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OPERATING PARAMETERS IN HIGH-PERFORMANCE DISPLACEMENT CHROMATOGRAPHY*

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SUMMARY

Preparative scale separation of basic amino acids, dipeptides and nucleic acid fragments was carried out by displacement chromatography on microparticulate octadecyl-silica and siliceous strong cation-exchanger columns of dimensions generally employed in analytical high-performance liquid chromatography (HPLC). Neat aqueous carriers were used along with the following displacers: tetrabutylammonium and benzyltributylammonium salts and *n*-butanol. The concentration of products in the column effluent was determined by HPLC analysis. The results reveal the complexity of the displacement process and illustrate the effect of operating conditions on the rate of production and the yield of pure products. A decrease in flow-rate reduces the zone overlap, but this increase in separation is at the expense of production rate. Separation efficiency improves at elevated temperature, but without the untoward effect on the rate of production. The selection of column length was found to be a critical parameter; best results are obtained with the shortest column that allows the system to reach isotachic conditions, *i.e.*, full development of the displacement train.

The solubility of the feed components imposes constraints on the chromatographic system as far as the obtainable throughput, maximum product concentration and efficiency of separation are concerned. The yield of a component is affected by its relative amount in the feed and generally increases with the feed load provided that the length of the column is increased accordingly. The amount that can be separated in a chromatographic run increases with the column length in displacement chromatography. Octadecyl-silica is preferred over siliceous ion-exchangers as the stationary phase owing to its higher capacity, when reversed-phase chromatography can be used for the separation.

INTRODUCTION

Advances in column technology and instrumentation are mainly responsible for the development of high-performance liquid chromatography (HPLC) as a lead-

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ing analytical tool of high efficiency and versatility[1]. In most applications of HPLC, as in those of gas chromatography, linear elution analysis is the predominant mode of operation. Although most mobile phase constituents exhibit non-linear chromatographic behavior in HPLC[2,3], the elution of the sample components, which migrate with different velocities, is nearly a linear process. As a result they elute as quasi-Gaussian peaks whose retention and shape are independent of their concentration and they are assumed to move through the column without interference[4]. For this reason together with the facility of monitoring and quantifying eluite peaks in the column effluent, the linear elution mode has great advantages in analytical work. These features account for the wide popularity of both gas chromatography and HPLC as preeminent analytical tools.

Recent work from our laboratory, however, suggests that the high efficiency columns and precision instrumentation of HPLC can also be employed for chromatographic separations in the displacement mode to separate mixtures at significantly higher concentrations than possible in the linear elution mode under otherwise comparable conditions. Thus the displacement mode appears to be particularly suited to preparative scale separations. The potential of displacement chromatography was recognized two score years ago by Tiselius[5], its feasibility has been amply demonstrated and its theoretical foundation is well established[6]. Yet the wide popularity and simplicity of elution analysis, the lack of suitable columns in liquid chromatography and certain complications associated with the non-linear nature of displacement chromatography have hindered the development of the technique. In fact, the displacement mode has rarely even been mentioned in recent monographs on chromatography.

Displacement chromatography is characterized by sample component concentrations high enough to lie in the non-linear region of the distribution isotherm. In order to support these high concentrations the mobile phase carrier liquid is selected for high solubility of the sample components as well as adequate retention on the stationary phase in use. The column is equilibrated with the carrier and thereafter the feed mixture followed by a solution of a "displacer" compound is pumped into the column. The technique demands solute concentrations that are high relative to those in elution chromatography so that separation will be driven by strong interferences among the sample components in their adsorption behavior. This interference causes weakly retained components to move out ahead of those that are more strongly retained, until, in a well-designed process, the mixture separates into adjacent bands ranked in the order of their affinity for the stationary phase. The displacer interferes with the rear of the feed zone and prevents it from tailing, which would occur if the system were operated in the elution mode. In order to carry out this task the displacer must have higher affinity for the stationary phase than any of the sample components. As the displacer continuously enters the column its front drives the sample zones ahead of it at a high enough velocity that they remain adjacent to, and so in a position to interfere with, one another. This requirement is met by using a displacer concentration above some minimum value that depends, in principle, only on the relative positions of the individual adsorption isotherms[7]. Upon complete development of the displacement train the adjacent zones containing pure solutes reach their characteristic concentrations and travel with the same velocity as the displacer front. Even in the presence of slight kinetic and mass-transfer resistances and flow maldistribution

which cause bandspreading in elution chromatography, the zones may maintain their rectangular shape during further passage through the column due to the boundary sharpening effects of interference between zones[6]. The width of the zones after reaching this isotachic state is proportional to the amount of the respective feed components.

Here we report results of our continuing research in this field. Besides demonstrating the potential of displacement chromatography to separate preparative quantities of peptides, amino acids and nucleic acid constituents by using analytical HPLC columns, our main goal is to illustrate the effect of operating parameters on separation efficiency. The findings presented here are in agreement with the results of mathematical modeling based on a rigorous theoretical approach to non-linear chromatography[6] that are being prepared for publication[8].

EXPERIMENTAL

Materials, columns and reagents

Partisil SCX columns having dimensions of 250 × 4.6 mm or 200 × 4.6 mm packed with 10-μm siliceous cation-exchanger were obtained from Whatman (Clifton, NJ, U.S.A.). Supelcosil LC-18 columns (150 × 4.6 mm or 250 × 4.6 mm) packed with 5-μm octadecyl-silica were obtained from Supelco (Bellefonte, PA, U.S.A.). Octadecyl-Spherisorb having a carbon load of 15% (w/w) was prepared from 5-μm Spherisorb silica gel by the procedure of Kováts and Boksányi[9] and packed into 150 × 4.6 mm and 250 × 4.6 mm stainless steel columns. Histidine, arginine, adenosine, deoxyadenosine, inosine, deoxyinosine, adenosine-2′-, -3′- and -5′-monophosphates (AMPs), L-methionyl-L-proline, L-phenylalanyl-L-proline, L-valyl-L-valine, L-glycyl-L-leucine, L-leucyl-L-valine and L-phenylalanyl-L-valine were purchased from Sigma (St. Louis, MO, U.S.A.). Cytosine, cytidine, adenine sulfate, tertrabutylammonium bromide (TBA), and benzyltributylammonium chloride (BTBA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile, methanol, *n*-butanol, H_3PO_4, NaH_2PO_4, Na_2HPO_4, acetic acid and sodium acetate were from Fisher Scientific (Pittsburgh, PA, U.S.A.). EDTA disodium salt was from Baker (Phillipsburgh, NJ, U.S.A.). Distilled water was prepared with a Bronsted distilling unit.

