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LIQUID CHROMATOGRAPHIC RESOLUTION OF RACEMIC β-3,4-DIHYDROXYPHENYLALANINE

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SUMMARY

The D- and L-isomers of β-3,4-dihydroxyphenylalanine were effectively resolved by liquid chromatography. The separation was best accomplished using an asymmetric support prepared by bonding L-arginine through a cyanuric chloride linkage to Sephadex G-25. In an analytical column, with water as the eluent, a separation factor of 1.60 was obtained. Subsequently, the separation was verified by polarimetric measurements made on cuts from a preparative-scale column. Partial resolution of the enantiomers of tyrosine, but not of phenylalanine, verified the importance of "three-point contact" between sorbent and sorbate for resolving these enantiomers.

INTRODUCTION

The L-enantiomer of β-3,4-dihydroxyphenylalanine (DOPA) is an essential intermediate in the neurochemistry of the brain, and recently has attained significance in the chemotherapy of Parkinson's disease[1,2]. With the estimated half-million afflicted patients requiring up to 5 g daily, the need for large amounts of L-DOPA at reasonable prices is obvious. An important route for manufacturing large quantities of L-DOPA is via synthesis of the racemic amino acid which of course requires an efficient separation and recovery process to secure the L-enantiomer. Separation of racemic mixtures of optical isomers has of course long been a difficult and expensive problem. Significant breakthroughs in scaling up analytical chromatography separations to large-diameter columns, and the increasing use of preparative-scale chromatography for industrial separations, suggest that an important and new approach may be available[3–5]. Therefore, we felt that the development of a packing capable of a laboratory scale, liquid chromatographic resolution of this amino acid racemate would perhaps provide the key to low-cost production of L-DOPA.

The desired separation would be suitable for the direct resolution of racemic DOPA mixtures. That is, there would be no need to form a derivative, separate the derivative, and then reform the isomers. This derivative approach of course is popular for gas chromatographic (GC) separations but means added expense for preparative chromatography and may not be possible in the case of DOPA, owing to oxidative in-

stability. Unfortunately, few chromatographic attempts have been made to resolve racemic D,L-DOPA directly or after derivative formation. DALGLIESH[6] used paper chromatography in an attempt to resolve various substituted phenylalanine enantiomer pairs, including D,L-DOPA. In this work, which was later confirmed by LAMBOOY[7], he found that the only hydroxylated phenylalanine isomer pairs which could be resolved were those in which the aromatic hydroxyl and methyl or methylene substituents were adjacent to one another. Racemic D,L-DOPA could not be resolved.

While little effort has been devoted to the chromatographic separation of DOPA isomers, a great deal of work has been devoted to resolving other racemates by liquid chromatography. Most of this work was reviewed by KARGER[8] in 1967, BUSS AND VERMEULEN[9] in 1968, and LOSSE AND KUNTZE[10] in 1970. In general, separations were marginal with few exceptions. Also, a great amount of the work was devoted to resolving D- and L-mandelic acid, mainly because the large optical rotation of the antipodes of this compound made polarimetric measurements of the eluent less difficult.

As pointed out above, it is economically more desirable to separate the isomers directly rather than try to resolve a derivative. Consequently, several criteria seem essential in the preparation of a packing for the direct chromatographic resolution of amino acid isomers without derivative formation. First of all, the packing needs to be asymmetric, that is, to contain "built in" optically active sites. The literature[11–19] consistently shows that where resolution was accomplished to any extent, a natural optically active packing was used or a synthetic packing was chemically altered to contain optical activity.

A second criterion in the preparation of a suitable packing is the availability of "three-point contact" between the solute species and the optically active portion of the packing. Several workers have emphasized the importance of this concept[6,9,20]. Since enantiomers differ only in their three-dimensional structures, it is logical, therefore, that a three-point interaction must be established between sorbent and sorbate for a packing to effectively "distinguish" between mirror-image isomers. Thus, in the case of DOPA, the three points that would interact with a stereoselective support would be the carboxyl group, the α-amino group and the aryl hydroxyl group(s). A suitable packing to resolve the enantiomers of DOPA would then contain an optically active site having at least three charge centers, capable of interacting with the three functional groups of DOPA. A logical support-tailoring candidate would be an optically active amino acid with an ε-carbon charge center in addition to the two sites on the α-carbon atom. Thus, the asymmetric sites on the packing would provide three charge centers for interaction with DOPA.

