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HIGH-PERFORMANCE LIGAND-EXCHANGE CHROMATOGRAPHY OF α-AMINO ACID ENANTIOMERS

STUDIES ON MONOMERICALLY BONDED 3-(L-PROLYL)- AND 3-(L-HYDROXYPROLYL)PROPYL SILICAS

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SUMMARY

Three packings with the following surface composition were synthesized on LiChrosorb Si 100, d_p = 10 μm: [Cu(II) 3-(L-prolyl)propyl]$^+$ (packing I), [Cu(II) 3-(L-hydroxyprolyl)propyl]$^+$ (packing II) and [Cu(II) 3-(L-hydroxyprolyl)propyl]$^+$ with remaining excess 3-(iodopropyl) groups (packing III). The separation of α-amino acid enantiomers was studied in eluents containing a constant concentration of copper(II) acetate (10^{-4} *M*). The pH of the buffered eluent was varied in the range 4–6 and the ammonium acetate concentration was varied from 0.001 to 0.1 *M*, and these were established as dominating parameters in controlling the retention and enantioselectivity. In contrast, the addition of an organic solvent such as methanol, acetonitrile or tetrahydrofuran gave only minor changes. The same was observed for the changes in k' and α on increasing the methanol content up to 30% (v/v) and by varying the column temperature between 298 and 323°K.

The order of elution of α-amino acid enantiomers was generally found to be L- < D-; there were, however, a series of exceptions, depending on the type of packing and the eluent composition. Enantioselectivity can be understood in terms of complexation of the enantiomer to a *trans*-bis(amino acidato)–copper complex and additional interaction through complexation of free ligand sites, hydrophobic interaction between the radical R and the *n*-propyl spacer of the bonded ligand, hydrogen bonding, etc.

The columns permitted the separation of six racemic α-amino acids into the corresponding enantiomers, using common high-performance liquid chromatographic equipment with a UV photometer (254 nm).

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INTRODUCTION

The separation of racemates into enantiomers by means of column liquid chromatography is a rapidly developing field covering both theoretical studies on the underlying retention mechanism and practical applications[1–3]. Thorough attempts have been made using high-performance liquid chromatography (HPLC) to generate enantioselectivity based on complex formation with soluble chiral reagents in the eluent[4,5] and by adsorbing appropriate chiral ligands on to the surface of a common reversed-phase silica[6]. A much more promising approach consists in the covalent binding of the chiral ligand to the surface of the silica support, yielding a stable and selective phase system. Studies have been reported on L-proline and L-hydroxyproline as optically active complexing ligands bonded either by the carboxyl[7,8] or the amino functional group[9–13] to a hydrocarbon spacer, which is anchored in turn by a siloxane group to the silica surface. Complexation was carried out with Cu^{2+} as metal ion. Critical evaluation of the data, however, shows that most of the chiral phases (except those reported in refs. 10–12) offer poor selectivity, low column performance and limited peak capacity.

The purpose of this work was to synthesize chiral bonded silica phases as monomeric coatings, composed of L-proline and L-hydroxyproline amino-linked to an *n*-propyl spacer anchored to the surface. Surface modification was accomplished either by direct reaction of the chiral silanizing reagent or by bonding the hydrocarbon spacer followed by the fixation of the chiral ligand. The three packings were tested under widely different eluent compositions using α-amino acids as model substances. The main concern was to assess the variation in retention and selectivity as a function of process variables and, based on these results, to elucidate the retention mechanism.

EXPERIMENTAL

Amino acids

The α-amino acids (supplied by Sigma, Munich, G.F.R., and Degussa, Hanau, G.F.R.) were aspartic acid (Asp), glutamic acid (Glu), histidine (His), alanine (Ala), asparagine (Asn), glutamine (Gln), serine (Ser), proline (Pro), citruline (Cit), threonine (Thr), valine (Val), 3,4-dihydrophenylalanine (Dopa), lysine (Lys), norvaline (Nval), tyrosine (Tyr), methionine (Met), arginine (Arg), isoleucine (Ileu), leucine (Leu), norleucine (Nleu), ethionine (Eth), phenylalanine (Phe) and tryptophan (Trp).

Preparation of packings

LiChrosorb Si 100 (particle size, d_p = 10 μm; E. Merck, Darmstadt, G.F.R.) was reacted with L-prolylpropyltriethoxysilane (product I) and with L-hydroxyprolylpropyltriethoxysilane (product II). Product III was obtained by a modification of the LiChrosorb Si 100 with 3-chloropropyltriethoxysilane and subsequent treatment with sodium iodide and L-hydroxyproline. Typical elemental analyses were C 7.37, H 1.44 and N 1.62% (w/w) for product I and C 5.31, H 1.06 and N 1.14% (w/w) for product III.

Chromatographic measurements

Columns were packed applying the high-viscosity slurry technique and *n*-hep-

tane as second liquid. After washing with 2-propanol the columns were loaded with Cu^{2+} by flushing the column with concentrated aqueous copper acetate (reagent grade; E. Merck). The loading procedure was always repeated when the eluent composition was changed.

The eluent contained 0.0001 *M* copper(II) acetate in all instances. The pH was varied between 4 and 6 by adding acetic acid/ammonium acetate. The ammonium acetate concentration used was either 0.001, 0.01 or 0.1 *M*. Methanol, acetonitrile and tetrahydrofuran were employed as organic solvents at a constant composition of 10% (v/v) in aqueous solution. The methanol content was also increased stepwise from 10 to 20 and 30% (v/v).

The liquid chromatograph was a Hewlett-Packard Model 1084 A fitted with a fixed-wavelength UV photometer (254 nm). The column temperature was set at 298 or 323°K. The enantioselectivity (α) was expressed by the ratio k'_D/k'_L, where k'_D is the capacity factor of the D-enantiomer and k'_L that of the L-enantiomer, t_0 (the elution time of an unretained solute) was measured applying a slightly modified mobile phase.

RESULTS AND DISCUSSION

Structure of chiral bonded phases

As trifunctional silanes were applied in the surface modification, on average between one and two surface hydroxyls are assumed to react with one silane molecule. Tentative models of the surface structure of products I–III are shown in Fig. 1. Product III may contain an amount of unreacted $\equiv Si(CH_2)_3I$ surface groups owing to incomplete reaction with L-hydroxyproline, and this leads to a lower surface concentration of bonded chiral ligands than for products I and II. When loaded with copper the chiral ligand forms a complex that is assumed to possess the structure indicated in Fig. 2.

