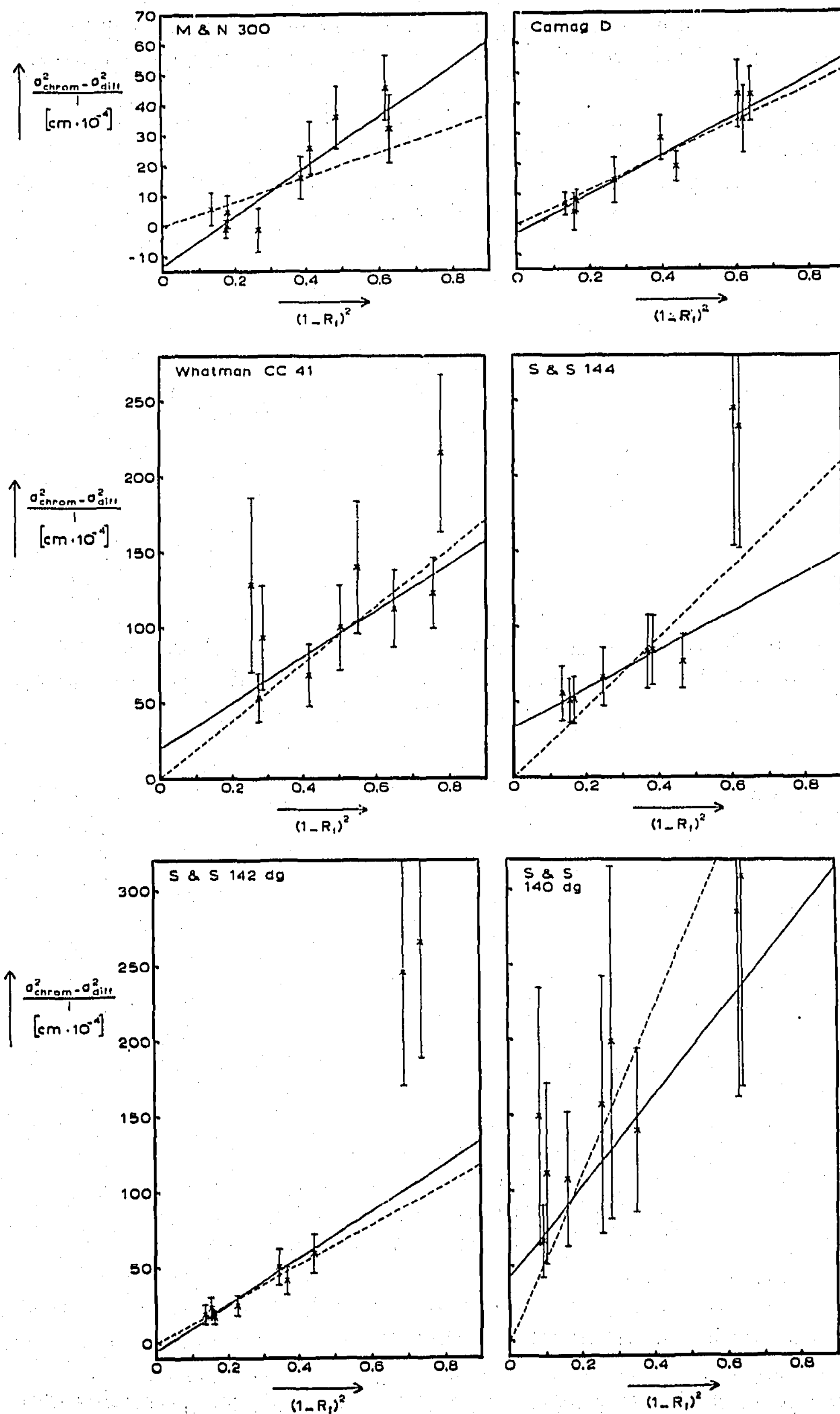
Reasoning that the variance of σ^2_{chrom} probably depends mainly on the magnitude of σ^2_{chrom}, log var (σ^2_{chrom}) was plotted *vs.* log σ^2_{chrom} and the best straight line through the points was determined, assuming that the accuracies of log var (σ^2_{chrom}) and log σ^2_{chrom} are unknown[11]. It appeared that var (σ^2_{chrom}) is proportional to $(\sigma^2_{chrom})^{3.3}$. From this graph var(σ^2_{chrom}) was read and combined with var(σ^2_{diff}) to yield var$\{(\sigma^2_{chrom}-\sigma^2_{diff})/l\}$.

