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LIGAND-EXCHANGE SEPARATION OF AMINO ACIDS

II. INFLUENCE OF THE ELUENT COMPOSITION AND OF THE NATURE OF THE ION EXCHANGER

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SUMMARY

The ligand-exchange separation of amino acids in protein hydrolysates has been studied in ammoniacal media. The various factors influencing the chromatographic behaviour have been investigated.

The selectivity of the process and the column efficiency depend appreciably on the resin matrix and the nature of the functional groups. The best results have been obtained with the acrylic-type Bio Rex 70 resin, in the copper (II) form. The zinc-(II) form of this support has also been tested; the elution order of glycine, alanine and leucine is the same as with the copper(II) form, but the affinities of these molecules for the zinc(II) resin are weaker.

The influence of the concentrations of copper(II) and ammonia on the elution volumes obtained for all the amino acids with the copper(II) Bio Rex 70 resin has been studied. The general variation of retention and selectivity with eluent composition confirms the theoretical expressions established previously.

Ligand-exchange chromatography has been compared to the conventional ion-exchange process using acidic or basic species. This study reveals analogies and differences between both techniques, and shows that ligand exchange can provide new opportunities for the separation of amino acids.

INTRODUCTION

An earlier study[1] was carried out on the theoretical investigation of the fixation of monoaminomonocarboxylic acids in an ammoniacal medium by resins containing complex-forming groups in the copper(II) form. This work led us to

describe the distribution of an amino acid between both phases in a simplified manner by means of two equilibria. One is relative to the formation of mixed complexes $CuA(NH_3)_x^+$ in solution (where $A^- =$ amino acid molecule) and the other to the ion exchange that takes place between these mixed complexes in solution and the cupriammine complexes that saturate the resin. Simple mathematical expressions that predict the general effect of the eluent composition on the fixation of the amino acids and on the selectivity of the process have been established. These show that the distribution coefficient is nearly always a decreasing function of the ammonia concentration and that the variation with the copper(II) concentration in solution exhibits a maximum at fairly low concentrations of the metallic cation, the actual position of the maximum being determined by the ammonia concentration and the nature of the amino acid. High values of the distribution coefficient are therefore obtained when both these constituents are diluted in solution. The variation with the composition of the eluent of the selectivity factor, which may be expressed as the ratio, D_2/D_1, of the distribution coefficients of two amino acids A_1 and A_2, is more complex and mainly depends on the nature of the amino acid. For molecules with similar structures, this expression predicts a maximum selectivity in dilute copper(II) media, the most favourable ammonia concentration varying according to the amino acids pair under consideration.

We have applied these theoretical assumptions to the study of the separation of the amino acids usually found in proteins hydrolysates. As yet, the separation of amino acids by ligand-exchange chromatography has received little attention. If we consider only the cases where the eluent, usually ammonia, is effectively an exchangeable ligand, only the separations of amino acids from peptides[2,3], from amino sugars[4,5] and the resolution of optical isomers[6–12] have been described. However, these studies have never really included the separation of the various amino acids from each other. While this problem has already been well solved by ion-exchange chromatography using acidic or basic species, it would seem that new solutions may be obtained by the application of a technique involving phenomena of an other nature. The present paper shows how the various parameters involved in the ligand-exchange process affect the chromatographic behaviour of amino acids.

EXPERIMENTAL

Reagents and chromatographic supports

The compounds in chromatographic effluents were detected by means of ^{14}C-labelled amino acids. These molecules, mainly in the L-configuration, were supplied by the C.E.N., Saclay, France. Only tryptophan and cystine were in the form of a mixture of the two enantiomorphs.

Amino acids for biochemical applications (Merck, Darmstadt, G.F.R.) were most often used as carriers. In a few cases (cystine, threonine, serine and phenylalanine) the Schuchardt products for analysis were preferred. With the exception of DL-tryptophan, all these acids were of L-configuration.