In addition to having asymmetry and appropriate charge sites, the steric location of these charge sites with respect to the sorbent and sorbate molecules is also important. Naturally, if molecular sizes are such that the charge centers cannot significantly contact or overlap, the resolving power of the packing is likely to be decreased or non-existent. Finally, the optically active tailoring species must be bound to the support in such a way that the backbone polymer will not sterically block the charge centers of the active component, inhibiting the three-point interaction and hence the resolution.

With these criteria as guidelines, a series of optically active, synthetic supports

were prepared and evaluated for the liquid chromatographic separation of racemic DOPA. The L-enantiomer of arginine was selected as the optically active species with which several polymeric organic supports were chemically tailored.

EXPERIMENTAL

Reagents

Chemicals other than the following were reagent grade. Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and L-arginine were purchased from Aldrich Chemical Co., Inc. Sephadex G-25 (gel filtration medium), 20 to 80 μm particle size, was purchased from Pharmacia Fine Chemicals, Inc. The following amino acids were purchased from Nutritional Biochemicals Corporation: L-, D-, and D,L-DOPA; L- and D-tyrosine (β-4-hydroxyphenylalanine); and D- and L-phenylalanine. N-Acetyl-L-phenylalanine was purchased from Pfaltz and Bauer.

The N,O,O-triacetyl-D,L-DOPA derivative was prepared by use of acetic anhydride. D,L-DOPA was dissolved in chilled, nitrogen-purged, 2.5 *N* sodium hydroxide. A five-fold excess of chilled acetic anhydride was incrementally added over a period of 1 h with stirring. The mixture was acidified to pH 2.5 with hydrochloric acid and stored at 0° C overnight. Upon rubbing the cold solution with a glass rod, the derivative crystallized in 60% yield. The product was readily recrystallized from water. The derivative was very soluble in alcohol and quite soluble in water. The melting point was 120° C. An IR spectrum revealed characteristic substituted amide and ester bands, the ester at 1763 cm^{-1} being especially prominent. The molecular weight was determined to be 323, equal to the theoretical value. The carbon and nitrogen contents were 55.5% and 4.3%, respectively, *versus* theoretical values of 55.7% and 4.3%, respectively.

Apparatus for chromatographic evaluation

A Milton Roy Instrument Minipump (Milton Roy Co.) with a maximum pumping capacity of about 3 ml/min was used without pulse dampening to deliver the eluent. Chromatographic columns (Chromatronix Inc.) measured 2.8 × 500 mm, 2.8 × 300 mm and 0.5 × 300 mm. The same sample injection port, Chromatronix No. 107B25, was used in conjunction with the above columns. The detection system consisted of a Beckman DK-2A spectrophotometer equipped with a microflow cell, having an internal volume of 0.125 ml and a path length of 5 mm and a reference beam attenuator. The amplifier of this spectrophotometer was wired to a Sargent Model XXI Polarograph recorder.

Packing preparations

L-Arginine on Sephadex. Dry Sephadex (50 g) was added to a 400-ml beaker, containing 250 ml of distilled water. The Sephadex was heated and maintained at the boiling point of water and was agitated by magnetic stirring. A solution composed of 20 g of cyanuric chloride in 200 ml of acetone was then prepared. About 50 ml of distilled water were added and the pH of the solution was adjusted to about 6 with sodium hydroxide. The hydrated Sephadex was then added to the cyanuric chloride solution in small increments, maintaining the pH at 6. When all the gel had been added, the pH was adjusted to 11 and maintained there for 1 min. Then 100 ml of

glacial acetic acid were added and the gel was filtered and washed well with distilled water.

