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FULLY AUTOMATED HIGH-SPEED ION-EXCHANGE CHROMATOGRAPHY OF AMINO ACIDS

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SUMMARY

A procedure based on ion-exchange chromatography was developed which separates the amino acids present in protein hydrolysates with an automatic elution cycle which is repeated every 63 min. Forty samples in a row can be run completely unattended. Short separation times were achieved by optimizing chromatographic parameters and by reducing extra column band widening caused by sample loading and the delay time in the analytical system. A linear relationship exists between plate height and carrier velocity in the flow rate range investigated. Thus, diffusion coefficients in the stationary phase could be calculated for a number of amino acids.

INTRODUCTION

Since the pioneering work of SPACKMAN, STEIN AND MOORE on the separation of all twenty protein amino acids by automated ion-exchange chromatography became known[1], their technique has been constantly improved and refined to such an extent that the original analysis time of 21 h has been reduced to about 2 h. This has been achieved without any deterioration in separation and reproducibility of the data obtained[2–5]. Recently, HRDINA[6] reported that the separation of the protein amino acids can be achieved in less than 1 h. The important study of HAMILTON *et al.*[7] on most of the physical parameters involved in the chromatographic process was a main stimulant for this progress. On the other hand, recent progress in gas chromatography in the area of amino acid separation[8], and the successful use of pellicular resins for ion-exchange chromatography[9], have caused investigators to explore further the potential of ion-exchange chromatography with spherical resins.

THEORETICAL PART

Optimization of column parameters

Within a given set of limitations, determined by the strength of the glass columns as well as the leak-proofness of the valves and connections, attempts were made to optimize the system with respect to separation time and resolution. Basically the elution buffers, column temperature and the chemical character of the sulfonated,

polystyrene–divinylbenzene co-polymer resin were not changed. The dimensions of the resin bed, the buffer flow rate, and particle size of the resin were regarded as variable parameters.

A range of linear flow rates was chosen where the main contribution to the total plate height of the columns is determined by the solid phase resistance term H_s (ref. 11) and the eddy diffusion term A.

$$H = A + H_s = 2\lambda d_p + \frac{1}{30} R(1 - R) \frac{{d_p}^2 v}{D_s} \tag{1}$$

As the separation time is defined by

$$t_s = \frac{NH}{Rv} \tag{2}$$

substitution of (1) into (2)

$$t_s = \frac{N(1 - R){d_p}^2}{30 D_s} + \frac{2\lambda d_p N}{Rv} \tag{3}$$

shows that the particle size d_p and the ratio d_p/v have to be decreased as much as possible.

The resolution of two peaks in our case is defined by

$$R_s = \sqrt{\frac{L\Delta R}{16HR}} = \frac{1}{4}\sqrt{\frac{L\Delta R}{R}} \frac{1}{2\lambda d_p + \frac{1}{30} R(1 - R) \frac{{d_p}^2 v}{D_s}} \tag{4}$$

neglecting all other terms of the plate height expression. Whereas a decrease in particle size enhances separation time and resolution, an increase of flow rate, as required for shorter separation times, is detrimental to resolution.

Once the selection of a workable column diameter is made, a resin of optimum particle size is chosen in order to avoid excessive pressure. One has to be mindful that at a given temperature, the pressure drop in packed columns (Δp) depends on the column length, the linear flow rate in the column and the particle size.

$$\Delta p \sim \frac{Lv}{{d_p}^2} \tag{5}$$

It was therefore advisable to decrease the flow rate and to shorten the column as much as eqn. (3) permitted.

The longitudinal diffusion term $H = 2\gamma D_m/v$ is completely negligible at the chosen flow rate.

Though little is known of the quantitative relationship between column diameter and plate height, experience shows that resolution improves with decreasing column diameter. This is particularly evident when the ratio of column diameter to d_p is high. However, limits are imposed by the capacity of the resin bed, the sample size and the sensitivity of the detection system.

The plate height of the column (H) contributes to the variance of the total system by

$$\sigma_C^2 = HL \tag{6}$$

the variance of the total system being the sum of the column's and all extra column contributions to band spreading σ_E^2

$$\sigma_T^2 = \sigma_C^2 + \sigma_E^2 \tag{7}$$

It is evident that the extra column contributions to band spreading become increasingly significant when σ_C^2 is small. With this in mind, particular efforts were made to reduce σ_E^2 to a minimum.