The eluent solutions were prepared from Prolabo RP ammonia solutions and Prolabo for analysis pure copper nitrate (Prolabo, Paris, France).

All our investigations were carried out on Bio-Rex 63, Bio-Rex 70 and Chelex 100 resins containing, respectively, phosphonic, carboxylic and iminodiacetic func-

tional groups, supplied by Bio-Rad Labs. (Richmond, Calif., U.S.A.). The 50–65-μm fraction was chosen after sieving for the chromatographic separations. Before use, all these resins were treated as described previously[1].

Columns and detection apparatus

All the investigations carried out on the Bio-Rex 70 resin were performed with a Jobling column (45 × 0.4 cm I.D.) fitted with a septum injector. For the tests on Bio-Rex 63 and Chelex 100 resins, we used a Chromatronic column (50 × 0.28 cm I.D.) fitted with a Chromatronix looped injector. The flow-rate was controlled by means of a Varioperpex 1200 type peristaltic pump.

The chromatographic peaks were detected by the continuous flow liquid scintillation method, using a plastic scintillating cell. A detailed description of the detection apparatus and of tests carried out to check its performances has been published elsewhere[13].

Operating conditions

The amounts injected were chosen so as to satisfy the limit distribution conditions and to ensure that the total concentration of the amino acids contained in the sample was weak or negligible compared with that of the copper(II). The injected amounts of amino acid were therefore in the range from $4 \cdot 10^{-3}$ to 0.1 μmoles. The amount of injected activity was determined so as to ensure correct detection of the chromatographic peaks while reducing as far as possible the risk of contamination. The minimum activity that can be injected is a function of the cell efficiency, the flow-rate and the counting time[13]. Furthermore, the injection of different amounts of labelled material enabled us to identify the amino acids in a variety of mixtures. All these factors led us to inject an amount of activity in the 0.015–0.1 μCi range for each amino acid.

The counting time is important as it can affect the chromatographic resolution. Furthermore, it depends on the flow-rate. Most of experiments carried out on the Bio-Rex 70 resin were performed with counting times of 5, 8 or 10 sec, with a flow-rate in the 15–17 ml/h range. A number of separations were carried out using the same support but a flow-rate of 8.8 ml/h. With the Bio-Rex 63 and Chelex 100 resins, we worked with counting times of 25 and 20 sec, respectively, and with flow-rates of 1.25 and 4.35 ml/h. All these experiments were performed at room temperature.

RESULTS AND DISCUSSION

The factors likely to affect the relative affinity of the molecules for the chromatographic support and therefore the selectivity of the process are numerous and rarely work in a simple manner. They depend on the nature of the support, the composition of the eluent and the complex-forming ability of the amino acids towards the cation present in both phases. The way in which the structure of the molecules, *i.e.* the nature, number, position and properties of the functions they contain, affects their reactivity towards the metallic cation is not reported in this paper, which only discusses the eluent composition and the nature of the stationary phase.

Influence of the eluent composition

Fig. 1 collects all the results obtained with the copper(II) carboxylate resin, and shows the effect of the eluent composition on the chromatographic behaviour of all the amino acids under investigation. Some of the chromatograms illustrate the importance for the chromatographic resolution of the concentrations of copper(II) (Figs. 2–4) and ammonia (Figs. 5–8).

It is clear that, whatever the ammonia concentration in solution, the dilute copper(II) media generally offer the highest selectivity, particularly when the amino acids have related structures. However, the opposite effect can also be seen, especially when the molecules are of a different nature such as tryptophan and cystine (Fig. 4).