Apparatus

Two fractionator units were used for displacement chromatography. The system used for the peptide separations was described earlier[10]. It consists of two pumps connected via a switching valve to the injection valve in order to minimize dead volume and eliminate flow interruption during the switching operations from carrier to feed and feed to displacer solution. The only modification to this system was the addition of a Lauda Model WB-20/R recirculating water-bath (Brinkmann, Westbury, NY, U.S.A.) which allowed for temperature control of the jacketed column. The fractionator unit used for the other separations consisted of a Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.) and Model 7010 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a stainless steel loop of appropriate volume for each experiment. The solvent flow to the column —carrier, displacer or regenerant solution— was switched by the stop-flow method. The column effluent

was monitored by a Model LC-55 variable wavelength detector or a Model 250 A fixed wavelength UV detector at 254 nm (Perkin-Elmer, Norwalk, CT, U.S.A.). The detector signal was recorded by a Model SR-204 strip-chart recorder (Heath, Benton Harbor, MI, U.S.A.). Fractions containing approximately 250 μl or 100 μl, depending on the type of experiment, of the effluent were collected with an Ultrarack II, No. 2070 fraction collector (LKB, Rockville, MD, U.S.A.).

Two analytical HPLC systems were used to analyze the fractions. In the experiments with peptides the instrument consisted of an Altex Model 100 solvent metering pump connected through a Rheodyne Model 7010 sampling valve with a 20-μl loop to a Perkin-Elmer LC-65T detector-oven. The signal was recorded on an Electronik 194 strip chart recorder (Honeywell, Ft. Washington, PA, U.S.A.). In the other experiments fractions were analyzed by an HPLC unit containing a Model LC 250/1 pump (Kratos, Westwood, NJ, U.S.A.), a Rheodyne Model 7010 sampling valve with a 20-μl sample loop and a Kratos Model 770 variable wavelength UV detector. Chromatograms were obtained with a Heath Model SR-206 dual pen strip-chart recorder. Fractionation was carried out with two [(250 + 200) × 4.6 mm] or three [(2 × 250 + 200) × 4.6 mm] Partisil SCX columns in series for cation-exchange displacement chromatography and two [(250 + 150) × 4.6 mm] or three [(2 × 150 + 250) × 4.6 mm] Supelcosil LC-18 or one (250 × 4.6 mm) or two [(2 × 250) × 4.6 mm] octadecyl-Spherisorb columns in series for the reversed-phase displacement chromatography. All analytical separations were carried out by using octadecyl-Spherisorb columns (150 × 4.6 mm) at room temperature unless otherwise noted.

Procedures

Fractionation. In experiments for peptide separations, the feed loop was filled with the feed mixture and then pressurized by the displacer pump while the carrier flowed through the temperature-regulated column at a measured flow-rate. When the pressure in the feed/displacer stream matched that of the carrier flow, the switching valve was turned so the feed solution entered the column ahead of the displacer. Effluent fractions were collected at time intervals such that the fractions measured 100 μl or less. After turning the switching valve the effluent was collected in a graduated cylinder in order to measure the flow-rate. In the other displacement experiments, the loop of the feed injector valve was filled with the feed solution dissolved in the carrier. At the same time, the reservoir of the displacer solution was connected to the pump by a stop-flow method. Subsequently the displacer solution was pumped into the column at a flow-rate of 0.1 ml/min and the injector valve was moved into the feed position. Fractions of the column effluent were collected in 2.5- or 1-min intervals depending on the type of experiment. The collection and numbering of fractions began upon turning the feed valve.

Column regeneration. After the emergence of the displacer front, the column was regenerated. In experiments with Partisil SCX columns each of the following solutions was pumped through the column at a flow-rate of 3 ml/min for 15 min: buffer solution having the same components as the carrier previously used in displacement but 200 times greater in ionic strength, distilled water, 0.5 *M* phosphoric acid solution in water, distilled water, 0.1 *M* EDTA disodium salt solution, distilled water, methanol or acetonitrile and distilled water. Finally the carrier solution was applied before the next displacement run. In reversed-phase displacement with benzyltributyl-

ammonium chloride (BTBA) or tetrabutylammonium bromide (TBA) used as displacer, regeneration of the column was less complicated. The column was first washed with distilled water for 15 min at a flow-rate of 3 ml/min, then with 100 ml of acetonitrile containing 0.5 *M* phosphoric acid. The column was then washed with distilled water and re-equilibrated with the carrier to prepare for the next run. Reversed-phase displacement with *n*-butanol as displacer needed only a one-step regeneration with 100 ml acetonitrile at 3 ml/min. After this step the carrier solution was directly applied to equilibrate the column before the next displacement run. The regeneration of the column was tested by elution analysis of the feed mixture using the carrier as eluent and a 20-μl loop in place of the preparative loop in the injection valve of the fractionator unit. The column was considered as properly regenerated when the respective retention factors did not deviate more than $\pm 2\%$ from the original values.

HPLC analysis of fractions. The analyzer unit was equipped with a 15 cm long octadecyl-Spherisorb column and the eluent was selected for rapid separation and quantification of the sample components. For instance, in the case of displacement separation of histidine and arginine on cation-exchange, the two amino acids were determined by using 50 m*M* phosphate buffer, pH 2.0, containing 5 m*M* sodium octyl sulfate and 17% (v/v) acetonitrile as the eluent at a flow-rate of 1.5 ml/min. The column effluent was monitored at 210 nm. No attempt was made to enhance the sensitivity of detection by using a post column reactor since the displacement development yielded highly concentrated fractions. In fact, each fraction was diluted 40-fold with a solution of alanine used as the internal standard and 20-μl aliquots were injected. For the analysis of fractions containing nucleic acid fragments the eluent was 50 m*M* acetate buffer, pH 3.5, at a flow-rate of 1.5 ml/min and the column effluent was monitored at 258 nm. The fractions were diluted 200-fold with a solution of cytosine used as the internal standard and 20-μl aliquots were injected. Analysis of the peptides was carried out with a mixture of 50 m*M* phosphate buffer, pH 2.0, and methanol as the eluent at a column temperature of 60°C.

Construction of displacement diagrams

The displacement diagrams illustrating product composition of the effluent fractions from a displacement run are constructed as histograms in which the overall length of the bar for each fraction represents the total concentration of product in that fraction, as indicated by the scale to the left of the diagram. The bars representing fractions containing more than one substance are broken into differently shaded areas according to the fraction of the concentration represented by each species. A bold line is drawn through the mixed region to further demarcate each product. In most cases the breakthrough of the displacer front is illustrated by a vertical line without showing the displacer content of the fractions.

Yield and overall yield

The yield of a pure product is expressed by the percentage of a feed component that is recovered without contamination by the other components as measured from the displacement diagram. The overall yield is the mass-average value of the yields calculated for all components.

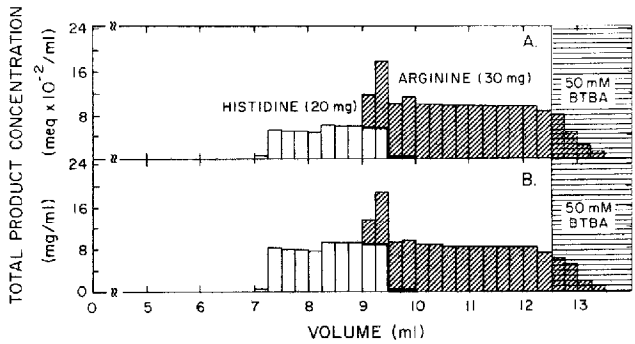

Fig. 1. Displacement diagrams of histidine and arginine constructed from data obtained by cation-exchange chromatography and HPLC analysis of the effluent fractions. Total product concentrations are expressed by mequiv./ml (A) and mg/ml (B). Column: 10-μm Partisil SCX [(200 × 4.6 mm) + (250 × 4.6 mm)]. Carrier: 1 m*M* phosphate buffer, pH 6.5. Displacer: 50 m*M* benzyltributylammonium chloride in the carrier. Flow-rate: 0.1 ml/min. Fraction volume: 250 μl. Temperature: 22°C. Feed: 20 mg histidine and 30 mg arginine in 0.5 ml of carrier.

