Journal of Chromatography, 155 (1978) 285–293

CHROM. 10,968

LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

V. SEPARATION OF OPTICAL ISOMERS OF AMINO ACIDS ON A POLYSTYRENE RESIN CONTAINING L-HYDROXYPROLINE

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(Received November 17th, 1977)

SUMMARY

An asymmetric resin containing copper(II) ions with L-hydroxyproline ligands fixed in a macronet isoporous polystyrene matrix has been used for the separation of optical isomers of amino acids. The resin selectivity and the column efficiency were determined and their dependence on the chromatographic conditions (the degree of saturation of the resin with copper(II) ions, ammonia concentration, column parameters) discussed.

INTRODUCTION

Ligand-exchange chromatography (LEC), which has recently been reviewed[1], has proved to be a powerful method for separating compounds with very similar molecular structures, including geometrical and optical isomers. For example, asymmetric resins with residues of optically active α-amino acids fixed on a polystyrene matrix can be used to separate the enantiomers of amino acids, provided the resin contains metal ions capable of forming complexes with both the fixed and the mobile amino acid ligands[2]. Earlier we have studied some general features characteristic of LEC of enantiomers, including its dependence on the amount of the complex-forming metal ion[3], on the temperature and concentration of the eluent[4] and on the structure of the fixed ligands[5].

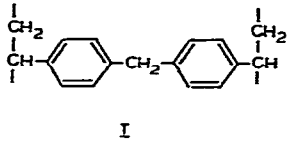
However, in order to evaluate the possibilities of practical application of ligand-exchange separation of optical isomers, a more detailed and quantitative study is needed of the interaction of enantiomers with the resin phase. In the previous papers[6,7] we discussed the method and results of calculating the stability constants of the fixed (stationary) complexes formed by the fixed ligands with the metal ions and of mixed-ligand sorption complexes containing both the fixed and the mobile ligands co-ordinated to the metal ion.

The present report deals with results of a systematic chromatographic study of the interaction of the enantiomers of amino acids with a resin loaded with copper(II) ions and containing L-hydroxyproline as the fixed ligand. The chromatographic

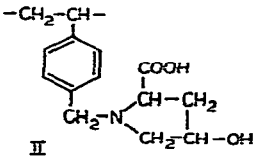
experiments were performed on a scale which would permit evaluation of the technique for use in both analysis and preparation.

EXPERIMENTAL

The asymmetric resin was prepared by aminating a chloromethylated polystyrene containing 11 mol% of cross-links of structure I with methyl L-hydroxyprolinate hydrochloride[6]. According to the nitrogen content and potentiometric titration of the resin obtained, the content of fixed ligand of structure II amounted to 3.44 mmol per gram of dry resin, taken in the zwitterionic form. The resin particles were of irregular shape and had an average size in the swollen state of 100 μm.



I



II

On treatment with excess copper–ammonia solution, the resin was saturated with copper(II) ions to an extent of 92% of the theoretical capacity calculated for the fixed complexes containing two fixed ligands per copper(II) ion. The equilibrium water content of the copper-containing resin in neutral media amounted to 200%. To maintain the copper saturation of the resin at 92%, the eluents used contained copper(II) ions in the following concentrations: $1.2 \cdot 10^{-5}$ *M* in 0.1 *M* NH_4OH for the chromatography of neutral amino acids; $2.5 \cdot 10^{-5}$ *M* in 0.025 *M* $Na(NH_4)_2PO_4$ solution of pH 8.3 for acidic amino acids and $2.0 \cdot 10^{-4}$ *M* in 1.5 *M* NH_4OH for basic amino acids.

The resin (6.3 ml) was packed into a column of 140 × 7.8 mm I.D. Portions of 1.0–1.5 mg of optically active or 2–3 mg of racemic amino acids were introduced into the column and eluted at a rate of 10 ml/h. Elution curves were recorded with a Uvicord III (LKB) detector at 206 nm. The void column volume of 4.7 ml was determined using acetone, which was assumed to be able to enter the resin phase without being strongly retained.

The retention volumes, V_L and V_D, of amino acid enantiomers L and D were determined from the distance between their peak maxima and that of acetone and expressed in void column volumes. The ratio of V_D to V_L is the enantioselectivity, α, of the sorption process and was used for calculating the difference, $\delta\Delta G^\circ$, between the free energies of the two diastereomeric sorption complexes formed in the resin phase:

$$\delta\Delta G^\circ = \Delta G^\circ_{R-Cu-D} - \Delta G^\circ_{R-Cu-L} = -RT \ln \alpha = -RT \ln \frac{V_D}{V_L}$$

If insufficient resolution of the racemate rendered a precise determination of the maxima positions or calculation of HETP values impossible, a separate chromatography of D- and L-enantiomers was performed.