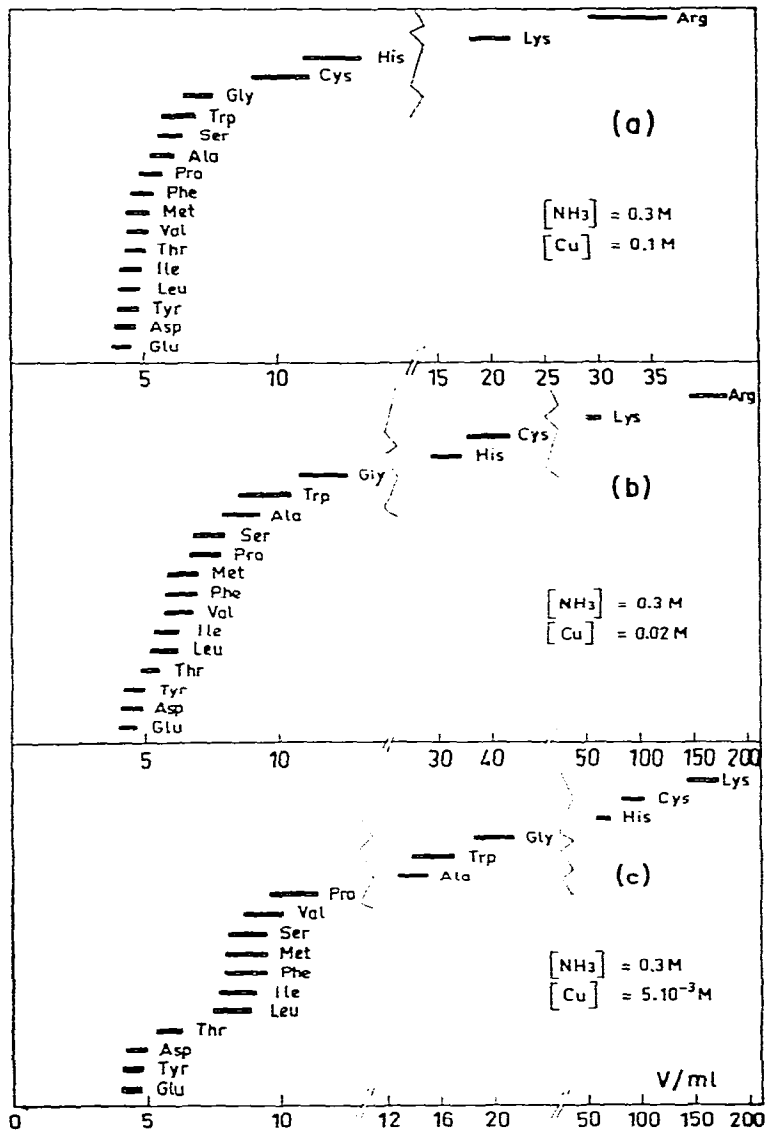

Fig. 1.

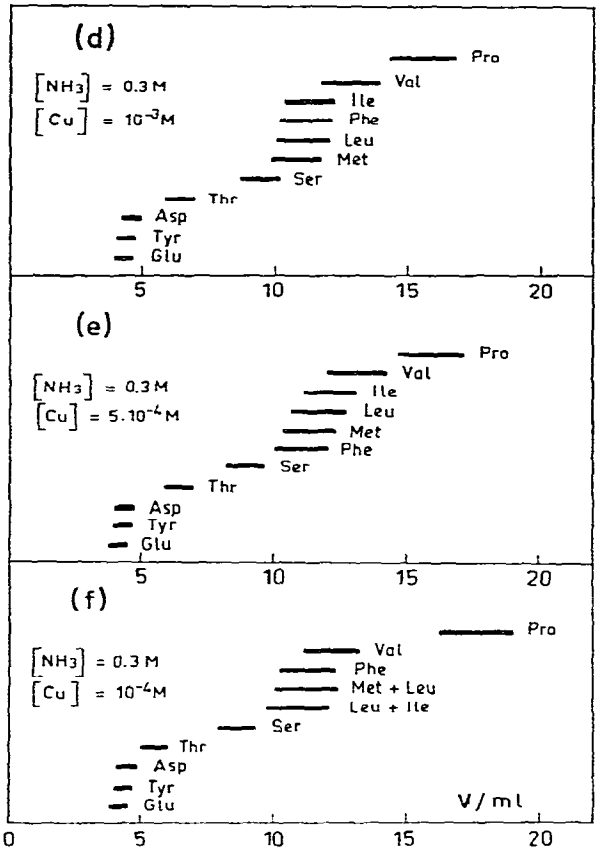

Fig. 1

(Continued on p. 42)