Production rate or throughput

The rate of production for a component is calculated as the amount recovered in pure form, according to the displacement diagram, divided by the sum of the feed-loading time and the breakthrough time of the displacer. The overall production rate is the total amount of pure products in the effluent divided by the sum of feed time and displacer breakthrough time. The rate of production is sometimes referred to as the throughput.

RESULTS AND DISCUSSION

Applications

Separation of arginine and histidine on cation-exchanger. Displacement chromatography of amino acids may play an important role in fractionation of protein hydrolysates. In fact, Bendall *et al.*[11] and Partridge and co-workers[12–14] used this technique more than 30 years ago to separate amino acids on both cation- and anion-exchange resins by using salts or acids as the displacer. However, the overall efficiency of separation was rather poor and their results suggest that elution and displacement took place concurrently.

We have examined the separation of arginine and histidine on a siliceous microparticulate cation-exchanger column, which is generally used in HPLC for separating basic amino acids, with benzyltributylammonium chloride (BTBA) as the displacer. Typical results are shown in Fig. 1 and it is seen that the two amino acids are present in essentially pure zones when the displacement train is fully developed. The tailing of arginine into the displacer zone may be due to backmixing in the feed loop after switching from feed to displacer.

In Fig. 1 the displacement diagrams were constructed with two concentration scales for the amino acids in the column effluent: mequiv./ml (A) and mg/ml (B). When the concentration is expressed on a milliequivalents basis the first product zone has a lower concentration than the second as is usually observed in displacement chromatography. The concentrations shown in Fig. 1A were calculated by assuming

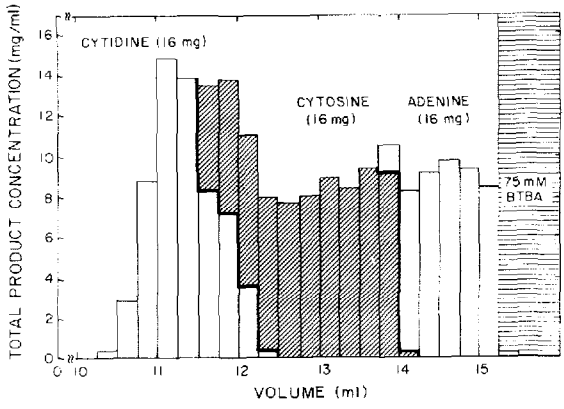

Fig. 2. Displacement diagram of cytidine, cytosine and adenine, constructed from data obtained by cation-exchange chromatography and HPLC analysis of the effluent fractions. Column: 10-μm Partisil SCX [2 × (250 × 4.6 mm) + (200 × 4.6 mm)]. Carrier: 1 mM phosphate buffer, pH 2.0. Displacer: 75 mM benzyltributylammonium chloride in the carrier. Flow-rate: 0.1 ml/min. Fraction volume: 250 μl. Temperature: 22°C. Feed: 16 mg of each component in 2.0 ml of carrier.

that histidine has only one protonated amino group, *i.e.*, the imidazole group is neutral, whereas arginine has two positive charges at pH 6.5 (ref. 15).

Due to the low capacity of the siliceous cation-exchanger a 45 cm long column was required to obtain satisfactory separation of the mixture containing 20 and 30 mg of histidine and arginine, respectively. The carrier was 1 mM phosphate buffer, pH 6.5. With this buffer as the eluent the respective retention factors of the two amino acids were 15 and 46 on the same stationary phase.

Separation of nucleic acid constituents on cation-exchanger. Fig. 2 shows the displacement diagram of cytidine, cytosine and adenosine. The carrier was 1 mM phosphate buffer, pH 2.0, and displacement chromatography was carried out on a

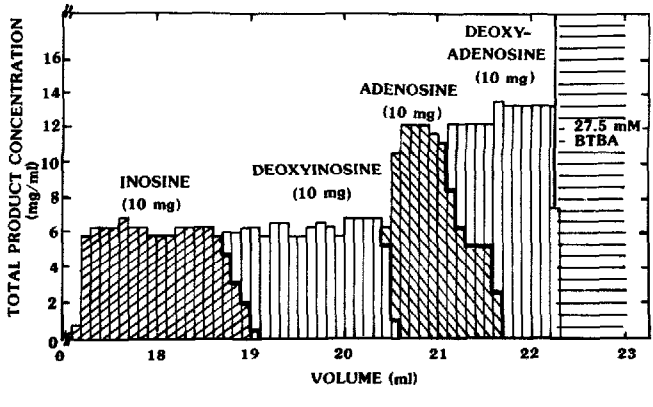

Fig. 3. Displacement diagram of inosine, deoxyinosine, adenosine and deoxyadenosine from data obtained by reversed-phase chromatography and HPLC analysis of the effluent fractions. Column: 5-μm Supelcosil LC-18 [(250 × 4.6 mm) + (150 × 4.6 mm)]. Carrier: 10 mM acetate buffer, pH 5.0. Displacer: 27.5 mM benzyltributylammonium chloride in the carrier. Flow-rate: 0.1 ml/min. Fraction volume: 100 μl. Temperature: 22°C. Feed: 10 mg of each component in 2.0 ml of carrier.

mixture containing 16 mg of each nucleo-base in 2.0 ml of carrier using a 70 cm long siliceous cation-exchanger column obtained by connecting two 25 cm and a 20 cm long column in series. The displacer was 75 m*M* BTBA in the carrier. In Fig. 2 the concentrations are not expressed on a milliequivalent basis because of the uncertainty as to their degrees of ionization[16] at pH 2. This may be the reason that product concentrations in the early zones appear to be higher than in the last zone of adenine.

With the carrier as the eluent the retention factors, k', on the cation-exchanger of cytidine, cytosine and adenine were 9, 19 and 31, respectively, in the elution mode. Thus the order of elution and displacement is the same for all components.

It will be shown later that the amount of feed and the degree of separation can be increased by employing a longer column. Nevertheless the relatively low capacity of siliceous ion-exchangers may be an impediment to the effective use of these columns for preparative-scale separations. The high capacity of conventional ion-exchange resin may be of advantage, though kinetic and mass transfer resistances in such column materials and the possibility of flow maldistribution as a result of swelling and shrinking of the column packing might offset the benefits of higher capacity.

Separation of nucleosides on octadecyl-silica. A displacement diagram of four nucleosides and deoxynucleosides is depicted in Fig. 3. The separation was obtained on a 40 cm long column packed with 5-μm octadecyl-silica by using a 10 m*M* acetate buffer, pH 5.0, and 27.5 m*M* BTBA as the carrier and displacer, respectively. It is seen that satisfactory separation of a 40-mg mixture could be accomplished by an analytical column.