Optimization of the analytical system

In a gas segmented stream of liquid moving at a constant velocity through a cylindrical tube, a constant transfer of liquid occurs from a given segment into the following segment. The transfer of solute m from segment n into segment $n+1$ along the distance x is described by the following equation (see Fig. 1):

$$\Delta m = \Delta C_{n+1}(\lambda - \mu)r^2 = C_n \Delta x \delta 2\pi r - C_{n+1}\Delta x \delta 2\pi r \tag{8}$$

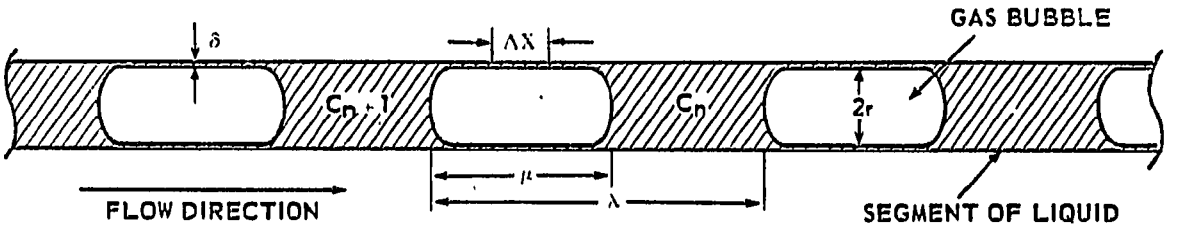

Fig. 1. Mass transfer in a gas segmented flowing liquid stream. m = mass of solute in stream; C_n = concentration in liquid segment n; k = number of segments past initial segment; μ = length of bubble; λ = length of total segment; $\lambda - \mu$ = length of liquid segment; δ = thickness of hypothetical uniform layer of liquid on tube wall; L = total length of tube; x = length parameter; r = radius of tube. For further explanation, see text.

In differential form we obtain eqn. (9)

$$\frac{dC_{n+1}}{dx} = (C_n - C_{n+1})\frac{2\delta\pi r}{(\lambda - \mu)r^2} = (C_n - C_{n+1})\frac{2\delta\pi}{(\lambda - \mu)r} \tag{9}$$

This equation neglects longitudinal diffusion in the tubular layer on the wall and it is assumed that instantaneous and complete mixing occurs in the liquid segments.

It has been demonstrated[6] by integration of the mass transfer process occurring in each segment over the total length of the system that the process can be described by a Poisson distribution,

$$P\ (x = k) = \frac{C_k}{C} = e^{-q}\frac{q^k}{k!} \tag{10}$$

where k is the serial number of segments following the segment which initially contained the total amount of solute m in a concentration C. The parameter q is a characteristic geometric and physical constant, which is defined by

$$q = \frac{2\delta L}{(\lambda - \mu)r} = \frac{2\delta\pi L r}{(\lambda - \mu)\pi r^2} = \frac{V_{\text{Film}}}{V_{\text{Liquid segment}}} \tag{11}$$

δ is the thickness of the hypothetical layer of liquid between gas bubble and glass tube, which summarizes all physical phenomena related to wall effects. q is also the ratio between the volume of the liquid film on the tubing wall (V_{Film}) to the volume of the liquid segment between two bubbles. For values of $q > 1$ the distribution has a maximum at values of $k = q$ and $k = q - 1$.

A plot of this function for various values of q shows that q determines the slope of the distribution and should therefore, be kept as small as possible. According to the laws of the Poisson distribution the variance σ_A^2 due to the spreading in the segmented analytical stream equals q.

EXPERIMENTAL

A Technicon Sequential Multi-Sample Amino Acid Analyzer-TSM in a modified form was used for the experiments. Only those modules of the instrument will be described which differ essentially from the model described previously[5].

Sample loading

The sample is applied to 0.10 ml of packed cation exchange resin ($d_p = 18$–21μ) which has been equilibrated with citrate buffer of pH 2. The resin is placed as a plug between two porous teflon filters in a cylindrical cartridge of 4 mm I.D. The sample is aspirated into the resin and the low pH causes the amino acids to stick to the resin within a very thin layer, thus reducing the variance due to sample loading to a minimum.