Assessment of the variation of eluent composition and column temperature on the retention and enantioselectivity of α-amino acids

The objective of this examination was to establish general trends in the retention of α-amino acids under various conditions and to look for dominant and less dominant variables. The enantioselectivity of single amino acids in correlation with their chemical structure is discussed below.

pH of the eluent. The amino acid solutes are assumed to undergo a complexation reaction with the copper complex of the bonded chiral ligand ($[Cu(X\text{-}\overline{A}A)]^+$ in their amino acidato form AA^-, yielding a mixed ligand of bis(amino acidato)–copper complex ($[Cu(AA)(X\text{-}\overline{A}A)]$ according to

$$[Cu(AA)]^+ \; Ac^- \; + \; [Cu(X\text{-}\overline{A}A)]^+ \; Ac^- \; \rightleftharpoons \; [Cu(AA) \; (X\text{-}\overline{A}A)] \; + \; Cu(CH_3COO)_2 \quad (1)$$

where X represents the matrix and $\overline{A}A$ the bonded L-proline or L-hydroxyproline. According to eqn. 1, complexation of a given α-amino acid is favoured when the pH is increased. Depending on the nature of the α-amino acid, *i.e.*, on the p*K* value, the amino acid becomes increasingly negatively charged owing to the release of protons of the ammonium group with increasing pH. As a consequence, the concentration of

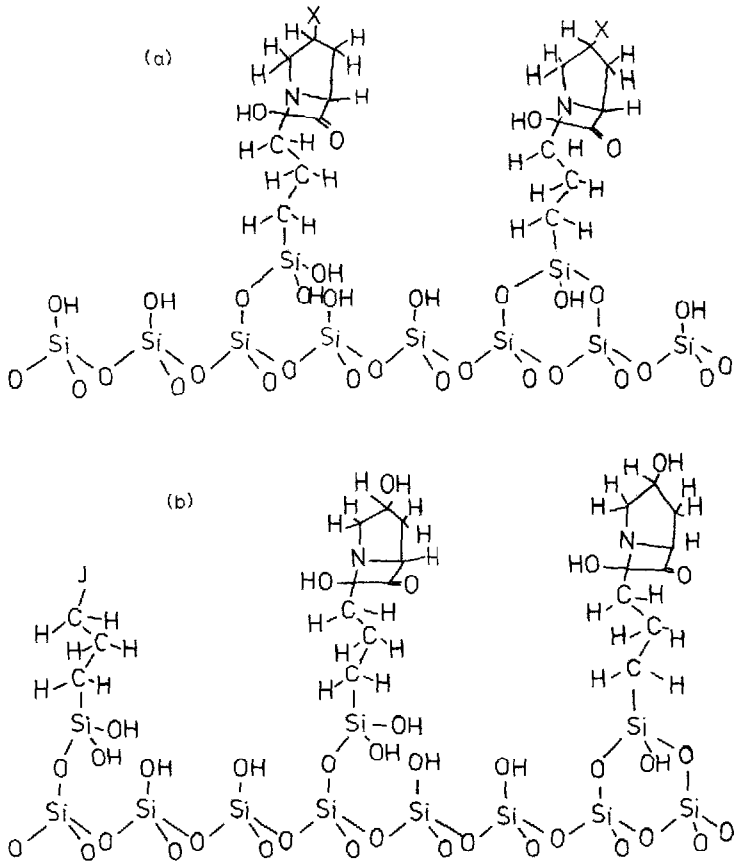

Fig. 1. (a) Model of surface structure of bonded phase I (X = H) and bonded phase II (X = OH); (b) model of surface structure of bonded phase III.

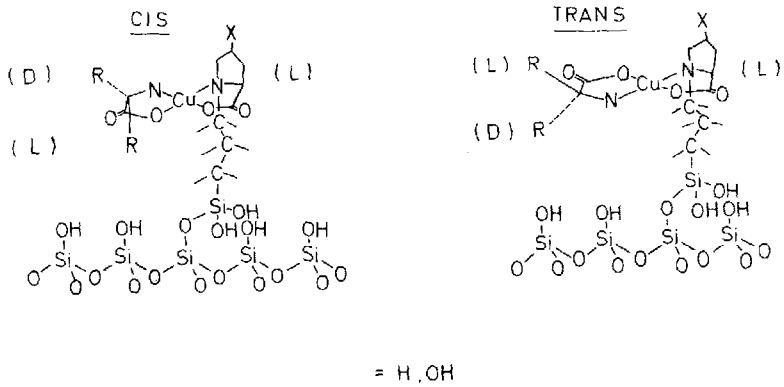

Fig. 2. Model of structure of mixed-ligand copper complex.

$[Cu(AA)]^+$ is increased, which results in a higher concentration of $[Cu(AA)(X\text{-}\bar{A}A)]$ and hence enhances retention. The stability constant, K, obtained by applying the law of mass action to eqn. 1, remains constant.

At pH <4 complexation occurs to only a small extent and hence the retention of α-amino acids is low. Although the retention of α-amino acids is enhanced considerably at pH >6, the peaks eluted are rather broad, *i.e.*, the efficacy of the column becomes poorer. Studies were therefore centred on the optimum pH range (4–6).

Table I lists the k'_D, k'_L and α values of α-amino acids on the three packings and columns in a buffered (packings I and III) and non-buffered eluent (packing II).

As discussed above, the retention of both enantiomers of a given α-amino acid becomes progressively larger with increasing pH. Basic α-amino acids were observed to be most retarded, in addition to those exhibiting a high hydrophobic character, *e.g.*, Phe, Nleu, Ileu and Nval; acidic α-amino acids were least retarded. On plotting k' against the pH of the eluent, the slopes were found to be different for the enantiomeric forms of most α-amino acids, thus providing enantioselectivity. This indicates that the pH of the eluent affects the interactions of the D- and L-forms with the bonded ligand in a distinctly discriminatory way. Changes in the α values of enantiomers with changing eluent pH were observed on all three packings.

Ammonium acetate content of the eluent. The effect of varying the ammonium acetate concentration in the eluent up to 0.1 M under otherwise constant conditions was studied on packings I and II (see Table II). As ammonia is the Brønsted base of the acid NH_4^+ ($NH_4^+ + H_2O \rightleftharpoons NH_3 + H_3O^+$), the former acts as a ligand competing with α-amino acids in the formation of the mixed-ligand copper complex. It then follows that retention of α-amino acids decreases with increasing concentration of ammonium acetate owing to displacement. Straight lines result on plotting the data in Table II as a graph of k' *vs.* $\log[NH_4OOCCH_3]$. Again, as observed in the dependence of k' on pH, the slopes of lines are different for the D- and L-forms of a given α-amino acid. It appears that the change in ammonium acetate concentration affects the interactions of both the enantiomeric solutes to different extents.

Methanol content of the eluent. Although the reversed-phase character of the chiral bonded packings is expected to be low owing to the shortness of the *n*-propyl spacer, the addition of increasing amounts of methanol to the eluent will provide a sensitive means of detecting any reversed-phase behaviour. It can easily be seen from the data in Table III that the retention of D- and L-enantiomers on packings I–III increases on increasing the methanol content under otherwise constant conditions. One may assume that three factors will operate simultaneously to control the retention of α-amino acids: complexation, adsorption to ionic surface sites and hydrophobic interactions. The addition of methanol diminishes the concentration of ammonia as a competitive ligand in complexation, thus causing an enhancement of the capacity factors with increasing dilution. In opposition to this, an increasing methanol content reduces the polarity of the eluent in a reversed-phase system so that the capacity factors tend to decrease. In adsorption interactions between solutes and polar surface sites (*i.e.*, silanols), the retention increases with increasing methanol content.