Separation of nucleotides on octadecyl-silica. The displacement diagram in Fig. 4 illustrates the separation of adenosine, 2′-AMP, 3′-AMP and 5′-AMP on a 40-cm octadecyl-silica column by using *n*-butanol as the displacer. In this case a neutral substance was chosen as the displacer in order to avoid untoward interactions with the nucleotides. Since *n*-butanol has relatively strong affinity to the hydrocarbonaceous stationary phase and is sufficiently soluble in the carrier it is a suitable

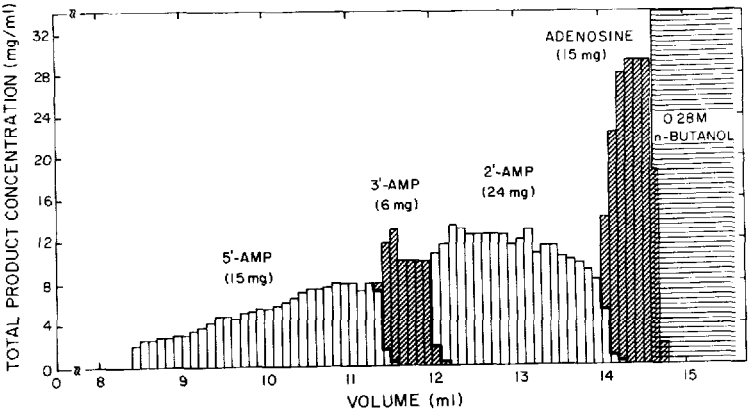

Fig. 4. Displacement diagram of 5′-AMP, 3′-AMP, 2′-AMP and adenosine from data obtained by reversed-phase chromatography and HPLC analysis of the effluent fractions. Column: 5-μm Supelcosil LC-18 [(250 × 4.6 mm) + (150 × 4.6 mm)]. Carrier: 10 m*M* acetate buffer, pH 5.0. Displacer: 0.28 *M* *n*-butanol in the carrier. Flow-rate: 0.1 ml/min. Fraction volume: 100 μl. Temperature: 22°C. Feed: 15 mg of 5′-AMP, 6 mg of 3′-AMP, 24 mg of 2′-AMP and 15 mg of adenosine in 1.5 ml of carrier.

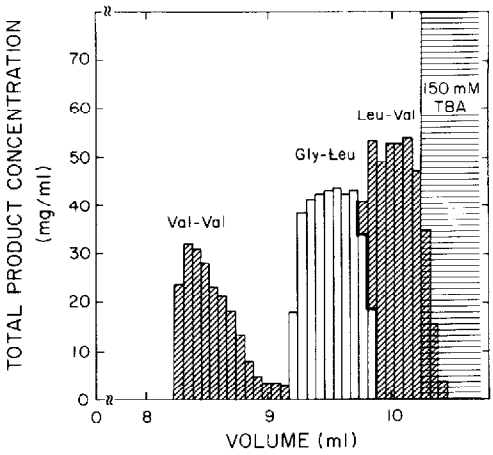

Fig. 5. Displacement diagram of three dipeptides constructed from data obtained by reversed-phase chromatography and HPLC analysis of the effluent fractions. Column: 5-μm Spherisorb-ODS [2 × (250 × 4.6 mm)]. Carrier: 50 mM phosphate buffer, pH 2.0. Displacer: 150 mM tetrabutylammonium bromide in the carrier. Flow-rate: 0.72 ml/min. Fraction volume: 72 μl. Temperature: 30°C. Feed: 20 mg of valylvaline, 30 mg of glycylleucine and 30 mg of leucylvaline in 1.0 ml of carrier.

displacer as was demonstrated earlier[10]. Another advantage of *n*-butanol is that it is volatile, so that it can readily be removed from the product if necessary.

The results shown in Fig. 4 were obtained with 0.28 M *n*-butanol in order to avoid the precipitation of adenosine on the column that had been observed at higher displacer concentration. On the other hand, the nucleotides themselves could be separated at higher butanol concentrations and no precipitation of them occurred when 0.5 M butanol was used as the displacer.

Separation of dipeptides. Peptides were among the first substances to be separated by displacement chromatography in a system that used a column packed with charcoal[17,18]. More recently, reversed-phase chromatography has become the preeminent method for separation of these and other biological compounds[19,20]. We have found that analytical columns packed with octadecyl-silica are also suitable for rapid separation of peptides in preparative amounts by using HPLC equipment. Fig. 5 shows the displacement diagram obtained with a mixture of three peptides. The carrier was 50 mM phosphate buffer, pH 2.0, and tetrabutylammonium bromide (TBA) was used as the displacer. The feed contained 80 mg of the dipeptide mixture. As seen, nearly 80% of this amount was recovered in pure form in less than 15 min by using a 50-cm column. This displacement diagram shows that the least retained component, valylvaline, does not have a rectangular shape, indicating that development of the displacement train is not complete although it is separated from the other components with a nearly 100% yield. Since further displacement development would result in lower product concentrations, the separation depicted in Fig. 5 can be considered more efficient for production of valylvaline than it would be under isotachic conditions.

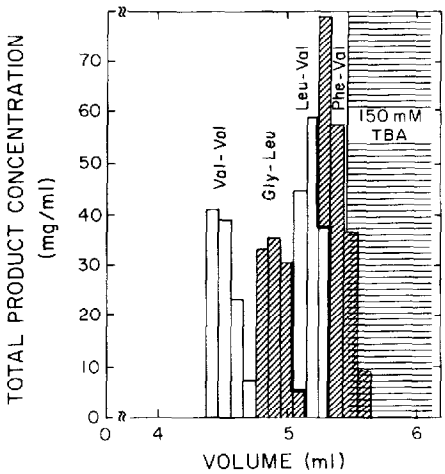

Fig. 6. Displacement diagram of a four-component dipeptide mixture constructed from data obtained by reversed-phase chromatography and HPLC analysis of the effluent fractions. Column: 5-μm Spherisorb-ODS (250 × 4.6 mm). Carrier: 50 mM phosphate buffer, pH 2.0. Displacer: 150 mM tetrabutylammonium bromide in the carrier. Flow-rate: 0.14 ml/min. Fraction volume: 98 μl. Temperature: 60°C. Feed: 10 mg of valylvaline, 12.5 mg of glycylleucine, 12.5 mg of leucylvaline and 15 mg of phenylalanylvaline in 0.5 ml of carrier.

Fig. 6 shows the separation of a more complex, four-component dipeptide mixture. In this experiment almost half the 50 mg of feed was recovered in pure form using a 25-cm analytical column packed with octadecyl-silica. As discussed later, an increase in both the feed amount and column length could have resulted not only in increasing production rate, but also in a higher yield for the individual components.