Sample introduction

The sample cartridge is sealed above a 0.3 mm I.D. stainless steel capillary by means of an O-ring seal. The end of the capillary is threaded at its lower end. A porous teflon filter is sleeved over the thread, followed by a screw with a conical head. An O-ring above the filter seals against the column. The conical head of the screw protrudes into the resin bed, thus causing an even distribution of the sample over the whole cross section of the resin bed without leaving any dead space between the fitting and the resin. The fitting is held in the column by means of a Thomas clamp (Fig. 2).

The columns and the resin

The column finally chosen for the separation of the acidic and neutral amino acids was 5 mm I.D. and the height of the resin bed 220 mm. The corresponding measurements for the basic column were 4 mm and 40 mm. The lower ends of the columns were enlarged in order to house the bottom fittings (Fig. 2). The heating jackets kept the whole resin bed at a constant temperature of 60°. The same spherical resin of 8% divinylbenzene cross-linked sulfonated polystyrene was used in both columns. The diameter of the particles was $8 \pm 0.5\,\mu$.

The bottom column fitting

A porous teflon disc, supported by a porous metal screen, both of the same diameter as the I.D. of the column, is placed into a cylindrical cavity of a Kel-F plug (see Fig. 2). The effluent from the column is collected at the bottom of the cavity and

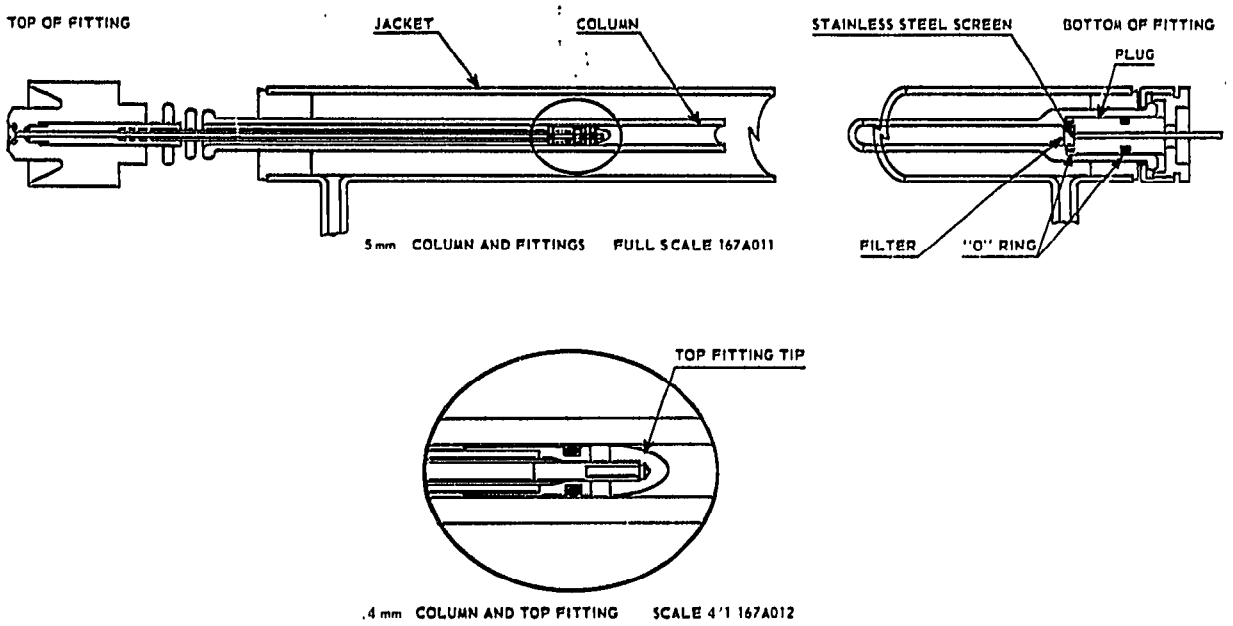

Fig. 2. Column and column fittings as used in the system. 1–5 mm column 300 mm long; 4 mm column 90 mm long.

fed into a stainless steel capillary. The fitting seals against the column bottom. This construction allows a maximum cross section of the filter to be in contact with the resin, avoiding higher flow rates and thus higher flow resistance in the porous filter material.

The analytical reaction

Advanced AutoAnalyzer technologies as described by SMYTHE *et al.*[10] were applied and adapted in order to reduce bandwidth broadening of the chromatographic peaks in the analytical system.