RESULTS AND DISCUSSION

Modern chromatographic methods can separate components that differ in sorption energy, $\delta\Delta G°$, by as little as 10 cal/mol (sorption selectivity $\alpha = 1.01$). For separation on a preparative scale, it is desirable that α should be not less than 1.5 (or $\delta\Delta G° \geqslant 250$ cal/mol). It is helpful to bear these figures in mind when considering the results (Table I) of the LEC of amino acid enantiomers on the resin containing L-hydroxyproline.

Aliphatic amino acids

As illustrated by Table I and Fig. 1, the retention volumes of the L-enantiomers increase steadily as the size of the α-C-atom substituent increases from methyl in alanine to butyl in norleucine. The retention times of the D-isomers increase faster still. This leads to a rise in the selectivity factor from 1.04 to 2.20, corresponding to $\delta\Delta G°$ values of 24 and 460 cal/mol. Among amino acids with the same number of carbon atoms, racemic compounds with a linear side-chain (norvaline; norleucine) are resolved better than their isomers with a branched one (valine; leucine, isoleucine). In the latter instance branching at the β-carbon atom (isoleucine) is more favourable than that in the γ-position (leucine). However, the presence of two substituents on the same α-C-atom has an adverse effect on the separation of enantiomers (compare isovaline with valine and norvaline).

The retention volumes of glycine (6.44) and β-alanine (0.28) clearly show the large difference in stability between five-membered and six-membered chelate rings.

Hydroxyamino acids

Substitution of a β-H-atom in alanine and aminobutyric acid by a hydroxy group (to give serine and threonine) results in a significant decrease in the retention volume (Fig. 2), probably reflecting the lower stability of copper(II) complexes of serine and threonine as compared with those of alanine and aminobutyric acid. However, the presence of a hydroxy group in the β-position enhances the enantioselectivity of the sorption process so that the $\delta\Delta G°$ values rise to 150 and 245 cal/mol for serine and threonine from 24 and 120 cal/mol for alanine and aminobutyric acid, respectively. Contrary to this, a hydroxy group in the γ-position has almost no influence on either the retention volumes of the enantiomers or their separation (compare homoserine with aminobutyric acid). Other functional groups in the γ-position (as in methionine and asparagine), just like the hydroxy group, do not increase the enantioselectivity above the value for aminobutyric acid. It is likely that β-hydroxy groups alone have the ability to participate in the coordination process. This suggestion explains the noticeable role of the β-C-atom configuration (compare threonine with allo-threonine) and the unusually high values of HETP for serine and threonine.

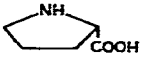
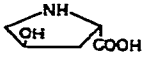
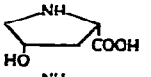
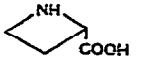
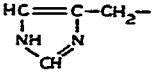
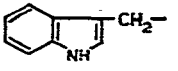
Aromatic amino acids

Amino acids containing a phenyl ring demonstrate comparatively high resolution ability and a high affinity towards the resin (large retention volumes). Some additional interactions with the polymer matrix must be increasing the stability of sorption complexes of these amino acids, especially β-phenyl-α-alanine (Fig. 3). Its

TABLE I

ELUTION PARAMETERS OF AMINO ACIDS ON THE L-HYDROXY-PROLINE RESIN IN THE COPPER(II) FORM

Eluents: 0.1 *M* NH_4OH (N = 1–28); 1.5 *M* NH_4OH (N = 29–32); 0.025 *M* $Na(NH_4)_2PO_4$, pH 8.3 (N = 33–36).