The Sephadex/cyanuric chloride derivative was re-suspended in 300 ml of distilled water and the pH adjusted to 6 with sodium hydroxide. L-Arginine (5 g) was then added and the pH was maintained between 10 and 11 during this addition and then for a further 16 h. The product was washed well with distilled water and was filtered and dried *in vacuo*.

An attempt was made to characterize the Sephadex/cyanuric chloride intermediate and the final L-arginine-tailored packing to determine the quantity of each substituent added and to estimate the capacity of this packing. Nitrogen analyses on both the intermediate and final products were performed and also an attempt was made to determine the quantity of L-arginine on the packing by non-aqueous titrations of the amine groups present. From the nitrogen data on the intermediate product, an approximate addition of 0.63% of cyanuric chloride (0.042 m*M*/g support) was obtained. This represents an addition of only one triazine group per hundred fifty repeating anhydroglucose units of the Sephadex. Nitrogen analysis and a non-aqueous amine titration of the final product indicated that an addition of 0.19% (0.011 m*M*/g support) of L-arginine had been achieved. This represents an addition of about one L-arginine molecule per four triazine units present. We made no attempt to increase this loading by employing such techniques as swelling in an alkaline medium or converting the Sephadex to an alkali species as is commonly done with cellulose. We do feel, however, that this low capacity was due at least in part to the fact that only secondary alcohol groups were predominantly available for bonding in the Sephadex structure.

L-*Arginine on cellulose*. The cellulose-based support was prepared in a manner similar to the Sephadex preparation except that no attempt was made to swell this material initially. Whatman Cellulose Powder, CF-1, was the starting material.

L-*Arginine on chloromethylated polystyrene (S·X-2)*. Biorad S·X-2, chloromethylated polystyrene beads (20 g), was added to 100 ml of pyridine. L-Arginine (5 g) was dissolved in 60 ml of distilled water and the dissolved amino acid was added to the pyridine–S·X-2 suspension. The mixture was transferred to a jacketed beaker and hot water was run through at 50° C. The mixture was stirred for several hours and 10 ml of triethylamine were added to assure deprotonization of the bonded L-arginine. The resin was filtered and washed thoroughly with acetone and water.

Chromatographic evaluations

Prior to evaluating the microbore columns, containing the L-arginine-tailored supports, the columns were conditioned by eluting degassed distilled water through them at least over a 16-h period at flow rates of about 3–4 ml/h. This conditioning was necessary to physically pack the support, and we also found that the retention volume of the enantiomers and hence separation factors increased during this period to constant values. Though the exact explanation has not been established, we feel that it was necessary to achieve a steady state of support protonation with degassed distilled water, such that the amino groups were just basic enough to provide for an attraction of the phenolic hydroxyl groups of DOPA. Deprotonation of the support via exposure to a tertiary amine resulted in complete retention of both DOPA enantiomers, and acidifying the support caused the DOPA enantiomers to elute at

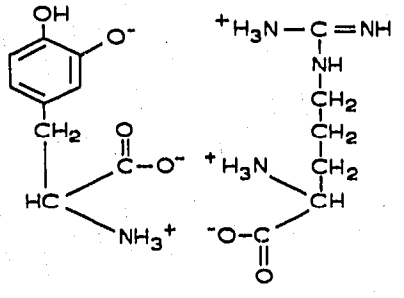
the solvent front. Extended periods of washing after both treatments re-established the activity of the column, however.