To a solution of 20 g ninhydrin in 1.0 l methyl cellosolve were added 100 ml of glacial acetic acid and 400 ml of sodium acetate buffer. The solution was diluted with distilled water to a total volume of 2 l.

A Technicon Peristaltic Pump III, which is provided with an air bar for precise bubble injection, was used to pump the reagents into the analytical system and the effluents from the photometer. A flow of 0.7 ml/min of ninhydrin reagent, segmented with nitrogen, was combined with 0.32 ml/min of a 0.002 *M* solution of hydrazine sulfate shortly before the column effluent was added. The unsegmented stream in the stainless steel capillary between column bottom and segmented reagent stream was shorter than 1 cm. The stream of reagents plus sample was fed into the reaction coil. According to eqn. (11), the diameter of the heating bath coil was chosen as large as possible without impairing the stability of the liquid segment due to gravity effects. A glass coil of 2.2. mm inner bore and 12 m length was selected. The glass coil was tapered at both ends in order to keep the flow pattern stable and smooth as the gas segments expand or contract at the ends of the coil. The coil was kept at 95 $\pm$ 0.01° in an oil bath. The total delay time in the analytical system was 9.5 min.

The photometer

The TSM photometer with four optical channels was used for the experiments. The light beams of 440 mμ and 570 mμ wavelength pass through sample and reference cells and reach two pairs of phototubes. Each phototube is generating a current proportional to the intensity of the entering light. Logarithmic amplification yields two voltages, the difference of which is proportional to the absorbance of the sample channel. Thus, a signal is obtained which varies in a linear way with the sample concentration. Range expansion permits readings at 0–0.1 absorbance for a full-scale pen deflection on the recorder.

The photometer is equipped with a high-efficiency flow cell as described previously[10]. The diameter of the tubular cuvette is 1.5 mm, its length 15 mm. This design prevents major contributions to band widening in the flow cell.

The recorder

A Technicon strip chart potentiometric type recorder was used for the experiments. The slide wire voltage of 60 mV was supplied by the TSM control box. The cross chart speed was 2 sec for a full-scale deflection.

Fail safe devices

In order to prevent loss of samples and reagents due to malfunctioning of the instrument when running completely unattended, a number of controls for proper operation were incorporated. The peristaltic pump which feeds the analytical reagents into the reaction coil is provided with a leak detector. If a pump tube breaks, the escaping liquid is collected in a cavity. At a given level of liquid, an electric contact is established within a safety circuit and the whole system is stopped. Two preset pressure relief valves are placed between the positive displacement pump and the column head. They are activated as soon as the column pressure exceeds 950 p.s.i. Flow rate drops caused by these mechanisms, or any other malfunctioning, are sensed by a detection device which is part of the photometer. A small fraction of the effluent from the photometer is fed through a flow cell into which light from the photometer lamp is focused. After passing the flow cell it strikes a photoresistor cell. The flow of liquid is balanced in such a way that a slight decrease in flow through the analytical system will reverse the flow through the detector flow cell. Air will shortly be aspirated into the cell. The photoresistor cell also monitors the photometer lamp. A change in photocurrent caused by either a low flow rate or a burned out lamp, activates the shut-off circuit. At two positions within each chromatographic cycle, the fail safe circuit is checked and if it is activated, the instrument is shut off.

Development of the chromatographic procedure

The separation characteristics of the resin had been established previously in the Technicon 2-hour hydrolysate system and the buffer program had been worked out[5]. Though higher flow rates and longer columns allow faster separation times (eqn. 3), pressure parameter set the upper limit. A resin bed of 220 mm at a flow the rate of 0.5 ml/min was finally chosen which resulted in 600 p.s.i. back pressure.

Under these conditions, a resolution of 0.9 was obtained for threonine and serine when citrate buffer of pH 3.25 + 6% methyl cellosolve was used as eluant. In order to separate glycine from alanine, the same buffer without methyl cellosolve was

then run through to cystine which was eluted with citrate buffer of pH 4.25. Valine, methionine, isoleucine, leucine, and norleucine followed. Citrate buffer of pH 5.23 eluted tyrosine and phenylalanine. The same buffer was used for the elution of the basic amino acids from the short column. This column provided sufficient theoretical plates to separate lysine, histidine, ammonia, and arginine at a flow rate of 0.5 ml/min.