The influence on selectivity of the ammonia concentration varies according to the amino acids under consideration (Figs. 1b and 1h). Nevertheless, it seems that, when the concentration of copper(II) is weak, dilute ammonia solutions are to be preferred (Figs. 1d and 1i). On the other hand, with those media where the selectivity is the highest, several groups of amino acids with similar behaviour can be observed. These groups are, in the order of elution, the acid amino acids group (glutamic acid and aspartic acid) plus tyrosine, a group of neutral amino acids (serine, leucine, isoleucine, methionine, phenylalanine and valine) separated from the first group by threonine, a group of neutral amino acids which are more strongly held within the resin (alanine, tryptophan and glycine) and lastly the basic amino acids (histidine, lysine and arginine, to which can usually be added cystine). Depending on the composition of the eluent, proline falls into one or other of the two groups of neutral amino acids.

These result show that, except for glutamic acid, aspartic acid and tyrosine, whose retention on the resin is weak and little influenced by variations in the composition of the eluent, the elution volumes are higher the lower the concentrations of copper(II) and ammonia. With very dilute copper(II) solutions, variation of the concentration of the metallic cation brings about little change in the retention volumes (Figs. 1d–f, 2c, 2d, 3c and 3d).

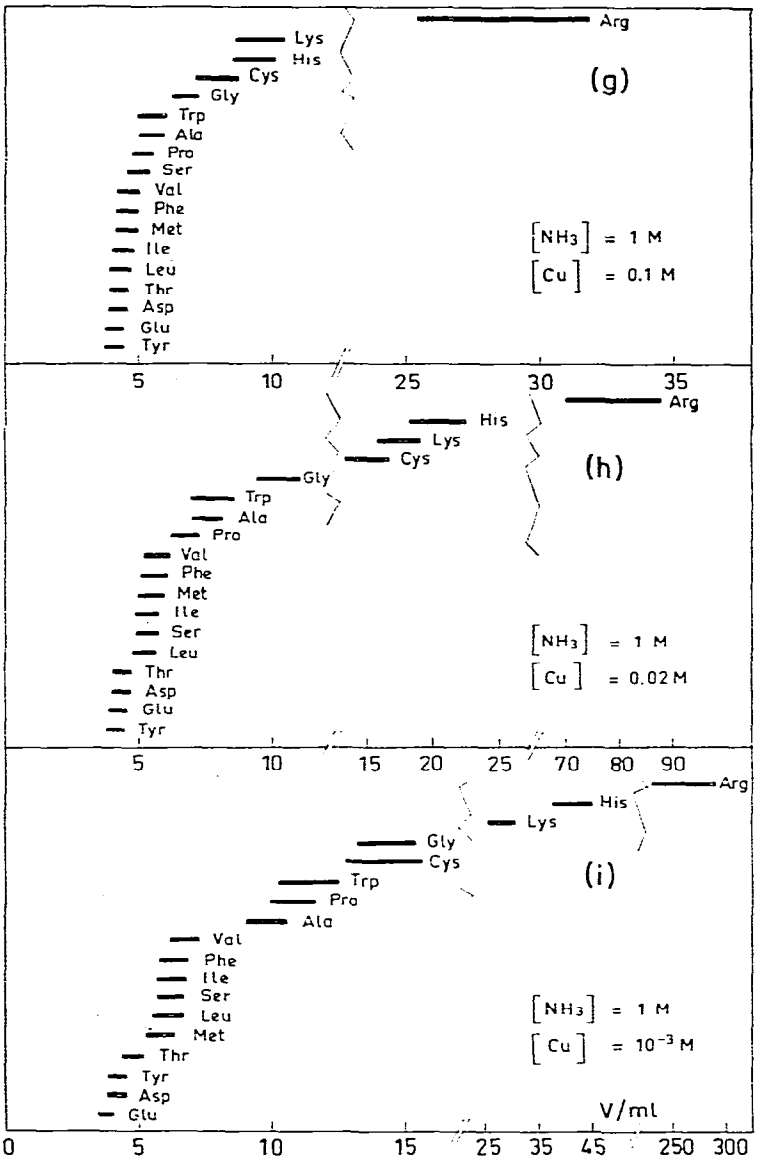

Fig. 1. Variation with the eluent composition of the retention volumes of 18 amino acids contained in protein hydrolysates. Width of the peaks corresponding to 99.7% of the injected substance. Bio-Rex 70 resin, copper(II) form, 50–65 μm; column 0.4 cm I.D. × 45 cm; flow-rates between 15 and 17 ml/h.