It is immediately clear from Table I that the values of $(\sigma^2_{chrom}-\sigma^2_{diff})/l$ strongly decrease when R_F increases. This suggests that the major part of the residual peak broadening is caused by the $C_M u$ term, which is, according to eqn. (5), proportional to $(1 - R_F)^2$.

Therefore, the best linear relationship between $(\sigma^2_{chrom}-\sigma^2_{diff})/l$ and $(1 - R_F)^2$ was calculated by the method of least squares, allocating to the values of $(\sigma^2_{chrom}-\sigma^2_{diff})/l$ statistical weights, inversely related to their variances. (See Figs. 6–11.)

The following points are noted:

(a) Only three out of the six intercepts are positive, which makes it probable that there are no measurable contributions to the residual peak broadening from other terms than the $C_M u$ term.



Figs. 6–11. Mean values of $(\sigma^2_{chrom} - \sigma^2_{diff})/l$ and their 90% probability intervals, as a function of $(1-R_F)^2$. (—) Best straight lines; (— — —); best straight lines constrained through the origin.

(b) The lines constrained through the origin (*i.e.*, the $C_M u$ terms alone) represent the measurements fairly well, passing through 37 out of the 54 90% probability intervals.

(c) From the slopes of the lines, constrained through the origin, the values of the effective particle diameter, d_p, can be calculated as follows:

In thin-layer (and paper) chromatography the flow rate of the eluent at the solvent front, u_f, is inversely proportional to the distance from the surface of the eluent in the tank to the solvent front, l_f [12]:

$$u_f = \frac{k}{2l_f} \tag{7}$$

From the known values of the distance from the surface of the eluent to the starting point, l_0, and l_f (1 and 11 cm, respectively, in our experiments) and of t_{elution}, the value of k can be calculated by:

$$t_{\text{elution}} = \int_{l_0}^{l_f} \frac{\mathrm{d}l_f}{u_f} = \frac{l_f^2 - l_0^2}{k} \tag{8}$$

The flow rate behind the front, u, is about 20% lower[12] than u_f (for $R_F < 0.8$).

As a consequence of the gradually decreasing flow rate, the observed mean plate height is equal to:

$$\bar{H} = \frac{\int_{l_0}^{l} H \mathrm{d}l}{\int_{l_0}^{l} \mathrm{d}l} = \frac{\int_{l_0}^{l_f} H \mathrm{d}l_f}{\int_{l_0}^{l_f} \mathrm{d}l_f} = \frac{1}{l_f - l_0} \int_{l_0}^{l_f} \left(A + \frac{B}{u} + Cu\right) \mathrm{d}l_f \tag{9}$$

This gives, with $u = 0.8\, k/2\, l_f$:

$$\bar{H} = A + B\,\frac{l_f + l_0}{0.8\,k} + C\,\frac{0.4\,k}{l_f - l_0} \ln \frac{l_f}{l_0} \tag{10}$$

Herein is $(l_f + l_0)/0.8\,k = 1/\bar{u}$ and $0.4\,k/(l_f - l_0)\ln l_f/l_0 = \bar{u}$.

It was concluded above that C is given by eqn. (5). Therefore, the slopes of the lines constrained through the origin in Figs. 6–11 are equal to $C_M\bar{u}/(1 - R_F)^2$.

The values of k, $\bar{u}$, $C_M\bar{u}/(1 - R_F)^2$, $C_M/(1 - R_F)^2$ and d_p are given in Table III, together with some manufacturer's data.

It is striking that the effective d_p values, though in excellent agreement among themselves, are more than an order of magnitude larger than the values given by the manufacturers.

The most probable cause of this discrepancy is that the cellulose particles clog together, thus forming large aggregates (*cf.* ref. 13).

TABLE III

RESISTANCE TO MASS TRANSFER IN THE MOBILE PHASE IN THIN-LAYER CHROMATOGRAPHY

Cellulose powder	k ($cm^2 \cdot sec^{-1}$)	$\bar{u}$ ($cm \cdot sec^{-1}$)	$C_M\bar{u}/(1-R_F)^2$ (cm)	$C_M/(1-R_F)^2 = 0.01\, d_p^2/D_M$ (sec)	d_p (cm)	d_p (*manufacturer's data*) (cm)
M & N 300	0.0168	0.0015	0.0040	2.6	0.028 ± 0.008	< 0.0040*
Camag D	0.0184	0.0016	0.0057	3.4	0.032 ± 0.002	0.001–0.0025*
Whatman CC 41	0.0286	0.0025	0.0189	7.3	0.047 ± 0.004	0.0008
S & S 144	0.0366	0.0032	0.0232	7.0	0.046 ± 0.005	0.0019
S & S 142 dg	0.0391	0.0034	0.0131	3.7	0.034 ± 0.003	
S & S 140 dg	0.0690	0.0060	0.0558	8.9	0.052 ± 0.006	

$D_M = 3.07 \cdot 10^{-6}$ [$cm^2 \cdot sec^{-1}$][6]
* Personal communication.