Operating procedure

As described previously[5], the chromatographic cycle of the TSM is automatically controlled by a peristaltic valve in conjunction with a tape timer. In valve position 1, the buffer pumps are switched off while the analytical system is being rinsed with 50% aqueous methyl cellosolve. One minute after the automatic sampler advances two cartridges on top of the columns, pump 2 is switched on to elute the sample from the basic column with pH 5.23 buffer. Six minutes later the valve changes to position 2, turning on pump 1 and feeding the analytical reagents into the reaction coil. The eluant of the short column is combined with the analytical reagents and the eluant of the long column is sent to waste. After 15 min, regeneration of the short column with 0.2 *N* sodium hydroxide starts. Buffer of pH 3.25 is supplied to the long column and its eluant switched into the analytical system. Regeneration of the short column continues until 25 min after the start of the run. At that time buffer of pH 4.25 is pumped on the long column, pH 5.23 buffer follows 13 min later and 10 min afterwards, 52 min after the start of run, the long column is regenerated with 0.2 *N* sodium hydroxide. Then pump 2 starts again to equilibrate the short column with pH 5.23 buffer. Four minutes later equilibration of the long column

TABLE I

ELUTION PROGRAM

Minutes	*Eluant column I*	*Eluant column II*	*Analytical system*
0	—	0.1 *M* citrate buffer, pH 5.23 0.35 *N* Na^+	Column II
6	0.1 *M* citrate buffer, pH 3.25 (6% methyl cellosolve) 0.2 *N* Na^+	0.1 *M* citrate buffer, pH 5.23 0.35 *N* Na^+	Column II
15	0.1 *M* citrate buffer, pH 3.15 0.2 *N* Na^+	0.2 N NaOH	Column I
25	0.1 *M* citrate buffer, pH 4.25 0.2 *N* Na^+	0.2 *N* NaOH	Column I
42	0.1 *M* citrate buffer, pH 5.23 0.35 *N* Na^+	0.2 *N* NaOH	Column I
52	0.2 *N* NaOH	0.1 *M* citrate buffer, pH 5.23 0.35 *N* Na^+	Column I
56	0.1 *M* citrate buffer, pH 3.25 (6% methyl cellosolve) 0.2 *N* Na^+	0.1 *M* citrate buffer, pH 5.23 0.35 *N* Na^+	Column II

with the first buffer starts and the analytical system is switched to the effluent of the short column. After 63 min, the elution cycle is terminated and after a delay time of 1 min the automatic sampler proceeds to the next sample (see Table I). In valve positions 4 and 11, the fail safe circuit is checked by the peristaltic valve.

RESULTS AND DISCUSSION

Two complete consecutive chromatograms obtained with the procedure described are represented in Fig. 3. The signals are linear in absorbance units and the areas under the peaks are directly proportional to the amount of amino acid in the sample. Due to the small values of σ_T^2 for the individual peaks, high absorbances are obtained for small amounts of amino acid. In other words, the minimum detectable limit of the method increases with its speed.

Special care is required when the sample is applied to the cartridge. A sample of 0.03 μmoles of each amino acid in 0.04 ml of pH 2 citrate buffer is pipetted onto the cartridge filter and aspirated into the resin, which previously has been equilibrated with pH 2 citrate buffer. A wash of 0.05 ml of the same buffer is then added to make sure that all the sample is removed from the top filter and completely fixed on the