The general evolution of the retention volumes, *i.e.* of the distribution coefficients, of the amino acids (especially monoaminomonocarboxylic acids) with the concentrations of copper(II) and ammonia in solution is in good agreement with the theoretical expressions previously established[1]. The specific chromatographic behaviour of polyfunctional amino acids (*i.e.* acid, hydroxylated and basic amino acids, cystine and tyrosine), arising from the formation of complexes with different charges

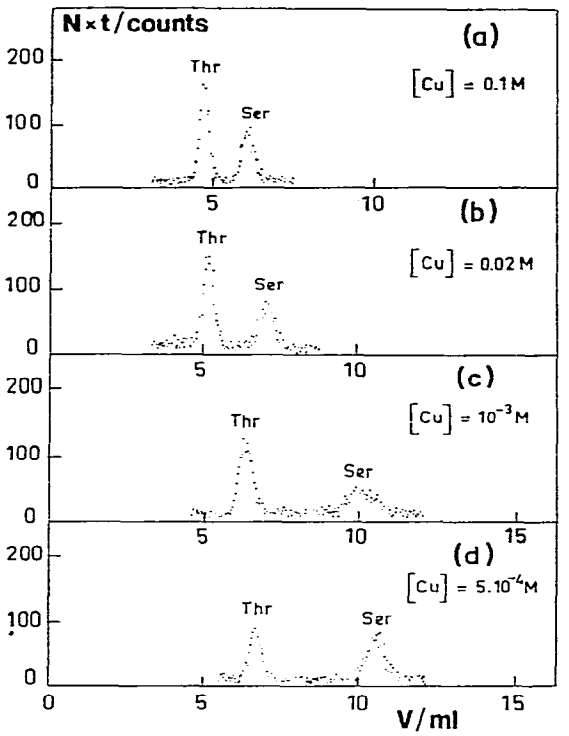

Fig. 2. Evolution of the separation of threonine–serine mixture with the copper(II) concentration in solution. Resin and column, see Fig. 1; $[NH_3]$ 0.3 *M*; flow-rates (a) 15.9 ml/h, (b) 16.7 ml/h, (c) and (d) 16.5 ml/h; counting time 10 sec.

and structures, can also be predicted by the theory[14]. Except for acid amino acids and tyrosine, the general shape of variation with the solution composition of the distribution coefficients of the polyfunctional amino acids is comparable with that obtained with monoaminomonocarboxylic acids.

This study indicates the various possibilities of separation offered by both simultaneous (Figs. 9 and 10) and selective (Fig. 11) elution, and allows the most favourable eluent to be selected. As a general rule, the fixation and separation of mixtures of amino acids with related structures, such as neutral amino acids, necessitate the use of media giving the maximum selectivity, *i.e.* dilute solutions of copper(II) and ammonia. In the case of basic amino acids and cystine, which are very strongly held by the resin but show more differentiated behaviour, concentrated cupric media are preferable because they reduce the elution volume.