N	*Amino acid*	*α-Radicals or molecular structure*	*V*		*α*	*δΔG°* (cal/mol)	*HEEP (cm)*	
			L	D			L	D
1	Glycine	H–	6.44		—	—	0.31	
2	β-Alanine	$H_2NCH_2CH_2COOH$	0.28		—	—	~100	
3	Alanine	CH_3–	5.82	6.04	1.04	24	0.38	0.56
4	Aminobutyric acid	CH_3CH_2–	6.48	7.95	1.22	120	0.29	0.32
5	Norvaline	$CH_3CH_2CH_2$–	11.2	19.9	1.65	290	0.30	0.21
6	Norleucine	$CH_3CH_2CH_2CH_2$–	21.4	47.4	2.20	460	0.26	0.17
7	Valine	$CH_3CH(CH_3)$–	7.27	11.8	1.61	280	0.31	0.43
8	Isovaline	CH_3CH_2–; CH_3–	6.8	8.5	1.25	130		
9	Leucine	$CH_3CH(CH_3)CH_2$–	14.2	24.2	1.70	310	0.44	0.51
10	Isoleucine	$CH_3CH_2CH(CH_3)$–	11.1	20.9	1.89	370	0.53	0.49
11	Serine	$HOCH_2$–	3.47	4.48	1.29	150	0.94	0.90
12	Threonine	$HOCH(CH_3)$–	3.47	5.27	1.52	245	1.03	0.63
13	allo-Threonine	$HOCH(CH_3)$–	2.65	3.85	1.45	220		
14	Homoserine	$HOCH_2CH_2$–	5.32	6.65	1.25	130	0.35	
15	Methionine	$CH_3SCH_2CH_2$–	11.7	14.3	1.22	120	0.53	0.53
16	Asparagine	H_2NCOCH_2–	4.60	5.37	1.17	90	0.39	0.38
17	Glutamine	$H_2NCOCH_2CH_2$–	2.46	3.70	1.50	240	0.52	
18	Phenylglycine	C_6H_5–	6.15	13.6	2.22	465	0.70	0.74
19	Phenylalanine	$C_6H_5CH_2$–	33.8	97.6	2.89	620	0.59	0.62
20	α-Phenyl-α-alanine	C_6H_5–; CH_3–	11.9	12.5	1.07	39	1.25	1.25
21	Tyrosine	$HOC_6H_4CH_2$–	8.95	19.8	2.23	465	1.25	0.83
22	Phenylserine	$C_6H_5CH(OH)$–	22.6	41.1	1.82	350	0.98	0.94
23	β-Phenyl-β-alanine	$C_6H_5CHCH_2COOH$ (NH$_2$)	1.25	2.23	1.79	340		
24	Proline		14.6	57.8	3.95	800	0.60	0.88
25	Hydroxyproline		9.18	29.1	3.17	680	0.67	0.48
26	allo-Hydroxy-proline		29.4	17.7	1.65	290	0.68	0.51
27	Azetidinecarboxylic acid		14.0	31.5	2.25	475	1.02	0.92
28	Ornithine	$H_2NCH_2CH_2CH_2$–	34.4					
29	Ornithine	$H_2NCH_2CH_2CH_2$–	2.0	2.0	1.0	0	1.2	1.2
30	Lysine	$H_2NCH_2CH_2CH_2CH_2$–	2.5	3.04	1.22	120	0.84	0.87
31	Histidine		14.6	5.22	2.80	600	0.49	0.51
32	Tryptophan		20.7	36.5	1.77	330	0.81	0.63
33	Aspartic acid	$HOOCCH_2$–	11.5	11.5	1.0	0	1.34	1.45
34	Glutamic acid	$HOOCCH_2CH_2$–	2.2	1.8	1.22	120	1.63	1.42
35	Iminodiacetic acid	$HN(CH_2COOH)_2$	32.6		—	—	1.28	
36	Serine	$HOCH_2$–	42.5					