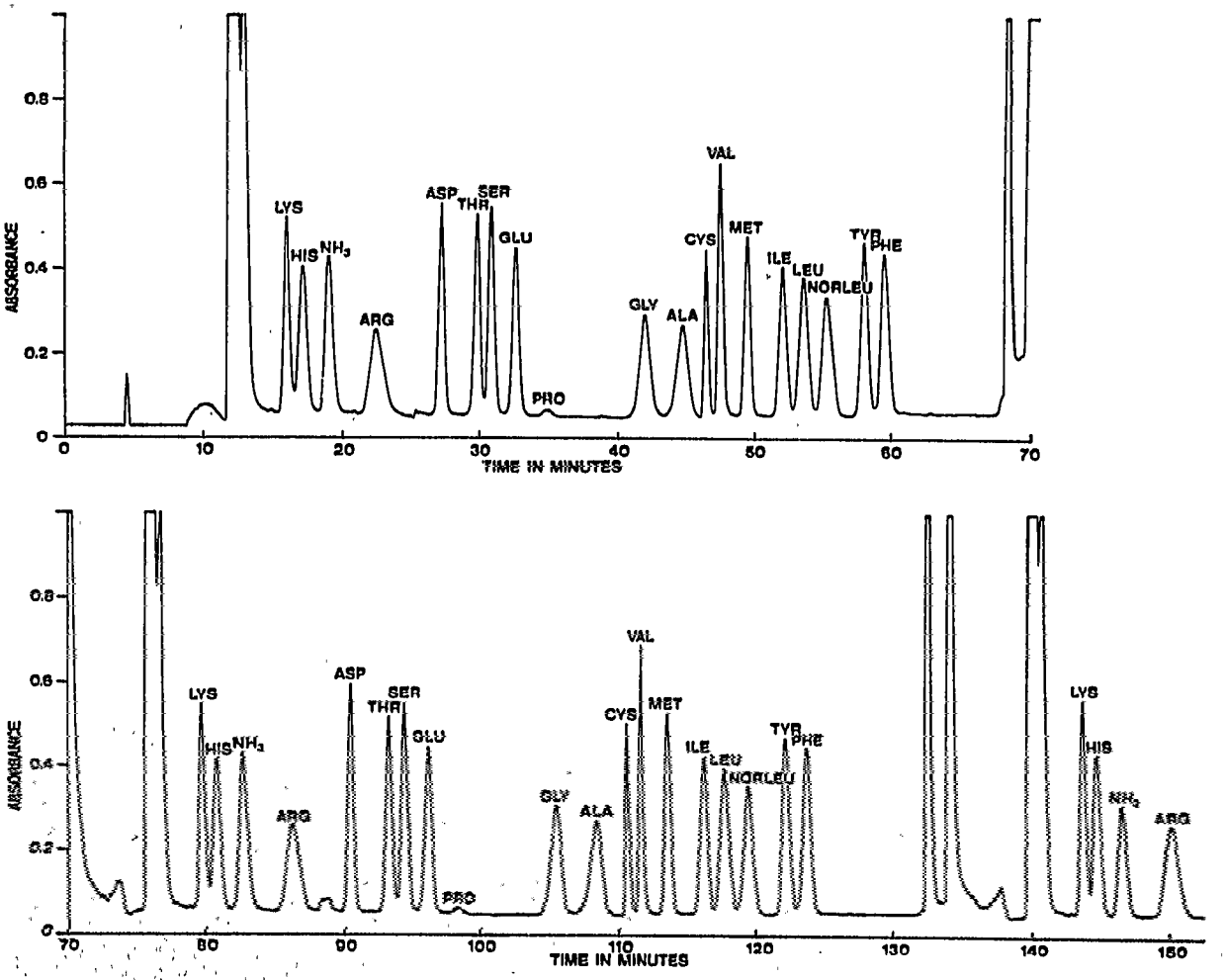

Fig. 3. Two sequential chromatograms of an amino acid standard run automatically. The sample contained 0.03 μmoles of each amino acid. Absorbance read at 570 mμ.

cartridge resin. On the other hand, we observed losses of aspartic acid, threonine and serine when more than 0.05 ml of wash buffer was applied. Sample residues in the filter led to increased σ_T^2 values for the initially eluted amino acids, in particular histidine and lysine. The peak broadening was further caused by a small gap which developed between the top column fitting and the resin bed during the first three runs. The column fitting had to be pushed into the resin again during operation and no gap ever developed again after more than 100 runs.

Fig. 4 shows a plot of the flow velocity and H_{obs} for the 5 mm column. H_{obs} was not corrected for extra column contributions to σ_T^2 and it is obvious from the data that the minimum value of H/v as required for the shortest separation time