Effect of operational parameters

Carrier. The carrier may be a pure solvent, a solvent mixture or a solution such as a buffer. The void space of the column is occupied by, and the stationary phase is equilibrated with, the carrier solvent prior to introduction of the feed components. In most cases the carrier serves as the solvent for the feed and displacer as well. Consequently it must be a good solvent for these substances, yet it has to allow relatively strong binding of them to the stationary phase. Preferably the carrier is also volatile in order to facilitate product recovery.

Elution analysis by HPLC or thin-layer chromatography[21,22] can be conveniently used in the search for an appropriate carrier to be used with the stationary phase selected for the separation by displacement chromatography. It is desireable that the components have high retention factors, k', or low R_F values and it is a *sine qua non* in displacement chromatography that the carrier is selected so that the retention of the displacer is greater than that of any of the feed components.

In both ion-exchange and reversed-phase chromatography aqueous carriers of appropriate ionic make-up and/or organic solvent content are preferable. For instance, in the displacement development of nucleo-bases and nucleosides described above on strong cation-exchange columns the pH and ionic strength of the carrier play an important role. The displacement of these substances as shown in Fig. 2 is

possible because the components are strongly bound to the cation-exchanger stationary phase from the carrier at pH 2.0. This was established by elution experiments that showed that phosphate buffer with a pH of 2.0 gives the highest retention for the feed components and the displacer under practicable operating conditions.

Displacement chromatography of nucleosides and nucleotides, *cf.*, Fig. 4, on the other hand, required a neutral displacer and we selected *n*-butanol for this purpose. Since these substances are weakly retained at low pH[23,24], the pH of the carrier was set at 5.0 where they are strongly retained. The advantage of a neutral displacer rests with its pH-invariant retention so that the carrier pH can be adjusted to maximize the retention of the feed components. The displacement of nucleosides on octadecyl-silica, *cf.*, Fig. 3, was accomplished by using an acetate buffer, pH 5.0, as the carrier because nucleosides are strongly retained from this medium by the nonpolar stationary phase, whereas the affinity of the benzyltributylammonium chloride displacer to the stationary phase is not affected by the pH of the carrier.

If the retention of the feed components and of the displacer from a neat aqueous carrier is too high in reversed-phase chromatography, an aqueous carrier containing a suitable organic solvent can be considered. In fact, a water-rich hydro-organic carrier was successfully used in displacement chromatography of polymyxins[22].

Displacer. In a displacement chromatographic run the zone concentration, degree and time of separation are determined primarily by the nature and concentration of the displacer[7] when all other parameters are kept constant. In reversed-phase or ion-exchange displacement chromatography, which are investigated in this report, the siliceous nature of the stationary phase limits the choice of displacer that can be used. In this work benzyltributylammonium chloride (BTBA) was used for the displacement of basic amino acids, nucleo-bases and nucleosides on a cation-exchanger column. BTBA is highly soluble in the carrier employed, its breakthrough is conveniently detected with UV detector and we have established by elution analysis that it is more retained than the feed components in the stationary phase-carrier systems investigated. It is believed that other quaternary ammonium salts could be successfully used in this application.

In reversed-phase chromatography as well, the displacement of positively charged species is conveniently carried out with positively charged amphiphilic substances such as quaternary ammonium salts having relatively long alkyl chains since these species have strong affinity to hydrocarbonaceous stationary phases. Therefore both BTBA and TBA were employed in reversed-phase displacement chromatography of peptides and nucleosides. When the feed contains negatively charged components, however, the displacer should not carry a positive charge but be neutral in order to avoid association between it and the feed components. Therefore the separation of a mixture of nucleotides and nucleosides on an octadecyl-silica column was carried out with butanol as the displacer.

Fig. 7 shows qualitatively the lower and the upper limits for the displacer concentration of a hypothetical binary mixture. As seen the lower limit of the displacer concentration is defined by the operating line on the left that just touches the isotherm of the least retarded feed component. If a lower displacer concentration is used the least retained component would elute ahead of the displacement train. The upper limit here is given by the saturation concentration for the more retarded feed

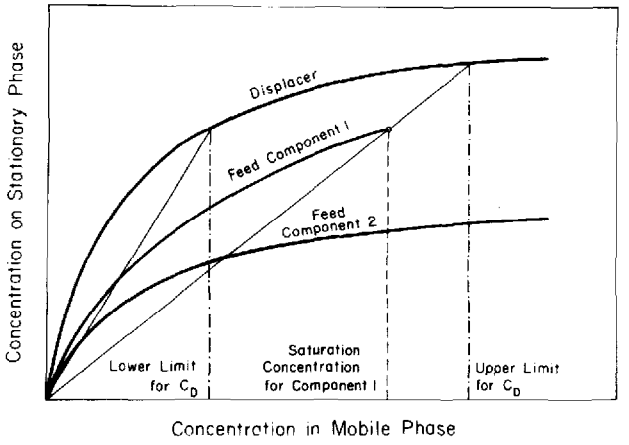

Fig. 7. Parameters defining the region of operation in displacement chromatography. The lower bound on displacer concentration is given by the point on the displacer isotherm that yields an operating line which coincides with the initial slope of the isotherm of the least-retained substance. An upper bound is the point where the operating line intersects any of the isotherms at the solute's mobile phase saturation concentration: beyond this point the isotherm is undefined.

component where the operating line on the right intersects its isotherm. Use of a higher displacer concentration would cause this component to precipitate.

Thin-layer chromatography as a tool for scouting for a suitable displacer was discussed in an earlier report[21]. Unfortunately neat aqueous carriers do not move on octadecyl-silica plates and plates coated with a siliceous cation-exchanger equivalent to Partisil SCX are not available. Consequently in our case elution analysis by using the appropriate column provided the retention data needed for the selection of the displacer and its concentration according to the operational requirements imposed on the system.

Generally, high displacer concentration is advantageous in order to reduce separation time and to increase the zone concentrations of the products since the isotachic product concentration increases with the displacer concentration. Therefore

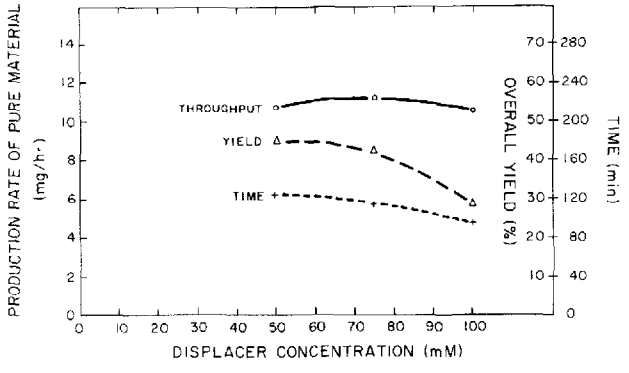

Fig. 8. Graph illustrating the dependence on the displacer concentration of overall yield, time of separation and rate of production for fractionation of a mixture of histidine and arginine by cation-exchange chromatography. Conditions, excepting displacer concentration, are the same as those in Fig. 1.