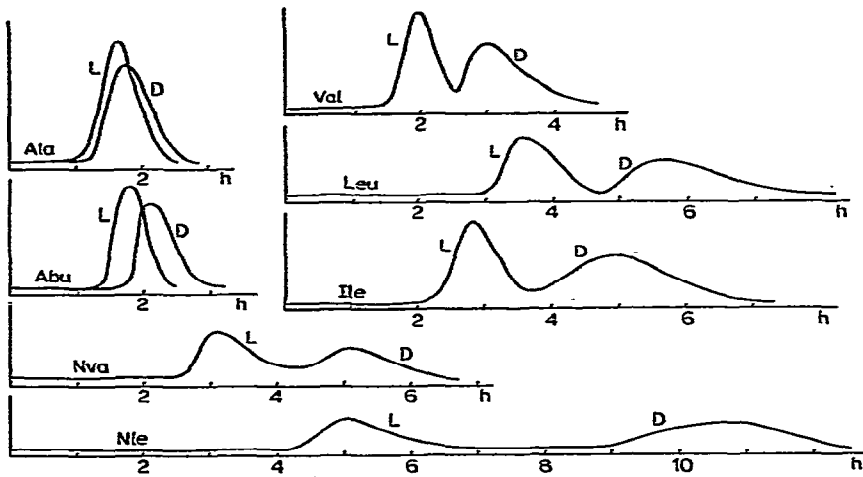


Fig. 1. Chromatography of the enantiomers of alanine ($\alpha = 1.04$), aminobutyric acid ($\alpha = 1.22$), norvaline ($\alpha = 1.65$), norleucine ($\alpha = 2.20$), valine ($\alpha = 1.61$), leucine ($\alpha = 1.70$) and isoleucine ($\alpha = 1.89$). Column 7.8 × 140 mm; 0.1 *M* NH_4OH; 10 ml/h. The degree of saturation of the L-hydroxyproline resin by copper(II) ions was 92%. Particle size *ca.* 100 μm.

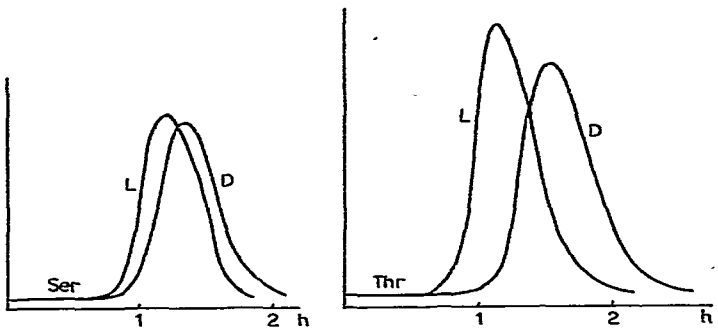


Fig. 2. Chromatography of the enantiomers of serine ($\alpha = 1.29$) and threonine ($\alpha = 1.52$). Conditions as given in Fig. 1.

hydroxy analogue, tyrosine, is partially ionized under the chromatographic conditions (0.1 *M* NH_4OH) and, to a certain extent, expelled from the negatively charged resin phase. A very low retention time of the β-phenyl-β-alanine isomers forming a six-membered chelate ring (but nevertheless resolving with a factor of $\alpha = 1.79$) indicates that there are no additional hydrophobic interactions between the aromatic sorbate and the resin matrix, other than in the coordination sphere of the sorption complex.

Among the aromatic amino acids, α-phenyl-α-alanine alone contains two substituents at the α-carbon atom. As was the case with isovaline, it is poorly resolved.

Cyclic amino acids

Proline enantiomers show the highest sorption selectivity on the resin containing L-hydroxyproline ($\alpha = 3.95$; $\delta\Delta G^\circ = 800$ cal/mol). Hydroxyproline ($\alpha = 3.17$) is also completely resolved into its enantiomers. Its diastereomer, allo-hydroxyproline,

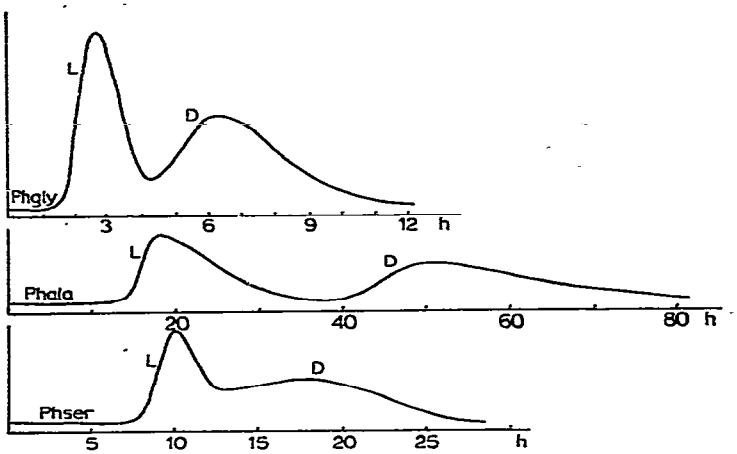


Fig. 3. Chromatography of the enantiomers of phenylglycine ($\alpha = 2.22$), phenylalanine ($\alpha = 2.89$) and phenylserine ($\alpha = 1.82$). Conditions as given in Fig. 1.

displays a lower selectivity ($\alpha = 1.65$) and surprisingly, a reversed elution order of the components: the D-isomer is eluted before the L-isomer (Fig. 4). The β-hydroxy group of the latter is undoubtly coordinated to the axial position of the copper(II) ion. The D-enantiomer cannot do this because the corresponding axial position is blocked by the *N*-benzyl group of the fixed ligand.