Collection of enantiomers for polarimetric measurements

Racemic DOPA was repetitively applied to a preparative column and separated. The column measured 0.5 × 300 mm and was packed and conditioned in a manner identical to the analytical column except that flow rates were increased to 3 ml/min. Three equal-volume cuts of the DOPA-containing eluate were taken from each run. Flash evaporation was used to concentrate these fractions to minimize the possibility of degradation of DOPA. Thus, daily, each pooled fraction was stripped of excess water and added to one of three 250-ml flasks wrapped in aluminum foil to eliminate UV radiation. The flasks were purged with nitrogen and were stored at 5°C. Flash evaporation was then used to reduce each fraction to about 50 ml. The DOPA was stabilized during evaporation by addition of several drops of concentrated hydrochloric acid. The concentrations were conducted under vacuum with the aid of the careful application of heat to permit evaporation, though the flasks never exceeded 30°C in temperature. The resulting solutions were purged with nitrogen and also stored at 5°C. Just before polarimetric measurements, a final concentration was made and consisted of flash evaporating the samples to a volume of 10 ml and permitting evaporation to dryness without applying heat. In this manner, the slight excess of hydrochloric acid was removed, leaving the hydrochloride of each resolved fraction. The dry residues were taken up in 2.00 ml of water at 25°C for determining the optical activities. Measurements were made at 25°C using a Perkin-Elmer Model 141 Polarimeter with a 3-ml capacity, 10-cm path length cell at 589 nm.

RESULTS AND DISCUSSION

On the basis of the criteria mentioned earlier for selecting candidate support-tailoring compounds, the amino acid, L-arginine, appeared to be the most likely to accomplish the resolution of DOPA. Diagramatically, the postulated three-point attraction between arginine and DOPA may be represented in the following manner:



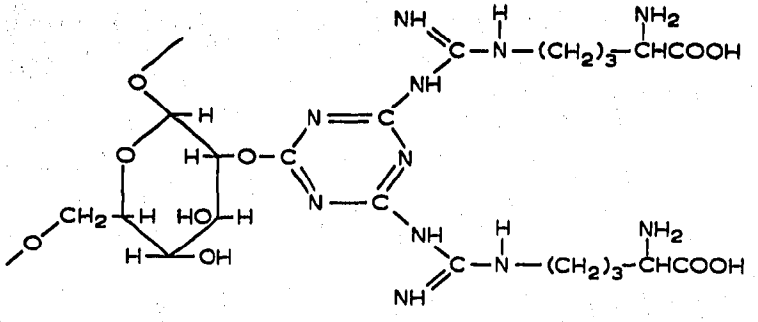
In this illustration, we have depicted one possible type of interaction between the phenolic hydroxyl group(s) of DOPA and the basic ε-amino groups of arginine, along with the quadrupole formation between the α-amino acid portions of these two molecules. In addition, spatially-accurate CPK molecular model representations of the two amino acids confirmed arginine to be an appropriate choice with respect

to steric considerations. Thus an apparently ideal three-point positional relationship existed between the two amino acids. Also, it was evident from the models that a spatially better fit was obtained with L-arginine and the D-isomer of DOPA. Because of this orientation, we would expect an L-arginine packing to retain the D-enantiomer, eluting the L-enantiomer first (a desirable situation for recovering L-DOPA).

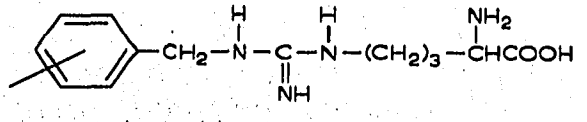
Packings prepared and used in this program included an L-arginine-tailored Sephadex support. Sephadex G-25 was swelled and reacted with an excess of cyanuric chloride. Then L-arginine was added to the Sephadex/cyanuric chloride derivative. Inasmuch as all the amine groups of L-arginine would be expected to react with the cyanuric chloride intermediate at least to some extent, the pH of the reaction was controlled in such a manner as to avoid protonation of the guanidine groups. In this manner, assurance was gained that at least the strongly basic ε-amino groups would be available for bonding and would be expected to be the predominant reaction site for bond formation.

A second support was prepared by bonding the L-isomer of arginine to cellulose in a similar manner, via cyanuric chloride. In both supports the terminal guanidine nitrogen of the arginine was thus connected through a secondary amino bridge to the support. As such, the three moderately basic amino groups available provided an apparently large area for forming the third contact point with the phenolic hydroxyl group(s) of DOPA.

The third candidate packing was prepared by bonding the L-enantiomer of arginine directly to a chloromethylated polystyrene (S·X-2). Reaction of the guanidine portion of arginine with the chloromethyl group predominated. While a secondary amino group again resulted, the basicity of the group was undoubtedly different (greater) than with the other two supports. Resonance of the guanidine residue, hence electron delocalization, was limited in this latter support, whereas the supports containing the cyanuric chloride bridge had significant resonance possibilities. These structural differences are illustrated below.