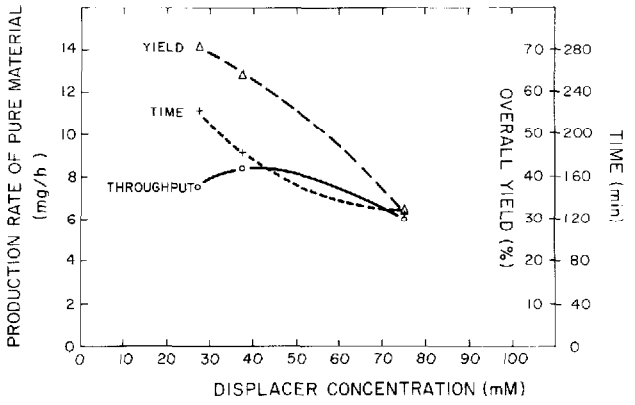

Fig. 9. The effect of displacer concentration in reversed-phase displacement chromatography of a mixture of inosine, deoxyinosine, adenosine and deoxyadenosine by reversed-phase chromatography. Conditions, excepting displacer concentration, are the same as those in Fig. 3.

the rate of production is strongly affected by the concentration of the displacer. The role of displacer concentration in determining the overall yield and time of separation is illustrated in Fig. 8 for the separation of histidine and arginine on siliceous strong cation-exchanger by using BTBA as the displacer. The results shown are the mean values of those calculated for the two amino acids individually. It is seen that in the particular range of BTBA concentration the production rate does not change appreciably although an optimum displacer concentration of approximately 75 m*M* can be discerned from the graph. It is seen in Fig. 8 that the time of separation defined by the breakthrough time for the displacer decreases from 125 to 96 min when the concentration of BTBA is increased from 50 to 100 m*M* whereas the yield of pure product decreases by approximately 60% for histidine and 70% for arginine. In this example there was no solubility problem since BTBA is quite soluble in water at 25°C and the saturation concentrations of histidine and arginine are about 42 and 150 mg/ml respectively. Slow displacement kinetics may contribute to the decrease in product purity when increasing the displacement speed.

The data obtained in the reversed-phase displacement chromatography of inosine, deoxyinosine, adenosine and deoxyadenosine were pooled to illustrate the overall rate of production and yield as well as the time of separation as shown in Fig. 9. The results demonstrate that there is an optimum displacer concentration with respect to maximum production rate even if its exact value is not known here because of the limited data. In these experiments the carrier was 10 m*M* acetate buffer pH 5.0 and the concentration of BTBA varied from 27.5 to 75 m*M*. Fig. 9 shows that the overall yield decreases from 70 to 30% when the displacer concentration is increased from 27.5 to 75 m*M*.

Experiments were also carried out with this system using 200 m*M* BTBA as the displacer. Under such conditions the four components emerged from the column as a colloidal suspension in less than 0.4 ml of the effluent and crystallized shortly thereafter. Of course, no separation was obtained under such conditions. Due to such solubility problems the displacer concentration in this type of separation is preferably low, *e.g.*, about 30 m*M* BTBA.

Since in both reversed-phase and cation-exchanger columns BTBA was used as the displacer we may compare the two systems. As expected, the capacity of the Partisil SCX column toward BTBA was found to be less than that of the Supelcosil LC-18 column as measured by the breakthrough volume of the displacer. The relatively low capacity of the siliceous ion-exchanger also manifested itself in the approximately three times lower zone concentration of arginine than of deoxyadenosine although the retention factors of the two substances were about the same on both columns. For the separation of small molecules, at least, the relatively high capacity of hydrocarbonaceous bonded phases appears to be a distinct advantage and the use of siliceous ion-exchanger is justified only if the feed components are too weakly or too strongly bound to non-polar bonded phases.

Flow-rate. The flow-rate in most separation processes is a key parameter in optimizing throughput. In displacement chromatography it has been demonstrated[10] that increasing the flow-rate within a practicable operating range has a deleterious effect on yield. On the other hand comprehensive theories of this process[6,25] have been based on certain assumptions of ideality, *viz.*, infinitely rapid mass transfer or displacement kinetics and uniform flow field, and does not predict the occurrence of such flow-rate effects. The observed non-ideal behavior, however, results in flow-rate-dependent separation efficiency and the effect of flow-rate is rather complicated since both flow-rate and column length influence separation. The experimental finding[10] that the residence time *per se* is a sufficient parameter has to be considered as fortuitous and most likely attributable to diffusional and kinetic resistances and flow maldistribution which may be exacerbated at the high concentrations pertaining in displacement chromatography. Fig. 10 shows the same separation as that depicted in Fig. 6 but at a higher flow-rate. Although the flow-rate of 0.86 ml/min employed for the separation shown in Fig. 10 is near the range employed in analytical HPLC, the separation is much less efficient than that achieved at a significantly lower flow-rate of 0.14 ml/min, *vide* Fig. 6. In fact, no pure fractions of leucylvaline or phenylalanyl-

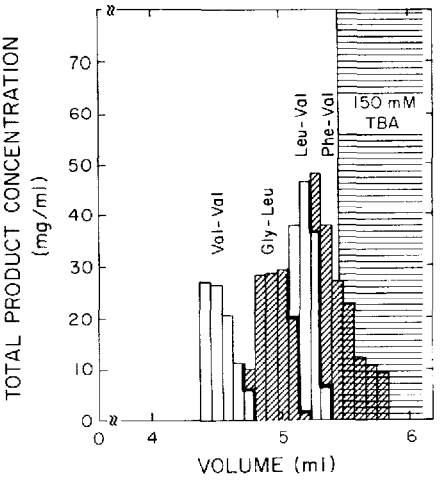

Fig. 10. Effect of flow-rate on resolution in displacement chromatography. Conditions are identical to those in Fig. 6, excepting flow-rate, 0.86 ml/min and fraction volume, 86 μl.

valine were obtained at the higher flow-rate. Thus departures from ideal chromatography manifest themselves by the dependence of the yield on the flow-rate in displacement chromatography and as a result, operation at high flow velocities is done at the expense of decreased yields. An important approach to improving the throughput to the system, therefore, is to discover means for reducing these untoward kinetic and mass transfer resistances and flow non-uniformities and thus permit the use of higher flow-rates. Therefore displacement chromatography greatly benefits from the development of microparticulate columns. At sufficiently low flow-rates neither the pressure drop across the column nor non-uniform radial temperature profiles due to viscous dissipation[26] are expected to be significant impediments to achieving high separation efficiency.

Column length. The volume is the characteristic column property of a given displacement system that determines whether, under the appropriate set of operating conditions, development is completed and the isotachic state reached, or it is arrested at some point in the transient period. At fixed column diameter, however, the column length is also an acceptable and more conveniently measured expression for the amount of stationary phase employed for a separation. Therefore, under a set of operating conditions which yields a fully developed displacement train it is appropriate to define a minimum column length required for the separation. With shorter columns, full development is not achievable without altering other system properties. If the column is longer than the minimum the displacement train traverses the excess length without change, in the absence of the above mentioned secondary effects. In practice, however, the movement of the displacement train through the excess column length results in a deterioration of the yield and an increase in separation time. One goal of investigating the course of development in the transient period[8] is to predict *a priori* the length of column needed to perform a separation so that the maximum production rate can be obtained.