The data in Table III indicate the highest retention for basic amino acids, *e.g.*, Lys, Arg and His, and also for hydrophobic amino acids, *e.g.*, Phe and Trp. This may support the assumption that complexation and hydrophobic interactions dominate. Comparison of the increase in k' with increasing methanol content shows a minor

TABLE I

EFFECT OF pH OF THE ELUENT ON THE RETENTION AND ENANTIOSELECTIVITY OF α-AMINO ACID ENANTIOMERS

Conditions: column dimensions, 250 × 4 mm I.D.; packing, products I–III, d_p = 10 μm; column temperature, 323°K; eluent, 10^{-4} *M* copper(II) acetate; pH adjusted with acetic acid.

Amino acid	*Packing I*						*Packing II*									*Packing III*					
	pH 4.75, 0.01 M NH_4Ac			*pH 5.0, 0.01 M NH_4Ac*			*pH 4.85*			*pH 5.0*			*pH 5.35*			*pH 5.0, 0.01 M NH_4Ac*			*pH 5.5, 0.01 M NH_4Ac*		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Asp	0.30	0.31	1.03	0.58	0.58	1.00	0.50	0.42	0.84	0.93	0.76	0.82	2.09	1.96	0.94	1.45	1.32	0.91	1.96	1.76	0.90
Glu	0.22	0.25	1.14	0.49	0.56	1.14	0.43	0.47	1.10	0.96	1.07	1.11	2.71	3.25	1.20	1.12	1.18	1.05	–	–	–
His	1.97	1.67	0.85	3.13	2.55	0.81	2.62	2.01	0.76	5.65	4.32	0.76		25.14		1.52	2.71	1.79	2.02	3.74	1.85
Ala	0.45	0.47	1.05	0.83	0.90	1.09	0.42	0.47	1.12	1.94	2.08	1.07	6.70	7.21	1.08	0.69	0.88	1.28	1.37	1.82	1.33
Asn	0.81	0.79	0.97	1.08	1.08	1.00	0.99	0.85	0.86	2.50	2.15	0.86	5.14	5.54	1.08	1.54	1.42	0.92	1.87	1.70	0.91
Gln	–	–	–	0.84	0.49	0.58	0.62	0.47	0.76	2.01	1.69	0.84	2.83	2.79	0.99	–	–	–	2.14	2.34	1.09
Ser	0.58	0.47	0.81	1.06	0.88	0.83	0.67	0.60	0.89	2.01	2.00	1.00	4.74	4.75	1.00	1.25	0.93	0.74	1.91	1.43	0.75
Pro	0.58	0.88	1.52	1.06	1.89	1.78	0.57	1.08	1.90	2.51	3.97	1.58	8.00	10.85	1.36	1.12	2.00	1.79	2.38	4.64	1.95
Cit		0.64	1.00		1.12	1.00		1.36	1.00		3.14	1.00		6.87	–	–	–	–	–	–	–
Thr	0.84	0.72	0.86	1.28	1.18	0.92	1.01	0.82	0.81	2.58	2.32	0.90	6.33	6.08	0.96	1.73	1.40	0.81	2.45	1.90	0.78
Val	0.74	0.94	1.27	1.69	1.86	1.10	0.87	0.94	1.08	2.74	3.08	1.12	8.53	9.72	1.14	1.89	1.54	0.82	3.35	3.15	0.94
Dopa							1.11	1.26	1.13		6.53	1.00	–	–	–	–	–	–	–	–	–
Lys	1.51	1.50	1.00	2.95	3.06	1.04	1.78	2.07	1.16	8.18	9.31	1.14	40.24	42.19	1.05	0.93	1.05	1.13	2.00	2.29	1.15
Nval		0.85	1.00		1.76	1.00		0.85	1.00		4.62	1.00	10.83	11.19	1.03	–	–	–	–	–	–
Tyr	1.16	1.04	0.90	2.26	2.01	0.89	1.13	0.78	0.69	2.96	2.14	0.72	7.12	6.05	0.85	3.95	2.73	0.69	6.21	3.94	0.63
Met	1.05	1.09	1.04	1.94	2.04	1.05	1.10	1.10	1.00	3.01	3.11	1.03	8.55	8.93	1.04	2.70	2.63	0.97	4.15	4.49	1.08
Arg	2.00	2.10	1.05	3.79	4.02	1.06	2.62	2.61	1.00	10.61	11.03	1.04	46.08	48.76	1.06	1.59	1.73	1.09	3.10	3.45	1.11
Ileu	0.89	1.09	1.23	1.76	2.11	1.20	1.07	1.12	1.05	3.11	3.56	1.14	8.93	10.07	1.13	2.79	2.48	0.89	4.18	4.96	1.19
Leu	0.92	1.05	1.14	1.77	1.94	1.10	1.07	1.10	1.03	2.83	3.21	1.13	8.51	9.31	1.10	2.25	2.22	0.97	4.2	4.02	0.96
Nleu		0.97	1.00		1.95	1.00		0.96	1.00		4.72	1.00	10.83	11.62	1.07	–	–	–	–	–	–
Eth		1.27	1.00		2.32	1.00		1.32	1.00		5.05	1.00		11.76	–	–	–	–	–	–	–
Phe	1.29	1.30	1.00	2.43	2.60	1.07	1.53	1.31	0.86	3.82	3.44	0.90	9.25	9.18	0.99	5.73	3.90	0.68	8.11	6.16	0.76
Trp	2.67	2.55	0.96	5.12	4.94	0.96	2.60	1.78	0.68	6.14	4.33	0.70	12.20	10.25	0.84	11.37	6.49	0.57	17.68	10.8	0.61

TABLE II

EFFECT OF CONCENTRATION OF AMMONIUM ACETATE IN ELUENT ON RETENTION AND ENANTIOSELECTIVITY OF α-AMINO ACID ENANTIOMERS

Conditions: column dimensions, 250 × 4 mm I.D.; packing, products I and II, d_p = 10 μm; column temperature, 323°K; eluent, 10^{-4} *M* copper(II) acetate and ammonium acetate adjusted to pH 5.0 with acetic acid.