L-Arginine bonded to Sephadex via cyanuric chloride



L-Arginine bonded to chloromethylated styrene

Thus, the DOPA diasstereoisomer pairs formed with the L-arginine on the S·X-2

support would be expected to dissociate less readily than the DOPA/L-arginine piars formed on the two less basic supports.

The three packings L-arginine on cellulose, on S·X-2, and on Sephadex, were evaluated under similar conditions for separation of D- and L-DOPA. Columns of 300 × 2.8 mm and 500 × 2.8 mm were used. Samples of 10 μg of each isomer and 20 μg total of D-,L-isomer were applied. Eluents of pure water, water–ethanol and salt solutions of various ionic strengths and pH were studied. All three of the L-arginine-tailored packings showed a stereo-selectivity for the D-enantiomer of DOPA, but the Sephadex support was superior to the other two preparations.

The DOPA isomers could not be eluted from the L-arginine–S·X-2 support with pure water or water/ethanol eluents. As expected, the nature of the chemical linkage between L-arginine and the support did affect the magnitude of the attraction between bonded L-arginine and DOPA. Bonding L-arginine to this chloromethylated polystyrene caused a very strong attraction between the packing and DOPA such that very high concentrations of salts or buffers were necessary to elute the amino acids. Eluents such as 0.5 *M* sodium chloride or acetate buffered to pH 6.5 were necessary to elute the DOPA isomers from the column. Marginal resolutions, however, were attained with this support.

The L-arginine–cellulose packing was evaluated with water–ethanol eluents of various proportions. Successful separation of the D- and L-isomers was obtained with this packing using ethanol–water (60:40) as an eluent at a flow rate of 0.3 ml/min. Based on runs of each isomer individually, a separation factor, α^*, of 1.18 $\pm$ 0.02 was measured on the 300 × 2.8 mm columns over a sample size range of 8 to 20 μg of each component. The D-isomer was always held up more than the L-isomer, as predicted from the stereochemical considerations. The separability of the two isomers was insufficient for observing two distinct peaks with mixtures of the two enantiomers although all other evidence indicated that separation had occurred.

More thorough evaluation of the cellulose packing may have led to better separation of the DOPA isomers. However, the L-arginine–Sephadex packings provided excellent resolution with a pure water eluent. With the L-arginine–Sephadex support, the direct link between the basic amines and the cyanuric chloride decreased the net overall basicity of the bonded guanidine group. A virtually ideal acid–base interaction formed such that water eluent was sufficient to provide the desired separation. Fig. 1 shows chromatograms of elutions of the individual DOPA enantiomers along with a commercial racemic preparation. A 300 × 2.8 mm column was used for this chromatogram with an eluent at a flow rate of 0.73 ml/h. It can be seen that the resolution of the DOPA enantiomers appears to be virtually complete, with only a small portion of the racemate unresolved. This "unresolved" portion of the chromatogram, however, was found to be due to the UV detector cell; the resolution was actually complete from the column. The cell was found to require five cell volumes of eluate to remove a species present. Also, with an internal volume of 0.125 ml, each peak would show a cell-induced tailing of approximately 0.6 ml before the baseline was reached. This tailing is especially evident in the D-DOPA portion of the chromatogram. Of course, modern detector cells with cell volumes of approximately 10 μl and wash volumes less than 2 μl would eliminate this problem

* $\alpha = (V_D - V_0)/(V_L - V_0)$, where V_D and V_L are elution volumes of the D- and L-DOPA enantiomers, respectively, and V_0 is the dead volume of the column plus the detector.