Tables I and II summarize the results of two experiments on ion-exchange and reversed-phase columns. It is seen that in both cases the use of a longer column improved the yield of pure material. These data indicate that the displacement development came closer to completion when the column length was extended. The tables reveal that the improvement in yield is greatest for the more strongly retained components, arginine and deoxyadenosine, for each set of data. Another measure of the extent of the system is the breakthrough volume of the displacer front, V_D^*, that

TABLE I

EFFECT OF COLUMN LENGTH IN DISPLACEMENT CHROMATOGRAPHY OF AMINO ACIDS ON SILICEOUS CATION EXCHANGER WITH BENZYLTRIBUTYLAMMONIUM CHLORIDE AS THE DISPLACER

C = Mean concentration (mg/ml) of pure product; P = yield (%); V_D^* = breakthrough volume of displacer front.

Column length (cm)	V_D^* *(ml)*	*His*		*Arg*	
		C	*P*	*C*	*P*
45	11.7	15.4	76	8.5	17
70	18.2	15.2	92	7.7	50

TABLE II

EFFECT OF COLUMN LENGTH IN DISPLACEMENT CHROMATOGRAPHY OF NUCLEOTIDES ON OCTADECYL-SILICA WITH BENZYLTRIBUTYLAMMONIUM CHLORIDE AS THE DISPLACER

Details as in Table I.

Column length (cm)	V_D^* *(ml)*	*Ino*		*d-Ino*		*Ado*		*d-Ado*	
		C	*P*	*C*	*P*	*C*	*P*	*C*	*P*
40	18.3	9.6	90	10.4	87	15.0	45	18.0	34
55	20.0	8.0	96	11.8	88	19.0	52	19.8	44

includes the volume of the sample loop. Just as in elution chromatography where the column length is frequently increased in order to provide a greater plate number and ensure separation, so in displacement chromatography the column length should be greater for more difficult separations, or as a peculiar feature of this mode of chromatography, when the amount of feed is increased.

The yield obtained depends on use of the optimal column length, however. Fig. 11 shows a separation similar to that depicted in Fig. 5, although with a column half as long and with half the amount of feed. It can be shown[8] that in ideal displacement chromatography the column length required for reaching the isotachic condition is proportional to the amount of feed. Thus the displacement diagrams in Figs. 5 and 11 appear to represent similar stages of development of the displacement train. However, the larger feed in the former experiment produces wider product zones without a significant increase in zone overlap as depicted in Fig. 5. As a result, the incremental load of feed permitted by use of a longer column increases the size of the pure regions in the product zones, so that the yield of pure material is higher than that obtained

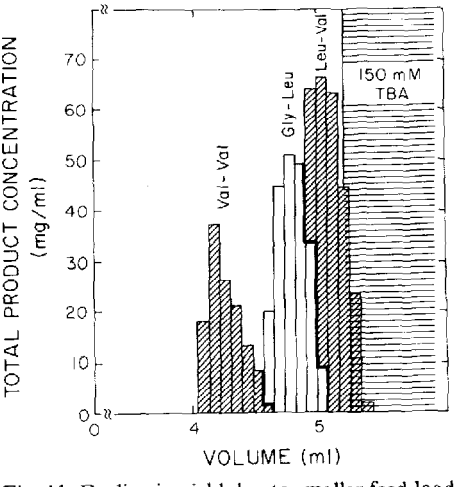

Fig. 11. Decline in yield due to smaller feed load for three-component dipeptide mixture. Conditions are identical to those in Fig. 5 except: feed, 10 mg of valylvaline, 15 mg of glycylleucine and 15 mg of leucylvaline in 0.5 ml of carrier; column, Spherisorb-ODS (250 × 4.6 mm). Flow-rate: 0.86 ml/min. Fraction volume: 86 μl.

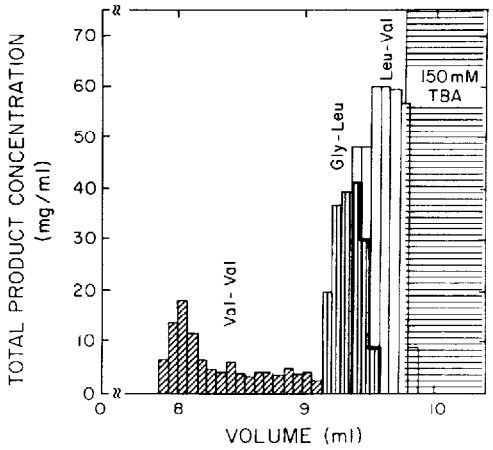

Fig. 12. Loss of efficiency in displacement chromatography as a result of excess column length. Conditions are identical to Fig. 11 except: column, Spherisorb-ODS [(2 × 250) × 4.6 mm]; flow-rate, 0.75 ml/min; fraction volume, 75 μl.

with the smaller feed load on the shorter column. For such a situation to occur it is important that the entire lengths of both columns be efficiently utilized. Fig. 12 shows a displacement diagram for separation under the same conditions stated in Fig. 11, but using a column of twice the length and slightly lower flow-rate. Despite the longer column the separation shown in Fig. 12 is worse than that depicted in Fig. 11. The excessive zone mixing is believed to arise from slow displacement kinetics and mass transfer in the excess length of the column that the displacement train had to traverse for the zones to emerge but where separation no longer occurs. This finding also underscores the need for proper matching of feed loading and column length for efficient process design and suggests that excess column length should be avoided. The zone shapes, especially for valylvaline, change in the two figures, indicating that de-

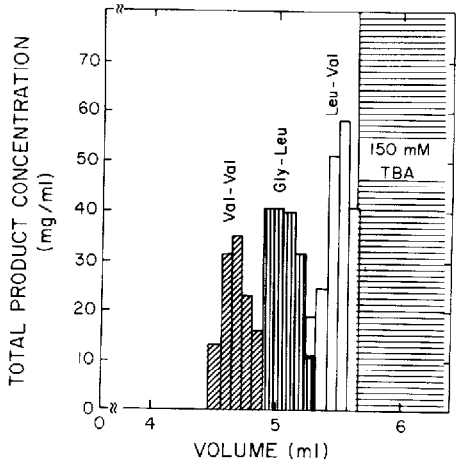

Fig. 13. Enhancement of separation efficiency with elevated temperature. Conditions were as in Fig. 11 except: temperature, 60°C.

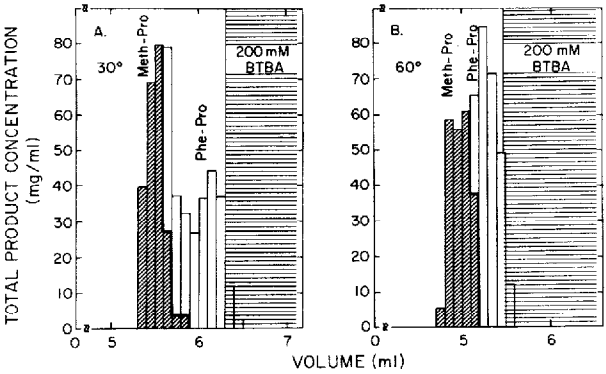

Fig. 14. Improvement in separation achieved by increasing temperature. Temperature: (A) 30°C; (B) 60°C. Column: 5-μm Spherisorb-ODS (250 × 4.6 mm). Carrier: 50 m*M* phosphate buffer, pH 2.0. Displacer: 200 m*M* benzyltributylammonium chloride in the carrier. Flow-rate: 0.5 ml/min. Fraction volume: 100 μl. Feed: 25 mg of methionylproline and 25 mg of phenylalanylproline in 1.0 ml of carrier.

velopment is not complete in Fig. 11. The separation, however, is virtually complete and the higher product concentrations and shorter breakthrough times in Figs. 5 and 11 constitute an operating advantage over those in Fig. 12..