Amino acid	Packing I									Packing II											
	0.001 M NH_4OOCCH_3			*0.01 M* NH_4OOCCH_3			*0.1 M* NH_4OOCCH_3			*0 M* NH_4OOCCH_3			*0.001 M* NH_4OOCCH_3			*0.01 M* NH_4OOCCH_3			*0.1 M* NH_4OOCCH_3		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Asp	2.19	2.12	0.97	0.58	0.58	1.00	0.20	0.20	1.00	0.93	0.76	0.82	0.60	0.50	0.83	0.36	0.33	0.92	0.47	0.47	1.00
Glu	2.51	2.91	1.16	0.49	0.56	1.14	0.06	0.06	1.00	0.96	1.07	1.11	0.67	0.74	1.10	0.19	0.22	1.16	0.28	0.33	1.18
His	8.62		1.00	3.13	2.55	0.81	1.08	0.78	0.73	5.65	4.32	0.76	4.25	3.47	0.80	3.43	5.39	1.56	2.08	1.18	0.57
Ala	4.37	4.60	1.05	0.83	0.90	1.09	0.11	0.14	1.27	1.94	2.08	1.08	1.63	1.74	1.07	0.58	0.64	1.10	0.36	0.39	1.08
Asn	4.27	4.16	0.97	1.08	1.08	1.00	0.32	0.32	1.00	2.50	2.15	0.86	2.18	1.76	0.81	1.49	1.32	0.88	0.83	0.74	0.89
Gln	4.29	4.22	0.98	0.84	0.49	0.58	0.17	0.06	0.35	2.01	1.69	0.84	1.67	0.71	0.42	0.89	0.24	0.27	0.54	0.35	0.65
Ser	4.21	4.15	0.98	1.06	0.88	0.83	0.23	0.24	1.04	2.01	2.00	1.00	1.61	1.21	0.76	1.07	0.81	0.76	0.60	0.47	0.78
Pro	5.39	6.86	1.27	1.06	1.89	1.78	0.16	0.28	1.75	2.51	3.97	1.58	1.82	3.14	1.72	0.76	1.42	1.87	0.51	0.96	1.88
Cit	–	–	–	1.12		1.00	–	–	–	3.14		1.00	1.83		1.00	1.07		–	0.61		–
Thr	4.65	4.51	0.97	1.28	1.18	0.92	0.33	0.29	0.88	2.58	2.32	0.90	1.99	1.87	0.94	1.51	1.33	0.88	0.83	0.72	0.87
Val	5.54	6.01	1.08	1.69	1.86	1.10	0.24	0.31	1.29	2.74	3.08	1.12	2.39	2.44	1.02	1.25	1.28	1.02	0.77	0.82	1.06
Dopa	–	–	–	–	–	–	–	–	–	6.53		1.00	4.00		1.00	2.08		1.00	1.57		1.00
Lys	14.4	16.3	1.13	2.95	3.06	1.04	0.31	0.31	1.00	8.18	9.31	1.14	6.61	6.75	1.02	3.05	3.12	1.02	0.90	0.96	1.07
Nval	–	–	–	1.76		1.00	–	–	–	4.62		1.00	2.60		1.00	1.17		1.00	0.68		1.00
Tyr	5.62	5.44	0.95	2.26	2.01	0.89	0.49	0.45	0.92	2.96	2.14	0.72	2.25	1.63	0.72	1.68	1.12	0.67	1.03	0.69	0.67
Met	5.70	5.94	1.04	1.94	2.04	1.05	0.38	0.41	1.08	3.01	3.11	1.03	2.49	2.54	1.02	1.55	1.49	0.96	0.90	0.89	0.99
Arg	18.46	18.70	1.01	3.79	4.02	1.06	0.46	0.46	1.00	10.61	11.03	1.04	8.07	8.69	1.08	3.87	4.12	1.06	1.15	1.24	1.08
Ileu	5.34	6.11	1.14	1.76	2.11	1.20	0.29	0.38	1.32	3.11	3.56	1.14	2.50	2.74	1.10	1.37	1.42	1.04	0.89	0.93	1.04
Leu	5.61	5.92	1.06	1.77	1.94	1.10	0.31	0.36	1.16	2.83	3.21	1.13	2.36	2.57	1.09	1.21	1.40	1.16	0.76	0.88	1.16
Nleu	7.57		1.00	1.99		1.00	–	–	–	4.72		1.00	2.74		1.00	1.36		1.00	0.80		1.00
Eth	7.69		1.00	2.32		1.00	–	–	–	5.05		1.00	2.82		1.00	2.00		1.00	1.12		1.00
Phe	6.50	6.55	1.00	2.43	2.60	1.07	0.55	0.60	1.09	3.82	3.44	0.90	2.87	2.63	0.92	2.08	1.69	0.81	1.28	1.08	0.84
Trp	10.67	10.32	0.97	5.12	4.94	0.96	1.30	1.27	0.98	6.14	4.33	0.70	4.29	3.07	0.72	3.57	2.28	0.64	2.18	1.42	0.65

TABLE III

EFFECT OF CONTENT OF METHANOL OF ELUENT ON RETENTION AND ENANTIOSELECTIVITY OF α-AMINO ACID ENANTIOMERS

Conditions: column dimensions, 250 × 4 mm I.D.; packing, products I–III, d_p = 10 μm; column temperature, 232°K; eluent, 10^{-4} *M* copper(II) acetate, 0.01 *M* ammonium acetate, adjusted to pH 5.0.

Amino acid	*Packing I*									*Packing II*		
	0% CH_3OH			*10% CH_3OH*			*30% CH_3OH*			*0% CH_3OH*		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Asp	0.58	0.58	1.00	1.05	1.05	1.00	2.12	2.13	1.00	0.36	0.33	0.92
Glu	0.49	0.56	1.14	1.00	1.10	1.10	2.00	2.16	1.08	0.19	0.22	1.16
His	3.13	2.55	0.81	3.81	3.10	0.81	6.38	5.10	0.80	3.44	5.39	1.56
Ala	0.83	0.90	1.09	1.23	1.32	1.07	2.61	2.82	1.08	0.58	0.64	1.10
Asn	1.08	1.08	1.00	2.08	1.96	0.94	3.30	3.17	0.96	1.49	1.32	0.88
Gln	0.84	0.49	0.58	–	–	–	2.64	1.91	0.72	0.89	0.24	0.27
Ser	1.06	0.88	0.83	1.95	1.96	1.00	3.09	2.64	0.85	1.07	0.81	0.76
Pro	1.06	1.89	1.78	1.90	2.70	1.42	2.96	4.18	1.41	0.76	1.42	1.87
Cit	1.12		1.00	–	–	–	3.19		1.00	1.07		1.00
Thr	1.28	1.18	0.92	2.06	1.96	0.95	3.21	2.87	0.89	1.51	1.33	0.88
Val	1.69	1.86	1.10	2.05	2.36	1.15	2.91	3.47	1.19	1.25	1.28	1.02
Dopa	–	–	–	–	–	–	–	–	–	2.08		1.00
Lys	2.95	3.06	1.04	4.14	4.20	1.01	7.88	7.75	0.98	3.05	3.12	1.02
Nval	1.76		1.00	2.22		1.00	3.02		1.00	1.17		1.00
Tyr	2.26	2.01	0.89	2.96	2.55	0.86	4.69	3.74	0.80	1.68	1.12	0.67
Met	1.94	2.04	1.05	2.36	2.43	1.03	3.57	3.57	1.00	1.55	1.49	0.96
Arg	3.79	4.02	1.06	5.03	5.33	1.06	8.95	9.79	1.09	3.87	4.12	1.06
Ileu	1.76	2.11	1.20	2.14	2.52	1.18	2.93	3.42	1.17	1.37	1.42	1.04
Leu	1.77	1.94	1.10	2.13	2.31	1.08	2.97	3.21	1.08	1.21	1.40	1.16
Nleu	1.99		1.10	2.33		1.00	3.01		1.00	1.36		1.00
Eth	2.32		1.00	2.60		1.00	3.66		1.00	2.00		1.00
Phe	2.43	2.60	1.07	2.86	2.95	1.03	4.02	3.93	0.98	2.08	1.69	0.81
Trp	5.12	4.94	0.96	5.78	5.32	0.92	8.00	6.68	0.83	3.57	2.28	0.64

enhancement of k' for Phe and Trp relative to that for Arg and Lys. Indeed, for Phe and Trp the hydrophobic interactions dominate more than do complexation effects, so that the enhancement of k' is less pronounced than that of basic amino acids.