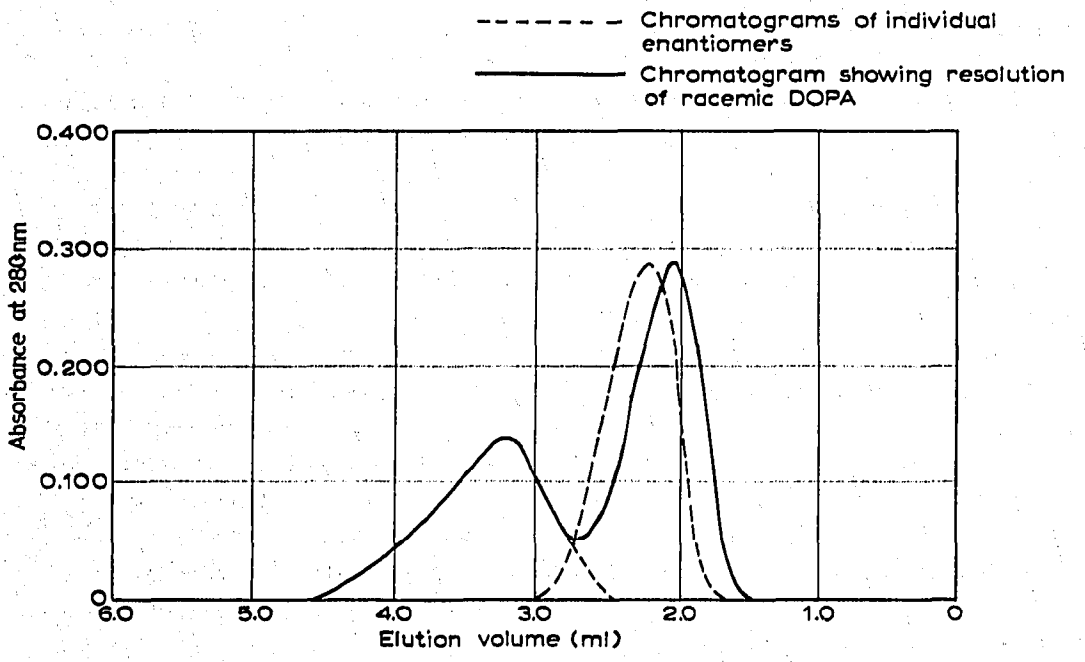


Fig. 1. Chromatogram of D-, L- and D,L-DOPA on L-arginine–Sephadex quantities, 10 μg D- 10 μg L-, and 20 μg D,L-DOPA; flow, 0.7 ml/h (linear, 5 mm/min); column, 300 × 2.8 mm glass microbore; solvent, distilled water.

completely. A separation factor of 1.60 was obtained, and despite the false tailing from the detector cell, the resolution value, R, for this separation was determined to be 0.9. An R value of unity is adequate for most purposes. To our knowledge, this is the first time a direct liquid chromatographic resolution of an underivatized amino acid has been observed as two distinct peaks.

In Fig. 1, a distinct difference was also observed in the retention of L-DOPA injected separately and in the racemic mixture. We suspected that, owing to the relatively low column capacity, a competition occurred between the two enantiomers for the L-arginine sites on the support, during the time in which the two antipodes occupied the same portion of the column. This created what could be termed a partial frontal elution in the early stages of the separation. Also, this occurrence was described or verified mathematically. A Fourier series of seventeen terms was determined for each of the three chromatograms. Constants for the series were found by computer analysis of the absorbance–retention time plots for each species. A good representation of the racemic DOPA chromatogram could be obtained by assuming the peaks of the D- and L-enantiomers to retain their shapes while eluting together in the chromatogram of the racemates, but to be separated by an even greater factor than when eluted separately. This was well represented by adding a phase angle to the L-DOPA retention volume in the Fourier series and adding this to the D-DOPA series.