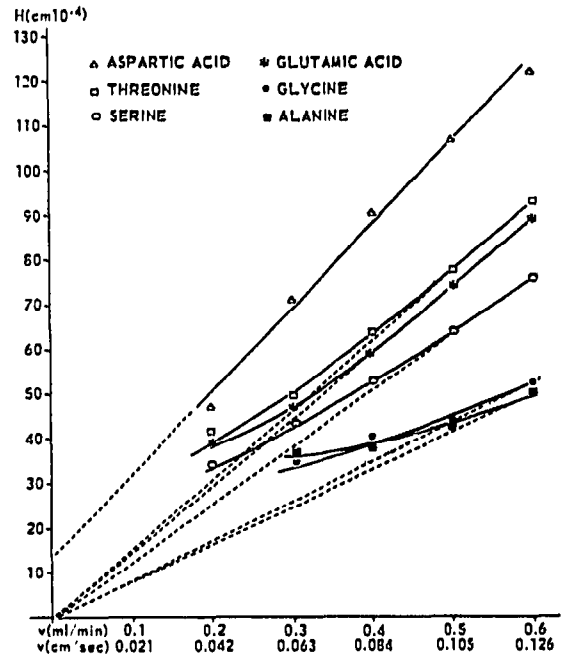

Fig. 4. Plot of H (H_{obs}) *versus* carrier velocity.

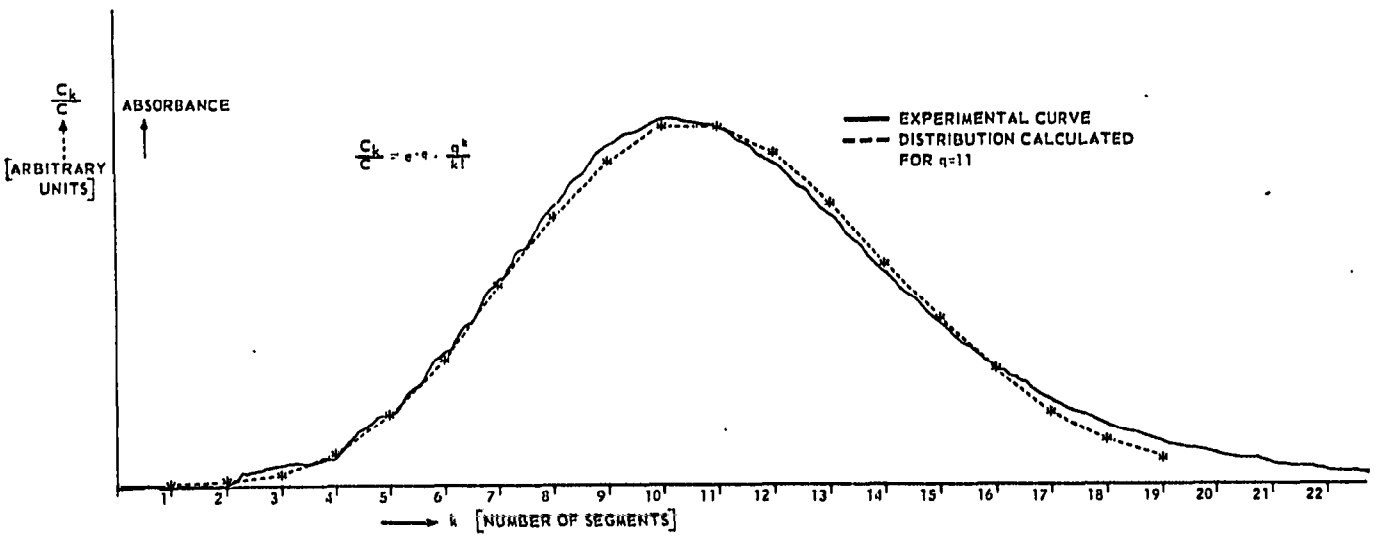

Fig. 5. Absorbance signal of amino acid sample injected into one liquid segment before entering reaction coil as compared to calculated distribution. For explanation, see text.

in the system (eqn. 2) is obtained at around 0.1 cm/sec within a practical flow rate and pressure range.

In order to prove that zone spreading in the analytical system occurs according to eqn. 10, a recorder with a high chart speed drive was used to trace the absorbance signal which resulted from a sample of amino acid solution injected between two bubbles before entering the analytical system (Fig. 5). It is obvious from the graph that the maximum of the curve is reached after approximately 11 segments. The Poisson distribution was therefore calculated for $q = 11$. The calculated curve fitted the experimental curve very closely. The slight deviation is due to the fact that $q = 11$ is an approximation only as well as to the small spreading contribution of the flow cell.