In contrast to the foregoing variables, the methanol content does not change α significantly except for a few α-amino acids.

Type of organic solvent. On comparing the retention of α-amino acids in the sequence methanol, acetonitrile, tetrahydrofuran at a constant content of 10/90 (v/v) and a constant composition of buffered eluent, one notices only slight changes in retention (Table IV). The eluents containing methanol or acetonitrile show slightly higher retention than does tetrahydrofuran. One may expect that variations in the type of organic solvent will primarily affect hydrophobic interactions, if these are present. In reversed-phase chromatography it is known that methanol generates higher retentivity than tetrahydrofuran at equal contents, which is also evidenced by the retention of α-amino acids on this type of packing.

Column temperature. Limited examinations on the influence of column tem-

Packing III															
10% CH_3OH			*30% CH_3OH*			*0% CH_3OH*			*10% CH_3OH*			*30% CH_3OH*		
k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
0.79	0.71	0.90	0.96	0.83	0.86	1.45	1.32	0.91	2.13	1.96	0.92	6.16	5.59	0.91
0.75	0.92	1.22	0.93	1.08	1.16	1.12	1.18	1.05	1.64	1.70	1.04	4.86	5.82	1.20
3.75	2.56	0.68	3.82	2.61	0.68	1.52	2.71	1.79	1.78	3.03	1.70	3.81	6.03	1.59
1.35	1.48	1.10	1.47	1.67	1.13	0.69	0.88	1.28	0.96	1.21	1.26	2.59	3.34	1.29
1.74	1.57	0.90	1.90	1.69	0.89	1.54	1.42	0.92	2.03	1.88	0.93	4.54	4.14	0.92
1.49	0.89	0.60	1.65	1.12	0.68	–	–	–	–	–	–	–	–	–
1.58	1.29	0.82	1.75	1.39	0.79	1.25	0.93	0.74	1.74	1.30	0.75	4.14	3.21	0.77
1.55	2.96	1.91	1.65	3.42	2.07	1.12	2.00	1.79	1.27	2.36	1.86	3.06	6.50	2.12
1.85		1.00	2.20		1.00	–	–	–	–	–	–	–	–	–
1.75	1.53	0.87	1.86	1.62	0.87	1.73	1.40	0.81	2.21	1.79	0.81	4.75	3.86	0.81
1.83	2.05	1.12	1.90	2.22	1.17	1.89	1.54	0.82	2.10	1.71	0.81	4.41	3.83	0.87
3.11		1.00	4.02		1.00	–	–	–	–	–	–	–	–	–
5.17	5.53	1.07	6.44	6.99	1.08	0.93	1.05	1.13	1.19	1.34	1.13	2.96	3.0	1.01
1.77		1.00	1.94		1.00	–	–	–	–	–	–	–	–	–
2.30	1.57	0.68	2.37	1.57	0.66	3.95	2.73	0.69	4.42	2.74	0.62	7.60	5.14	0.68
1.86	1.83	0.98	1.94	1.93	0.99	2.70	2.63	0.97	2.87	2.75	0.96	5.49	5.52	1.00
5.79	6.33	1.09	6.74	7.36	1.09	1.59	1.73	1.09	1.82	2.01	1.10	3.72	4.15	1.12
1.86	2.05	1.10	1.87	2.15	1.15	2.79	2.48	0.89	2.90	2.56	0.88	5.48	5.16	0.94
1.62	1.94	1.20	1.67	2.03	1.21	2.25	2.22	0.99	2.32	2.35	1.01	4.64	4.94	1.06
1.85		1.00	1.96		1.00	–	–	–	–	–	–	–	–	–
2.08		1.00	–	–	–	–	–	–	–	–	–	–	–	–
2.27	1.96	0.86	2.27	1.90	0.84	5.73	3.90	0.68	5.77	3.90	0.68	9.63	6.49	0.67
3.87	2.44	0.63	3.87	2.33	0.60	11.37	6.49	0.57	10.50	5.95	0.57	14.88	8.85	0.59

perature, T_c, on retention were carried out (see Table V). On increasing T_c from 298 to 323°K, three cases can be observed: decrease in k', increase in k' and no change in k'. Most α-amino acids show about a 20% decrease in retention at 323°K compared with 298°K. The decrease in k' may be understood from the fact that both complexation and hydrophobic interactions are weakened on increasing T_c. It is also apparent that the variations in k' of both enantiomers with T_c are similar, so that α scarcely changes. Exceptions are His and Gln.

Retention mechanism

A critical evaluation of the dependences derived above indicates that complexation is the dominant interaction. Although the exact composition and structure of the complex between the bonded chiral copper complex and the α-amino acid solute is not known, a model structure is adapted to explain the enantioselectivity of the phase system. We assume that the complex formed is a mixed-ligand bis(amino acidato)–copper complex, which exists in a *trans* and/or *cis* configuration (see Fig. 2).

TABLE IV

EFFECT OF TYPE OF ORGANIC MODIFIER ON RETENTION AND SELECTIVITY OF α-AMINO ACID ENANTIOMERS

Conditions: column dimensions, 250 × 4 mm I.D.; packing, products I and II, d_p = 10 μm; column temperature, 323°K; eluent, 10^{-4} *M* copper(II) acetate, 0.01 *M* ammonium acetate, pH = 5.0/organic modifier 90:10 (v/v). THF = Tetrahydrofuran.