The effect of flow rate on the D,L-DOPA separation with L-arginine on Sephadex was evaluated with the analytical column. A plot of height equivalent to a theoretical plate (HETP) and α against linear flow rate is shown in Fig. 2. The HETP, based on the L-enantiomer, is seen to decrease with decreasing flow rate while α remains constant at 1.60 with flow rates up to 20 mm/min. The ability of the support to

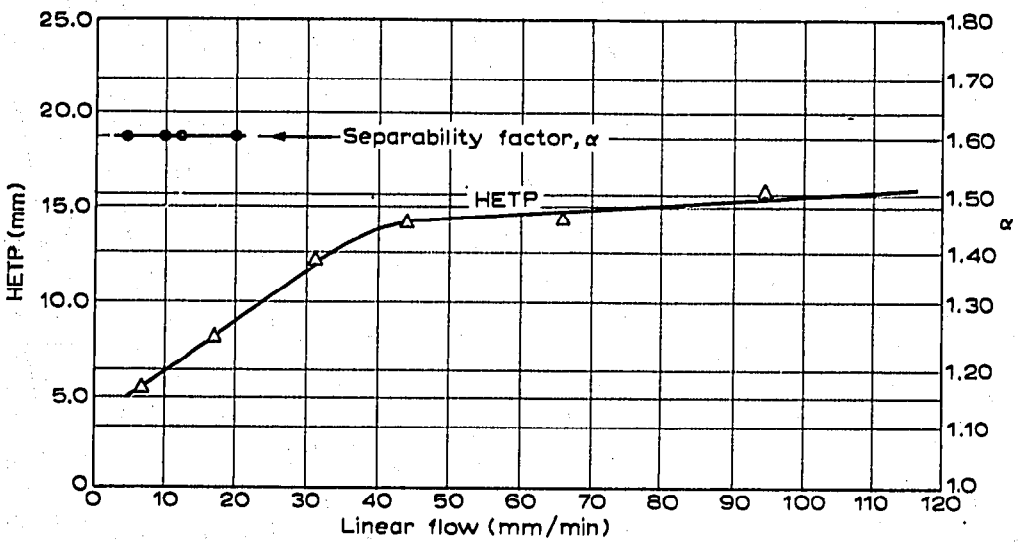
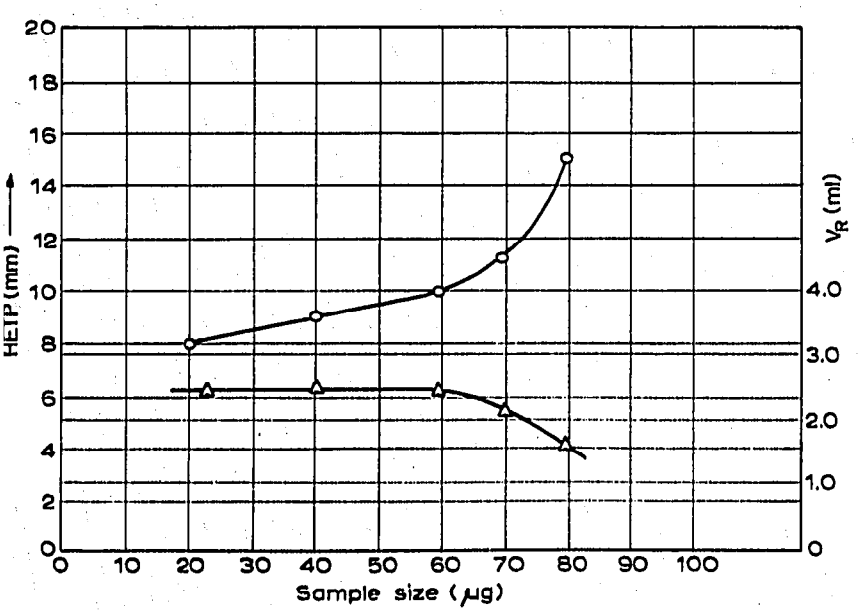


Fig. 2. Variation of HETP and α with flow rate. HETP determined using 20 μg L-DOPA; α determined using 20 μg D,L-DOPA.

function as both an asymmetric ion-exchange resin and a permeable, swellable gel must be weighed in considering these results.