Temperature. Increasing the throughput in displacement chromatography has two aspects: improving the yield and increasing the concentration of the product stream. The latter may frequently involve an enhancement of the solubility of the components in the carrier. In displacement chromatography at elevated temperature both of these aspects are affected so that the separation process becomes more efficient. Fig. 13 shows a displacement diagram obtained under conditions identical to those used in the experiment whose result is shown in Fig. 11, but at a temperature of 60°C. The increase in temperature greatly reduces the overlap between zones, so that the yield of pure material increases. The effect is attributable to an increase in the rates of displacement kinetics and mass transfer by increasing temperature. As noted above these "secondary" factors, which are responsible for the departure from ideal behavior, are highly detrimental to both yield and throughput in displacement chro-

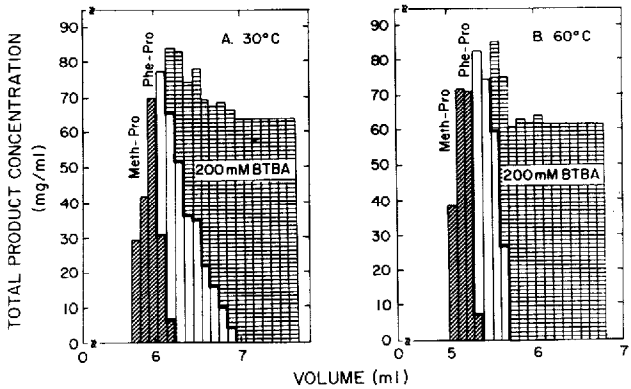

Fig. 15. Effect of increasing temperature on resolution in displacement chromatography. Details as in Fig. 14, except carrier pH 6.0.

matography. Comparison of Figs. 14 and 15 also highlights the effect of temperature on separation of a mixture containing phenylalanylproline that has been shown to undergo a conformational change[27]. This effect is the dominant contributor to bandspreading of phenylalanylproline in elution chromatography at room temperature but substantially vanishes at 60°C. Other gains with respect to throughput may be realized when the process is operated at elevated temperature, including enhanced solubility of all components in the carrier, and lower viscosity of the mobile phase. Solubility is a major factor limiting the utility of preparative liquid chromatography, so higher temperatures are advantageous in any mode. The reduction in liquid viscosity permits use of longer columns and higher flow-rates, and thus, according to the discussion above, permits an increase in throughput.

Yield and throughput. An optimization strategy for displacement chromatography, as for any separation process, may have various bases, including overall recovery of pure product or yield, overall production rate, yield or production rate of a particular feed component or production at a specified level of product purity, and the design of a process has to be tailored accordingly. By way of example, Table III lists the overall yield and production rate performance data for operation under a variety of conditions.

Separations of binary mixtures shown in Table III indicate that, at a fixed temperature, an increase in the flow-rate from 0.14 to 0.5 ml/min is preferable when the goal is to maximize throughput, despite the lower yield obtained at the higher flow-rate. The much shorter run-times at high flow-rates more than make up for the lower efficiency of each run, so while the increase in flow-rate causes the yield to decrease from 45.8 to 39.5%, it also results in a corresponding increase in the throughput from 30 to 84 mg/h. Furthermore, when the temperature is raised to 60°C both yield and production rate increase substantially, to 63.8% and 154 mg/h, respectively. The same influence of higher flow-rate in enhancing production rate despite the reduction in yield is shown by the behavior of the four-component feed mixture. As seen in Table III, a six-fold increase in flow-rate resulted in a 30% decline in yield but more than quadrupled the production rate. The beneficial effect of operating at high temperature is borne out by the separations of the three-peptide mixtures at 30 and 60°C and a flow-rate of 0.86 ml/min. There the temperature increase alone pushed the yield up to nearly 90%, and raised the production rate by 100 mg/h to 320 mg/h. These values were all obtained on a 25-cm long analytical HPLC column. In the two cases when a 50 cm long column was employed, varying effects on yield and production rate were noted. In one instance the column length was doubled, without change in the 40-mg load of the three-peptide mixture. Since the displacement train was fully developed with the 25 cm long column, the additional 25 cm was an excess column length. The excess length is shown to impair significantly the separation, causing a decline in the yield from 60 to 56.5% and a drop in production rate from 222 to 103 mg/h. The deterioration in these efficiency parameters is likely due to zone mixing effects caused by slow displacement kinetics and mass transfer, and the longer breakthrough time associated with the longer column. Since the 50 cm long column is demonstrably under-utilized by the 40-mg feed load, the feed amount was doubled to 80 mg in another experiment with this column. In this case the column is once again operated efficiently, and the yield and production rate were as high as 79.3% and 260 mg/h, respectively. Thus when the column is too long and the excess length impairs

TABLE III

EFFECT OF OPERATING PARAMETERS ON YIELD AND PRODUCTION RATE OF PEPTIDES

Feed		*Displacer*		*Flow-rate (ml/min)*	*Column length (cm)*	*Temperature (°C)*	*Overall* yield (%)*	*Production rate** (mg/h)*
Number of components	*Amount (mg)*	*Type*	*Concentration (mm)*					
Two***	50	BTBA	200	0.5	25	30	39.5	84.0
	50	BTBA	200	0.5	25	60	63.8	154
	50	TBA	200	0.14	25	30	45.8	30.0
Three §	40	TBA	150	0.86	25	30	61.0	222
	40	TBA	150	0.86	25	60	87.7	320
	40	TBA	150	0.75	50	30	56.5	103
	80	TBA	150	0.72	50	30	79.3	260
Four §§	50	TBA	150	0.14	25	60	47.6	32.4
	50	TBA	150	0.86	25	60	31.4	135

* Pure components in the product expressed as weight per cent of the feed mixture.

** Total amount of pure products obtained in a run divided by the time for the run.

*** Methionylproline and phenylalanylproline.

§ Valylvaline, glycylleucine and leucylvaline.

§§ Valylvaline, glycylleucine, leucylvaline and phenylalanylvaline.

separation, an appropriate increase in the feed load produces a more efficient separation.

CONCLUSIONS

Despite the relative complexity of the displacement chromatographic process owing to its non-linear nature the technique offers significant advantages when high product concentrations in the column effluent are desired in chromatographic separations. The separation of dipeptides and nucleic acid fragments obtained in the displacement mode by reversed-phase and ion-exchange chromatography demonstrate the wide applicability of the technique. The results shed light on the effect of the key parameters on the efficiency of separation and facilitate the selection of optimum conditions.

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