Amino acid	*Packing I*									*Packing II*								
	CH_3OH			*CH_3CN*			*THF*			*CH_3OH*			*CH_3CN*			*THF*		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Asp	1.05	1.05	1.00	0.95	0.95	1.00	0.86	0.86	1.00	–	–	–	–	–	–	–	–	–
Glu	1.00	1.10	1.10	0.80	0.95	1.19	0.72	0.83	1.15	–	–	–	–	–	–	–	–	–
His	3.81	3.10	0.81	4.08	3.35	0.82	3.62	2.88	0.79	3.82	2.13	0.56	4.18	2.55	0.61	3.69	2.04	0.55
Ala	1.23	1.32	1.07	1.35	1.60	1.18	1.17	1.30	1.11	1.35	1.61	1.19	1.74	2.04	1.17	1.41	1.71	1.21
Asn	2.08	1.96	0.94	2.07	2.00	0.97	1.96	1.87	0.95	–	–	–	–	–	–	–	–	–
Gln	–	–	–	1.35	0.83	0.61	1.15	0.74	0.64	–	–	–	–	–	–	–	–	–
Ser	1.95	1.96	1.00	2.00	1.95	0.97	1.84	1.79	0.97	1.46	1.21	0.83	1.86	1.59	0.85	1.54	1.28	0.83
Pro	1.90	2.70	1.42	1.77	2.49	1.41	1.34	2.21	1.65	1.63	4.24	2.60	1.91	4.68	2.45	1.52	3.91	2.57
Cit	–	–	–	1.97		1.00	1.74		1.00	–	–	–	–	–	–	–	–	–
Thr	2.06	1.96	0.95	2.12	2.00	0.94	1.94	1.80	0.93	1.65	1.39	0.84	1.97	1.71	0.87	–	–	–
Val	2.05	2.36	1.15	2.03	2.34	1.15	1.78	2.00	1.12	2.18	2.53	1.16	2.50	2.78	1.11	–	–	–
Dopa	–	–	–	1.31		1.00												
Lys	4.14	4.20	1.01	4.65	4.61	0.99	3.80	3.74	0.98	–	–	–	–	–	–	–	–	–
Nval	2.22		1.00	2.16		1.00	1.90		1.00	1.83	2.21	1.21	2.04	2.33	1.14	1.64	1.93	1.18
Tyr	26	2.55	0.86	2.51	2.51	1.00	2.86	2.38	0.83	2.86	1.88	0.66	3.20	2.07	0.65	3.37	1.92	0.57
Met	2.36	2.43	1.03	2.39	2.44	1.02	2.14	2.17	1.01	–	–	–	–	–	–	–	–	–
Arg	5.03	5.33	1.06	5.65	5.64	1.00	4.70	4.96	1.05	–	–	–	–	–	–	–	–	–
Ileu	2.14	2.52	1.18	2.09	2.44	1.17	1.86	2.10	1.13	–	–	–	–	–	–	–	–	–
Leu	2.13	2.31	1.08	2.13	2.28	1.07	1.83	1.96	1.07	2.27	2.71	1.19	2.48	2.87	1.16	1.83	2.14	1.17
Nleu	2.33		1.00	2.24		1.00	1.95		1.00	2.3	2.69	1.17	2.37	2.72	1.15	1.81	2.10	1.16
Eth	2.60		1.00	2.56		1.00	2.26		1.00									
Phe	2.86	2.95	1.03	2.77	2.83	1.02	2.48	2.53	1.02	3.4	3.03	0.89	3.4	2.99	0.88	3.4	2.44	0.72
Trp	5.78	5.32	0.92	5.31	4.74	0.89	5.17	4.57	0.88	6.81	4.24	0.62	6.4	3.85	0.60	5.80	3.35	0.58

TABLE V

EFFECT OF COLUMN TEMPERATURE ON RETENTION AND ENANTIOSELECTIVITY OF α-AMINO ACID ENANTIOMERS

Conditions: column dimensions, 250 × 4 mm I.D.; packing, products *II*, d_p = 10 μm; column temperature, 323°K; eluent, 10^{-4} *M* copper(II) acetate, 0.01 *M* ammonium acetate, pH 5.0.

Amino acid	*Packing II*					
	298°K			*323°K*		
	k'_L	k'_D	α	k'_L	k'_D	α
Asp	1.21	1.12	0.93	0.36	0.33	0.92
Glu	0.87	1.10	1.26	0.19	0.22	1.16
His	3.43	2.29	0.67	3.44	5.39	1.56
Ala	0.74	0.85	1.15	0.58	0.64	1.10
Asn	1.38	1.30	0.94	1.49	1.32	0.88
Gln	1.11	1.11	1.00	0.89	0.24	0.27
Ser	1.13	0.96	0.85	1.07	0.81	0.76
Pro	1.17	2.21	1.89	0.76	1.42	1.87
Cit	–	–	–		1.07	1.00
Thr	1.44	1.28	0.89	1.51	1.33	0.88
Val	1.50	1.62	1.08	1.25	1.28	1.02
Dopa	–	–	–		2.08	1.00
Lys	2.53	2.68	1.06	3.05	3.12	1.02
Nval	–	–	–		1.17	1.00
Tyr	1.99	1.37	0.69	1.68	1.12	0.67
Met	1.96	1.97	1.00	1.55	1.49	0.96
Arg	3.44	3.71	1.08	3.87	4.12	1.06
Ileu	1.71	1.87	1.10	1.37	1.42	1.04
Leu	1.65	1.85	1.12	1.21	1.40	1.16
Nleu	–	–	–		1.36	1.00
Eth	–	–	–		2.00	1.00
Phe	2.39	2.13	0.89	2.08	1.69	0.82
Trp	4.62	3.30	0.71	3.57	2.28	0.64

Of these the *trans* configuration is about 8 kJ/mol more stable than the *cis* form[6]. All mechanistic aspects are therefore related to this basic structure. The characteristic feature is the formation of planar quadratic configurations with copper as central metal ion. The two remaining axial positions of the octahedral copper complex are occupied by ligands, *e.g.*, ammonia. The formation of the mixed-ligand copper complex alone does not explain the enantioselectivity between D- and L-enantiomers of α-amino acids, but provides the necessary preconditions for increasing α by putting the D- or L-form of an α-amino acid in a most favourable position for additional interactions. Complexation, *i.e.*, the participation of chemical equilibria in retention, provides an effective means of increasing retention and of controlling retention simply by adjusting the pH, ammonium acetate concentration, etc.

In the bonded state the α-amino acid enantiomer can be involved in additional interactions that are weaker than those of complexation but, owing to the balance of complexation, will have a considerable effect on the α values of enantiomers.

The interactions taken into consideration are as follows:

(i) additional complexation of bonded α-amino acids having free interacting sites and occupying one of the axial positions of the octahedral copper complex;

(ii) additional tri- or polydentate complexation of α-amino acids with the free coordination sites of adjacent bonded copper complexes, provided the chain of the α-radical R is sufficiently long;

(iii) hydrophobic interactions between the organic radical R of the α-amino acid in its bonded state and the *n*-propyl spacer of the bonded ligand;

(iv) interactions between the organic radical R of the bonded α-amino acid and the hydroxy group of the bonded L-hydroxyproline phase;

(v) interactions between the organic radical R of the α-amino acid with residual silanol groups when R carries basic groups;

(vi) ionic interactions between the charged site chains of the α-amino acid and bonded ligands that do not participate in complexation.

At first sight it seems difficult to discriminate between these interactions and to correlate α. A limited number of instances will demonstrate the usefulness of the concept, however.

α-Amino acids of varying chain length of the radical R

Three candidates are considered: Ala[$CH_3CH(NH_2)COOH$], Nval [$CH_3(CH_2)_2CH(NH_2)COOH$] and Nleu[$CH_3(CH_2)_3CH(NH_2)COOH$]. These have the same basic structure but differ in the chain length of the radical R. Retention increases in the order $k'_{Nleu} > k'_{Nval} > k'_{Ala}$. As there is no significant difference in the stability constants of α-amino acids forming the bis(amino acidato)–copper complex, the increase in retention can be attributed to hydrophobic interactions between the radical R of the α-amino acid and the *n*-propyl group of the bonded ligand. Both enantiomers are, of course, involved in hydrophobic interactions; the D-enantiomer is particularly favoured, however, owing to its closer attachment to the propyl spacer than the L-enantiomer. Hence the elution sequence will be L- < D-.