The sample capacity of this support was evaluated by varying sample size and observing the corrected retention volume, V_R, and HETP. The capacity of a column has been defined[21] as the amount of sample causing the number of plates to decrease by 10% from the value for zero sample size. A plot of HETP and V_R *versus* amount of L-DOPA added is shown in Fig. 3. The flow rate was 12 mm/min and the capacity factor was 2.4. A sharp change in HETP was observed, at about 60–70 μg of L-DOPA. The retention volume was constant for samples up to about 60–70 μg and



then decreased indicating the overload point. The shape of the peaks at overload conditions indicated a Langmuir-type sorption isotherm. Of course, while HETP will vary with flow rate the overload point based on V_R should remain relatively constant.

In determining the dead volume of the column, it was not legitimate to use conventional techniques. In the first place, Sephadex is used in molecular size separations, retaining the small and excluding the large molecules. As such, the effective interstitial volume to which a large molecule is exposed is different from the volume in which a smaller molecule can permeate. Thus we decided that a compromise was necessary. A compound would have to be eluted through the column which approximated the size and shape of DOPA but without the three-point attraction. To meet these requirements, we prepared N,O,O-triacetyl-D,L-DOPA, and found that, of all the compounds checked, this compound had the shortest retention. A similar but slightly longer retention was obtained with N-acetyl-D,L-phenylalanine. Thus we used the retention of the triacetylated DOPA derivative as the closest approximation to the true effective dead volume.

No evidence of on-column DOPA decomposition was observed. The packing maintained its original performance for periods greater than one month under constant use. We did observe, however, that once a quantity of DOPA had been oxidized to quinoid or indole species in sample solutions, the L-arginine column retained these colored species irreversibly. This was evidenced by a darkening at the top of the support interface of the column.

To verify the resolution of D- and L-DOPA optically, a larger column was used to secure a sufficient quantity of each enantiomer for polarimetric measurements. Fifty microliters of D,L-DOPA (400 μg/ml) were added to the column via a microsyringe and three fractions were collected which were to have contained L-, D,L-, and D-DOPA, respectively. Approximately fifty resolutions were performed and were pooled in each of three fractions. After the fractions were concentrated, the pH of each was adjusted with hydrochloric acid in such a manner that the concentration of DOPA was exactly equal to the concentration of the acid present. The polarimetric measurements verified that the resolution of DOPA occurred as presented in the chromatographic data. As expected, optical activity was observed in the first and third fractions.

In addition to resolving the enantiomers of DOPA, two other amino acid resolutions were attempted on the Sephadex packing. This was done to elucidate the chemistry and mechanics of the observed DOPA separation. These amino acids were β-4-hydroxyphenylalanine (tyrosine) and β-phenylalanine. In this experiment, identical chromatographic conditions were used as in the analytical scale DOPA resolutions with the exception of sample concentrations and sizes. Tyrosine (2 mg/ml) was added in the quantity of 40 μg per injection and phenylalanine (10 mg/ml) was added in the quantity of 20 μg per injection. The tyrosine isomers were resolved in the same order as DOPA isomers with an apparent separation factor of 1.19; phenylalanine was retained to a lesser extent by the support and was not resolved.

This information lends further credence to the three-point contact theory. It shows that the addition of a third contact point to the sorbate provides a basis for resolution, and an increase in the hydroxy substituents from the 4-hydroxyphenyl to 3,4-dihydroxyphenyl either statistically increases the number of reactions occurring down the column or increases the stability of the sorbent–sorbate species. In con-

sidering the minute differences between the acidities of DOPA and tyrosine, the former postulate seems more likely.

In summary, this work demonstrates the feasibility of using properly tailored supports for the separation of optical isomers. At least for separating amino acid isomers, logical rules seem to exist for choosing the proper bonded species, the support, and the bonding mechanism to provide suitable asymmetric packings. The most important criteria are that the bonded species (1) provide the asymmetry, (2) allow for three points of significant electrostatic or electronic interaction, and (3) be of suitable steric shape (length) to allow appreciable overlap of the three contact points with the analogous electrostatic centers in the isomers to be separated. In addition, we feel that while the supports used here were satisfactory, hard-core (*i.e.* glass) supports coated with thin polymer films to anchor the asymmetric species should give equivalent separations, but better resolution.

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