The time which elapsed until the recorder had reached its maximum pen deflection was measured. Based on the laws of the Poisson distribution this value is the variance in time units.

The spreading contributions resulting from sample loading and connections were negligible.

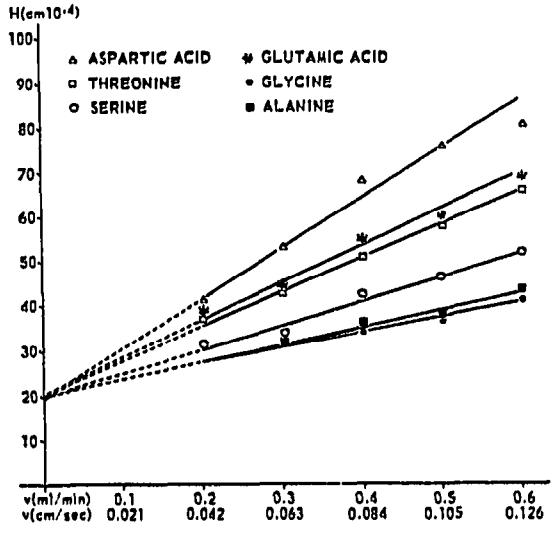

Fig. 6. Plot of H (H corrected for extra column contributions) *versus* carrier velocity.

In Fig. 6 the H values which were corrected for extra column contributions to band spreading are plotted against the flow velocity in the column. Though the chromatographic peaks are a complex superimposure of two spreading functions, *viz.* Gaussian for the column and Poissonian for the analytical system, the final peak was treated as if Gaussian because the column contribution in the flow rate range under investigation was in all cases at least twice that of the analytical system.

The values of H were calculated according to

$$H = L\frac{\sigma_T^2 - \sigma_E^2}{t^2} = L\frac{\sigma_C^2}{t^2} \qquad (12)$$

t was corrected for the delay time in the analytical system. The H values represent the mean of at least three measurements.

The graph shows that in accordance with earlier results[7] the data for an

individual amino acid lie on straight lines and their intercepts are approximately the same. If we assume "classical" eddy diffusion, $A = 2\lambda d_p$, we obtain a λ value of about unity which is in close agreement with the results of HAMILTON *et al.*[7]. Observations of PRETORIUS *et al.*[12] showed that the total mobile phase contributions to H in liquid chromatography columns of similar I.D. packed with uncoated glass beads become constant at flow rates which are in the range of those applied in our case. Their results indicate that the linear velocities at which a constant H is attained, decrease with the size of the particles, which are about twenty times larger than those used in our study. The constant H value for the smallest beads they used was around $2\,d_p$, which agrees with our findings. The results of PRETORIUS *et al.* strongly support the hypothesis of HAMILTON[7] and BOGUE[13] that velocity-dependent mobile phase effects are negligible in the flow rate range under investigation. Therefore, values for the solid phase diffusion coefficient of the individual amino acids could be calculated from the slopes of their curve and eqn. (11). The results are shown in Table II. The results agree with those of HAMILTON, though they were derived by different methods and obtained with resin material from different manufacturers.

We will report on precision data obtained for the system and its long-range reproducibility in a later publication.

TABLE II

DIFFUSION COEFFICIENTS IN STATIONARY PHASE

	Retention ratio	$D_s \times 10^7$ *cm·sec*$^{-2}$
Aspartic acid	0.28	1.15
Threonine	0.23	1.3
Serine	0.21	1.15
Glutamic acid	0.19	1.6
Glycine	0.125	1.7
Alanine	0.115	1.45

SYMBOLS

A = Eddy diffusion term.
D_m = Molecular diffusion coefficient in mobile phase.
D_s = Molecular diffusion coefficient in solid phase.
d_p = Mean particle diameter.
H = Plate height.
H_{obs} = Plate height not corrected for extra column effects.
H_s = Plate height due to solid phase resistance.
L = Column length.
Δp = Pressure drop.
N = Required number of theoretical plates for given resolution.
R = Retention ratio.
ΔR = Difference of retention ratios of peaks to be separated.
t = Retention time.
R_s = Resolution.

t_s = Separation time.
v = Linear flow velocity.
λ = Eddy diffusion coefficient.
σ_T^2 = Total variance, time units.
σ_E^2 = Extra column contributions to total variance, time units.
σ_C^2 = Variance of column, time units.

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