α-Amino acids having linear and branched chains in the organic radical R

Two series of α-amino acids were chosen for comparison: (a) Nleu[$CH_3(CH_2)_3CH(NH_2)COOH$], Leu[$(CH_3)_2CHCH_2CH(NH_2)COOH$] and Ileu[$CH_3CH_2CH(CH_3)CH(NH_2)COOH$] and (b) Nval[$CH_3(CH_2)_2CH(NH_2)COOH$] and Val$(CH_3)_2CHCH(NH_2)COOH$].

In both series, α-amino acids having a linear chain possess a higher retention than those having branched chains. Again, the enantioselectivity found is L- < D-, suggesting that for steric reasons the D-enantiomer exhibits a preferential orientation for stronger hydrophobic interactions. In addition, one notices a higher enantioselectivity for branched than for linear α-amino acids. This may be caused by a better accessibility of *n*-propyl groups in the ligand to the branched alkyl chains of the α-amino acids than would exist for linear chains.

On packing III the opposite elution order, D- < L-, is observed for some α-amino acids compared to packings I and II. In this instance the lower hydrophobic character of the surface of packing III (due to residual ≡ $Si(CH_2)_3I$ groups) may be responsible for this behaviour.

Bidendate vs. tridendate and polydendate α-amino acids

The basic α-amino acids compared with Nleu[$CH_3(CH_2)_3CH(NH_2)COOH$], *viz.*, Lys[$H_2N(CH_2)_4CH(NH_2)COOH$] and Arg[$H_2NC(NH)NH(CH_2)_3CH(NH_2)$-COOH], are taken to illustrate the dependence of the number of ligand sites in α-amino acids on retention and enantioselectivity. The retention increases in the sequence $k'_{Arg} > k'_{Lys} > k'_{Nleu}$ as a result of a stronger complexation in the same order. The retention order is observed to be L- < D- for these three α-amino acid enantiomers. The free ligand sites of Lys and Arg, bonded in the mixed-ligand copper complex, are capable of undergoing additional complexation with those free ligands of the copper complex $[Cu(X\text{-}\overline{A}A)]^+$ which are in the vicinity. Again for steric reasons, interactions of the D-enantiomer are more probable than those of the L-enantiomer. Interactions may also take place between the basic ligand sites of the α-amino acid and the residual silanol groups at the surface of the packings. α values of enantiomers of Arg and Lys are of the same order of magnitude but are higher than for those of Nleu.

Acidic α-amino acids

Asp[$HOOCCH_2CH(NH_2)COOH$] and Glu[$HOOC(CH_2)_2CH(NH_2)COOH$] were considered. The k'_D value of Glu is observed to be higher than k'_D of Asp, whereas k'_L of L-Glu and L-Asp are comparable. Sometimes k'_L of L-Asp exceeds that of k'_L of L-Glu. The latter observation indicates a similar behaviour of L-enantiomers in the strength of complexation.

The enhancement of k'_D of D-Glu relative to k'_D of D-Asp may be attributed to a better coordinative linkage of the carboxyl group of D-Glu to adjacent bonded copper ligands.

α-Amino acids carrying polar substituents in the organic radical R

The following pairs of α-amino acids were considered for comparison:
Phe[$C_6H_5CH_2CH(NH_2)COOH$] and Tyr[$HOC_6H_4CH_2CH(NH_2)COOH$];
Ala[$CH_3CH(NH_2)COOH$] and Ser[$HOCH_2CH(NH_2)COOH$];
Ala[$CH_3CH(NH_2)COOH$] and Asn[$H_2NCOCH_2CH(NH_2)COOH$];
Ala[$CH_3CH(NH_2)COOH$] and Asp[$HOOCCH_2CH(NH_2)COOH$]; and
Glu[$HOOC(CH_2)_2CH(NH_2)COOH$] and Gln[$H_2NCO(CH_2)_2CH(NH_2)COOH$].

For the pair Phe and Tyr, Phe is seen to be more strongly retained than Tyr owing to the loss of hydrophobicity of Tyr on introducing a hydroxyl group.

The elution order on packing I (L-proline bonded type) is L-Phe < D-Phe and D-Tyr < L-Tyr. In contrast, on packing II (L-hydroxyproline bonded type) the retention order was D-Phe < L-Phe and D-Tyr < L-Tyr. In the former instance, strong hydrophobic interactions between the phenyl ring of D-Phe with the *n*-propyl spacer of the bonded ligand may cause the preferential retention of D-Phe over L-Phe. Disappearance of the hydrophobic character as in the case of Tyr will reverse the elution order.

For the pair Ala and Ser the following results were obtained: k'_L of L-Ser > k'_L of L-Ala and k'_D of D-Ser < k'_D of D-Ala. For the D-enantiomers hydrophobic interactions govern retention and hence the sequence of elution will be k'_D of D-Ala > k'_D of D-Ser (owing to the loss of hydrophobicity of D-Ser stemming from the hydroxyl group in the α-radical). The L-Ala cannot undergo such complexation interactions. Thus the observed elution sequence is for Ala L- < D- and for Ser D- < L-.

The retention data of Ala and Asn are k'_L of L-Asn > k'_L of L-Ala and k'_D of D-Asn > k'_D of D-Ala at pH $\leqslant$ 5.0. The latter changes to k'_D of D-Asn < k'_D of D-Ala at pH > 5.0 (see Table I, packings II and III).

The stronger retention of L-Asn relative to L-Ala may be understood if the $CONH_2$ of L-Asn participates in additional complexation at the axial position of the octahedral Cu(II) complex. The longer retardation of D-Asn relative to D-Ala at pH $\leqslant$5.0 and the reverse of this effect of pH >5.0 indicate two opposing, pH-dependent interactions. The elution sequence for Asn is D- < L- for reasons already discussed above.

Comparing Ala with Asp one obtains k'_L of L-Asp < k'_L of L-Ala and k'_D of D-Asp < k'_D of D-Ala. This is valid for packings I and II provided that the concentration of ammonium acetate does not exceed 0.01 *M*. At 0.1 *M* ammonium acetate at pH 5.0 (see Table II), both enantiomeric forms of Asp are seen to be longer retained than those of Ala. The latter order is also generally established on packing III at all eluent compositions (see Tables I and III). The elution order is D- < L- for Asp on all packings studied and L- < D- for Ala.

The preferential retention of L-Asp relative to D-Asp is believed to be due to additional complexation of the bonded L-Asp to the copper complex via its carboxyl group, while the D-Asp is unable to undergo such interactions for steric reasons.

An interesting comparison is provided by the pair Glu and Gln: k'_L of L-Glu < k'_L of L-Gln and k'_D of D-Glu < k'_D of D-Gln. For both D-enantiomers there are exceptions: (i) on changing the pH of the eluent from 4.85 to 5.35 on packing II (see Table I), (ii) on changing the ammonium acetate concentration from 0.001 to 0.01 *M* on packing I (see Table II) and (iii) on changing the methanol content from 0 to 30% (v/v) on packings I and II (see Table III).