CONCLUSIONS

(1) Longitudinal diffusion in the stationary phase contributes to peak broadening in thin-layer chromatography.

(2) The tortuosity factors for longitudinal diffusion are $\gamma_M = 0.39 \pm 0.06$ and $\gamma_S = 0.03 \pm 0.02$.

(3) Peak broadening in thin-layer chromatography is exclusively caused by longitudinal diffusion and by resistance to mass transfer in the mobile phase.

(4) The values of the effective particle diameter d_p, calculated from the values of the mass-transfer term are much larger than the values given by the manufacturers. The reason is probably that the cellulose particles clog together, thus forming large aggregates.

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DISCUSSION

PROCHÁZKA (to Dr. DE LIGNY): You have mentioned that the greater sharpness of the spots in TLC is partly due to the greater flow rate of the solvent. It is obvious that the longer the chromatography, the greater will be the diffusion. However, in my opinion the predominant factor for the sharpness of the spots in TLC is simply

adsorption on the adsorbent preventing the substance from diffusion. Grain diameter will usually play an important role in the separation. It will affect the flow rate and the number of active sites per given amount of adsorbent.

DE LIGNY: We used, in thin-layer chromatography as well as in paper chromatography, the 4:1:5 butanol–acetic acid–water system, which separates into two layers, and equilibrated the cellulose powder or paper for 20 h with the vapour of the lower layer. So I think we had mainly partition, rather than adsorption, in both techniques.

As I showed, tortuosity factors in thin-layer chromatography are much smaller than in paper chromatography. This is an important cause for the smaller HETP values obtained in thin-layer chromatography.

PROCHÁZKA: How would you explain that substances on the same chromatogram, where all of them were in contact with the solvent for the same length of time, have different shapes? Those near the front are always more diffuse. I think that it is simply because they are less adsorbed and have more time to diffuse, since the proportion of time they are in the mobile phase is longer than the time they are on the adsorbent.

DE LIGNY: I agree with your explanation.

GÄNSHIRT: Are the terms for the various diffusions of the VAN DEEMTER equation in the case of crystalline cellulose similar to the conditions prevailing on the TLC sorption layers or to the diffusion conditions in the sheet paper chromatography?

DE LIGNY: We have no experience with papers made of crystalline cellulose, but we have started an investigation into dispersion in paper chromatography analogous to the investigation described for TLC. It would be of interest to include a paper made of crystalline cellulose in this study.

GRÜNE: Comparative studies between crystalline cellulose and paper might be misleading if considered from the point of cellulose chemistry, since crystalline cellulose has been pre-treated with acid and the amorphous micellary portion has been removed to a greater extent. Paper fibre has not been subjected to this pre-treatment and consequently still contains the amorphous micellary portion.

HAIS: The influence of flow rate can be studied independently of other properties of the sorbent, at least as far as the *reduction* of flow rate is concerned. The cross-section of the initial part of the flow bed can be limited and the flow slowed down by cutting or scraping off part of the material, thus making windows of appropriate size, or by attaching a slow-running paper to the starting edge.

FRANC: The VAN DEEMTER equation is well known in GC. This equation expresses the dependence of the number of theoretical plates on various factors. It shows that there exist optimum conditions, namely a certain grain size and carrier gas velocity. Thus, according to the conditions in the actual case, higher flow velocity of the mobile phase may improve or impair separation respectively.

BRENNER: It has been stated that better separations were often observed on prefabricated than on individually spread layers, and Mr. PETROWITZ has suggested that this might be ascribed to the lower flow velocity on prefabricated layers. According to Mr. FRANC, this suggestion is supported by the VAN DEEMTER equation. If in this connection better separation means smaller spot diameters, decrease of spot diameter takes place when migration rate of the solvent increases: If the most rapidly migrating dye from the STAHL test dye mixture is chromatographed in such a way that the distance between the immersion line and the spot is constant, then the distance be-

tween the immersion level and the front during the whole experiment obviously remains shorter than with the standard procedure. This causes an increase in the solvent migration rate. Result: After the same distance the dye spot is less diffuse than with the standard procedure (Kieselgel G). Here, Mr. FRANC, we seem to be on that branch of the VAN DEEMTER hyperbola which indicates an increase in the plate number accompanying an increase in flow velocity.