Azetidinecarboxylic acid, a four-membered heterocyclic homologue of proline, shows a sufficiently high selectivity ($\delta\Delta G^\circ = 475$ cal/mol) but unusually broad elution peaks (HETP *ca.* 1 cm).

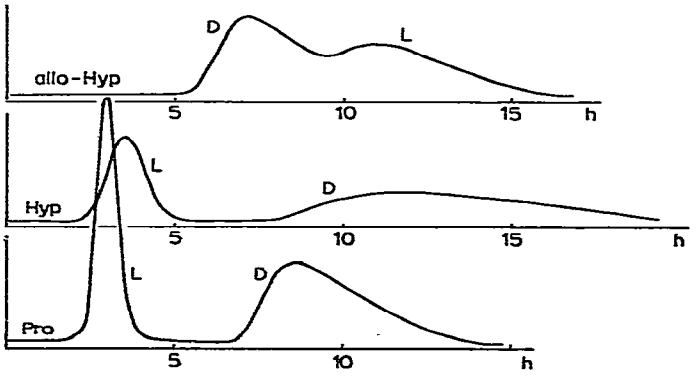


Fig. 4. Chromatography of the enantiomers of allo-hydroxyproline ($\alpha = 1.65$) and hydroxyproline ($\alpha = 3.17$) in 0.2 *M* NH_4OH at a flow-rate of 20 ml/h and proline ($\alpha = 3.95$) in 0.5 *M* NH_4OH at a flow-rate of 8 ml/h. Other conditions as given in Fig. 1.

Basic amino acids

Basic amino acids are strongly retained by the resin, so 1.5 *M* NH_4OH is used for their elution. This is probably due to the partial positive charge of these com-

pounds, which can strongly influence their distribution between the resin and the solution, rather than to the stability of the sorption complexes. Arginine, which contains a positively charged side-chain is not eluated even if 6 *M* NH_4OH is used.

Ornithine isomers are not resolved, and lysine isomers only poorly so. Tryptophan shows a high selectivity factor ($\alpha = 1.77$), but its peaks are too broad to be completely resolved under standard conditions (Fig. 5). The elution order of histidine isomers is reversed, which can be accounted for in terms of axial coordination of the imidazole group of the L-isomer.

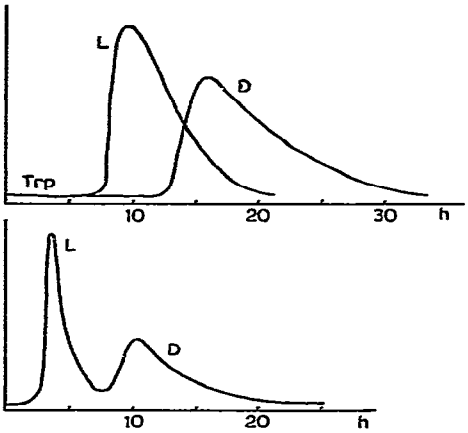


Fig. 5. Chromatography of the enantiomers of tryptophan. *Above:* in 1.5 *M* NH_4OH, other conditions as given in Fig. 1; $\alpha = 1.77$. *Below:* in 0.2 *M* NH_4OH at a flow-rate of 6 ml/h on a column of 4.5 × 280 mm with the L-hydroxyproline resin saturated by copper(II) ions to an extent of 15%, particle size 20–30 μm; $\alpha = 3.09$.

Monoaminodicarbonic acids

Acid compounds are eluted by ammonia within the void column volume in spite of the fact that the stability of their copper complexes does not yield to that of aliphatic amino acids —negatively charged species are expelled from the resin phase. However, the distribution coefficients of amino acids rise significantly at lower pH values[7]. Thus, chromatography of acid compounds can be carried out in an ammonium phosphate buffer solution at a pH of 8.3. The retention volumes increase in the series: glutamic acid, aspartic acid, iminodiacetic acid, in accordance with the increasing stability constants of their copper complexes. (On chromatography in the phosphate buffer, neutral amino acids show very high retention volumes.)

Unfortunately, no resolution was observed in the case of aspartic acid and only a small one ($\delta\Delta G^\circ = 120$ cal/mol) in the case of glutamic acid. From unknown reasons, D-glutamic acid forms more stable sorption complexes than the L-isomer does.