The substitution of the second carboxyl group of α-amino acids by the uncharged carboxamide group predominantly affects the retention only of the D-form, whose radical is closer to the surface than is that of the L-form. The dependence of the retention of the D-enantiomers of D-Glu and D-Gln on so many variables (*e.g.*, pH, concentration of ammonium acetate, methanol content) makes it extremely difficult to separate the net interaction into individual contributions.

It is interesting to follow the enantioselectivity of Glu and Gln on packing II, first on varying pH and secondly on varying the ammonium acetate concentration. The following α values are obtained: for Glu, 1.10 (pH 4.85), 1.11 (pH 5.0) and 1.13 (pH 5.35), and for Gln, 0.76 (pH 4.85), 0.84 (pH 5.0) and 0.99 (pH 5.35) (see Table I); and for Glu, 1.14 ($[NH_4OOCCH_3]=0$), 1.10 ($[NH_4OOCCH_3]$ = 0.001 *M*), 1.14 ($[NH_4OOCCH_3]$ = 0.01 *M*) and 1.19 ($[NH_4OOCCH_3]$ = 0.1 *M*), and for Gln, 0.84 ($[NH_4OOCCH_3]$ = 0), 0.42 ($[NH_4OOCCH_3]$ = 0.001 *M*), 0.27 ($[NH_4OOCCH_3]$ = 0.01 *M*) and 0.64 ($[NH_4OOCCH_3]$ = 0.1 *M*) (see Table II).

Examples of the separation of the enantiomers of Glu and Gln are given in Fig. 3. A chromatogram of the resolution of five pairs of α-amino acids into their enantiomers on packing II is shown in Fig. 4.

CONCLUSION

The main emphasis has been placed on illustrating the sensitivity of the phase system to variations of the eluent composition and column temperature with regard

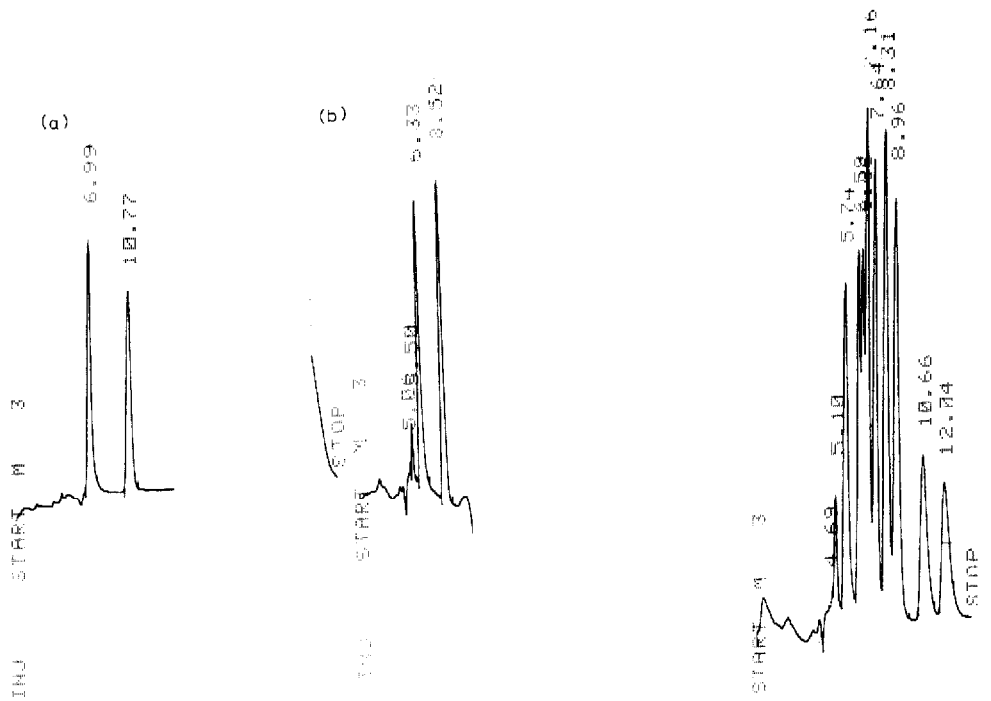

Fig. 3. Separation of racemic α-amino acids on bonded phase II. Column dimensions, 250 × 4.0 mm I.D.; packing, bonded phase II, d_p = 10 μm; column temperature, 323°K; eluent, 0.01 *M* ammonium acetate, adjusted to pH 5.0 with acetic acid, 10^{-4} *M* copper(II) acetate; flow-rate, 0.75 ml/min; detector, UV (254 nm). Elution sequence: (a) L-Glu, D-Glu; (b) D-Glu, L-Glu.

Fig. 4. Separation of racemic α-amino acids on bonded phase II. Column dimensions, 250 × 4 mm I.D.; packing, bonded phase II, d_p = 10 μm; column temperature, 323°K; eluent, 0.01 *M* ammonium acetate, adjusted to pH 4.3 with acetic acid, 10^{-4} *M* copper(II) acetate; flow-rate, 0.75 ml/min; detector, UV (254 nm). Elution sequence: DL-Gln, L-Ala, D-Ala, D-Ser, L-Ser, D-Thr, L-Thr, D-Phe, L-Phe.

to retention and enantioselectivity. The composition of the bonded chiral ligand was held constant with the exception of substituting L-proline by L-hydroxyproline and slight changes in the modification procedures. The significance of the variables of the eluent composition for the retention of enantiomers was established for the pH, the ammonium acetate concentration, the type of organic solvent and its content in the aqueous eluent. The dependences permit (to a first approximation) an estimate on the types of interaction that govern the retention of α-amino acids. It was clearly demonstrated that the phase system with its variations provides an enormous potential for gaining enantioselectivity. Compared with the phase system consisting of a reversed-phase packing and an adsorbed N-alkyl-L-hydroxyproline as chiral ligand (which gives elution of L-ahead of D-, except for His[6]), the bonded 3-(L-hydroxyprolyl)propyl silica phase (packing II) offers all kinds of variations in the elution of enantiomers, *e.g.*, D- ahead of L- and L- ahead of D- for many α-amino acids, depending on the conditions. α values obtained on bonded chiral phases are lower than those on the adsorbed N-alkyl-L-hydroxyproline phase (α values up to 16), but are sufficient for resolution of enantiomers because the efficiency of the columns is acceptable. In this

context it is worth adding that the bonded chiral phase is attached to the surface by a short-chain *n*-propyl spacer, which does not provide such a high potential for hydrophobic interactions as in the case of adsorbed *n*-C_7H_{15}-, *n*-$C_{10}H_{21}$- and *n*-$C_{16}H_{33}$-L-hydroxyproline systems.

It was further shown that the model assumed for the surface structure [a chiral ligand as a *trans*-bis(amino acidato)–copper complex] has certain limitations in explaining the retention mechanism. For some α-amino acids the retention behaviour is so complex that a discussion of different interactions contributing to retention on the basis of the model chosen appears to be highly speculative. Nevertheless, separations can be carried out.

Subsequent studies will include the variation of the structure and length of the hydrophobic spacer group bonded to L-proline and L-hydroxyproline and its effect on retention and selectivity of α-amino acids[14].

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