By the way, even if we apply the standard procedure on the test mixture, simple inspection gives us the impression that the separation process is more efficient in the initial stage ("start effect") than in the later stages when the front has progressed and the flow velocity has been slowed down accordingly. In this connection, I should like to remind of the centrifugation technique in PC.

GRÜNE: We have studied the loading (*i.e.* the amount of solvent held on the paper) of the ascendent paper chromatogram with the mobile solvent. Just above the line of immersion there is a very high loading, then follows a zone of a relatively constant loading, and finally, a rather steep drop. The separation thus depends on the distance from the line of immersion.

HAIS: Prof. BRENNER has mentioned a case where the separation of a dye mixture is best at the beginning of chromatography. At the beginning the concentration is higher and the solutes can compete for the adsorption sites and displace one another. In paper chromatography we can often observe initially formation of thin contiguous pigment zones, sometimes even resulting from a circular origin spot. Gradually, they then become more diffuse. On the other hand, there may be cases when an excessive rate of flow at the very beginning does not permit equilibrium to be achieved and causes blurring; the solutes only begin to separate in the subsequent stages.

BRENNER: The objection put forward by Dr. HAIS against my interpretation, namely that mutual displacement could account for the "start effect", seems to be ill grounded in view of the very small amount of substances applied; all the same, a verification seems indicated.

On the other hand, I agree with Dr. PROCHÁZKA that grain diameter might play an important role. Besides, the higher degree of homogeneity of the commercial layers might have a favourable effect. Notoriously, it is generally valid that the more homogeneous the layer, the higher the number of theoretical plates. If rapid flow is accompanied by bad separation this is not due to the rapid flow itself but rather to the inhomogeneity of the flow bed or coarseness of the grain which reduce the number of elementary plates.

HAIS: It is also conceivable that a steep gradient ("second front") passes the origin very early, thus concentrating the substances during the initial stages; only then they begin to diffuse. Dr. GRÜNE may have had this in mind when making her remark.

GEISS: In case of protracted development there is always the danger that any imperfection of the procedure becomes more pronounced; thus in the case of the chimney effect, when the chamber leaks at the bottom, or in the case of condensation the untoward effect increases proportionally with time. It may happen, *e.g.*, that for 150 min of development the front does not proceed beyond 10 cm, while nearer to the origin the solvent is still running and carrying the solutes, all this only because there is a difference of 0.5° between the plate and the chamber wall.

DE LIGNY: I would not agree with the general statement of Prof. BRENNER that

the flow rates are too small. We made experiments with the butanol–acetic acid–water system using different flow rates and plotted plate height as a function of flow rate.

From H–u curves obtained for Whatman No. 1 paper it follows that the optimum mean flow rate of the eluent, corresponding with the minimum H value, is about 0.001 cm.sec^{-1}, *i.e.*, an elution time of 6 h for a travelling distance of the eluent of 20 cm. This is about the mean flow rate obtained in practice.

GEISS: How did you measure the flow rate?

DE LIGNY: We used a sheet on which we applied the samples at different distances (ranging from 5 to 35 cm) from the surface of the eluent in the reservoir. Only the average flow rate for a travelling distance of the eluent of 5 cm was measured, yet we obtained a fairly good VAN DEEMTER curve.

BRENNER: If I understood correctly, Mr. DE LIGNY uses the formula $w^2/d = 16\,H$ for the estimation of the HETP. For a given plate, a given compound and a given solvent this formula yields, according to the time of measurement (or the length of d, respectively), different values, since the solvent does not advance at a linear rate in function of time, but in function of the square root of time. How is it thus possible to reach a definite result using this formula?

DE LIGNY: In paper and thin-layer chromatography the compound which is used to measure the plate height travels at an ever decreasing rate, because the flow rate of the eluent is gradually slowing down. Therefore, in the VAN DEEMTER equation

$$H = A + B/u + Cu$$

u is the *average* flow rate of the eluent.

BRENNER: If we pass to the columns we should like to obtain the same chromatographic migration as in the layers. A few years ago, FUCHS demonstrated that if the solvent is allowed to penetrate the column in the capillary fashion without being forced into it, a similar separation is obtained in the column as in the plate. It is difficult, however, to introduce the solvent on an adsorption column in a capillary fashion and nevertheless efficiently.