General remarks

Though there is a general correlation between the stability of copper(II) complexes of different amino acids and their retention volumes on the L-hydroxyproline resin, contributions from electrostatic interactions and hydrophobic interactions

undoubtedly play an important role in the resin phase. Besides, only mixed ligand (amino acidato) (N-benzyl-L-hydroxyprolinato) copper(II) complexes can be considered as adequate low molecular weight models for sorption complexes.

Sorption selectivity of many amino acid enantiomers on the L-hydroxyproline resin is sufficiently high to permit their complete separation under the conditions used and to indicate that the process can be successfully carried out on a preparative scale.

The efficiency of the column used was rather poor: the values of HETP amounted to 0.3–0.5 cm for aliphatic amino acids, 0.6–1.2 cm for aromatic, basic and hydroxy amino acids, and greater than 1 cm for acid compounds. However, the efficiency can be substantially improved by using resins with a smaller particle size (Fig. 6), which reduces the values of HETP by a factor of 2 or 3 and results in complete resolution of valine, leucine, tryptophan, phenylglycine and some other amino acids.

The LEC process can be improved in other ways. The very high retention volumes of basic amino acids can be reduced by lowering the degree of saturation

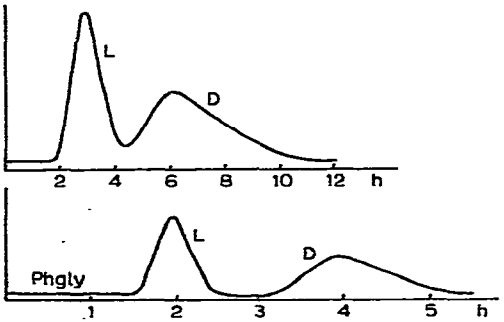


Fig. 6. Chromatography of the enantiomers of phenylglycine. *Above:* under standard conditions (see Fig. 1); HETP = 7 mm, $\alpha = 2.22$, $R_s = 0.75$. *Below:* on a column of 3.5 × 190 mm at a flow-rate of 8.2 ml/h, particle size 20–30 μm; HETP = 2.8 mm, $\alpha = 2.22$, $R_s = 1.22$.

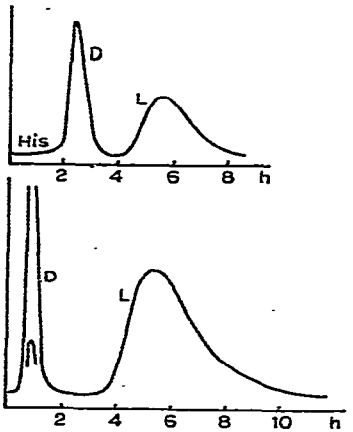


Fig. 7. Chromatography of the enantiomers of histidine. *Above:* in 1.5 *M* NH_4OH, other conditions as given in Fig. 1; $\alpha = 2.80$. *Below:* in 0.5 *M* NH_4OH at a flow-rate of 20 ml/h; the degree of resin saturation by copper(II) ions was 70%; $\alpha = 8.0$.

of the resin by copper(II) ions[3,7]. More dilute ammonia solutions can then be used for chromatography. Unexpectedly, a significant increase in sorption selectivity was observed in dilute ammonia for histidine (Fig. 7), tryptophan (Fig. 5) and proline. Obviously, this phenomenon indicates that ammonia molecules can take part in the formation of mixed-ligand sorption complexes, sometimes lowering the enantioselectivity.

Fig. 8 shows an example of how elution profiles can be improved by the use of ammonia gradients.

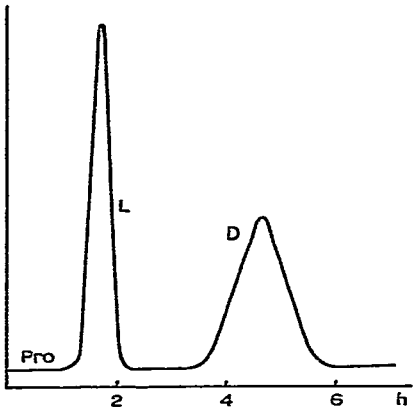


Fig. 8. Chromatography of the enantiomers of proline in a 0.5–1.5 *M* gradient of ammonia. Other conditions as given in Fig. 1.

Following further improvement of the LEC process, we hope to develop a simple and rapid method for the analysis of the enantiomeric composition of many amino acids, which would differ from the GLC method by making superfluous the preparation of volatile derivatives of amino acids.

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