In addition, a number of special problems such as the separation of a small number of amino acids with different chromatographic behaviour, the breakdown of a mixture into several groups or the isolation of one or more amino acids from the rest of the mixture, can be tackled by using specific eluting solutions. For example, Fig. 1 shows that the separation of threonine from serine or tyrosine from phenylalanine can easily be effected using a 1 *M* ammonia and 0.02 *M* copper(II) solution. The complete separation of alanine from glycine is also more rapidly performed in a concentrated copper(II) medium whatever the concentration of ammonia. Separa-

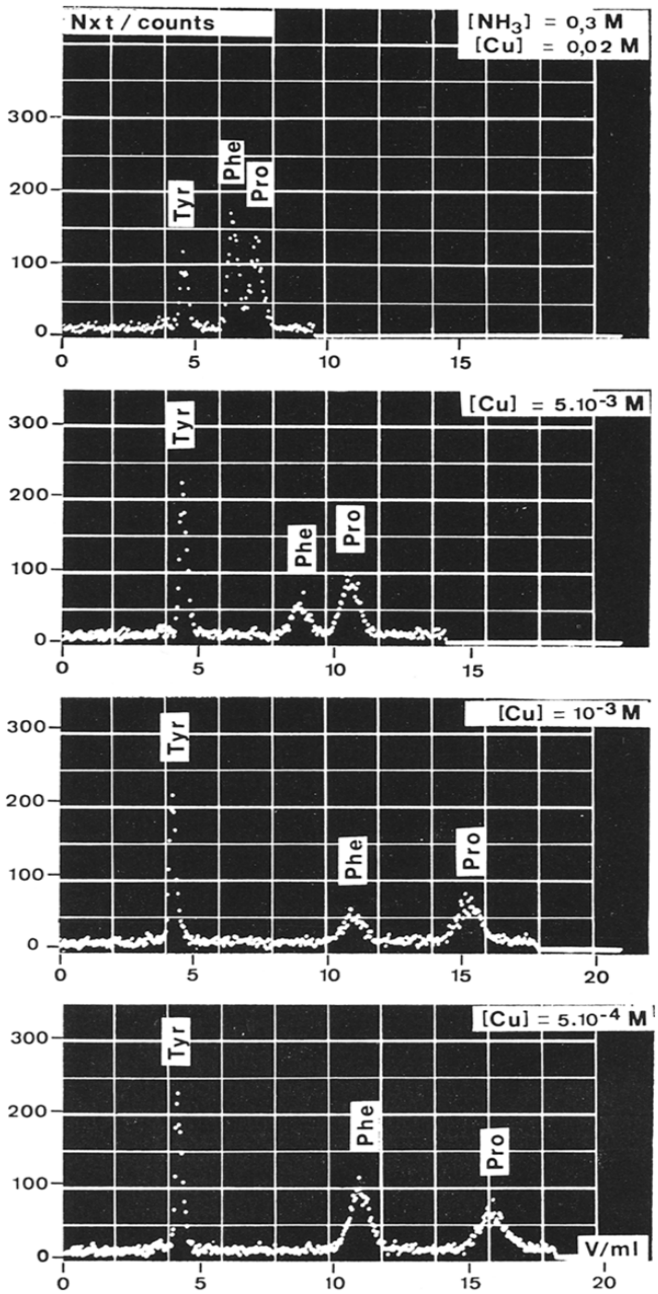

Fig. 3. Evolution of the separation of tyrosine–phenylalanine–proline mixture with the copper(II) concentration in solution. Resin and column, see Fig. 1; $[NH_3]$ 0.3 *M*; flow-rates (a) 16 ml/h, (b) 15.5 ml/h, (c) 15.8 ml/h; counting time 10 sec.

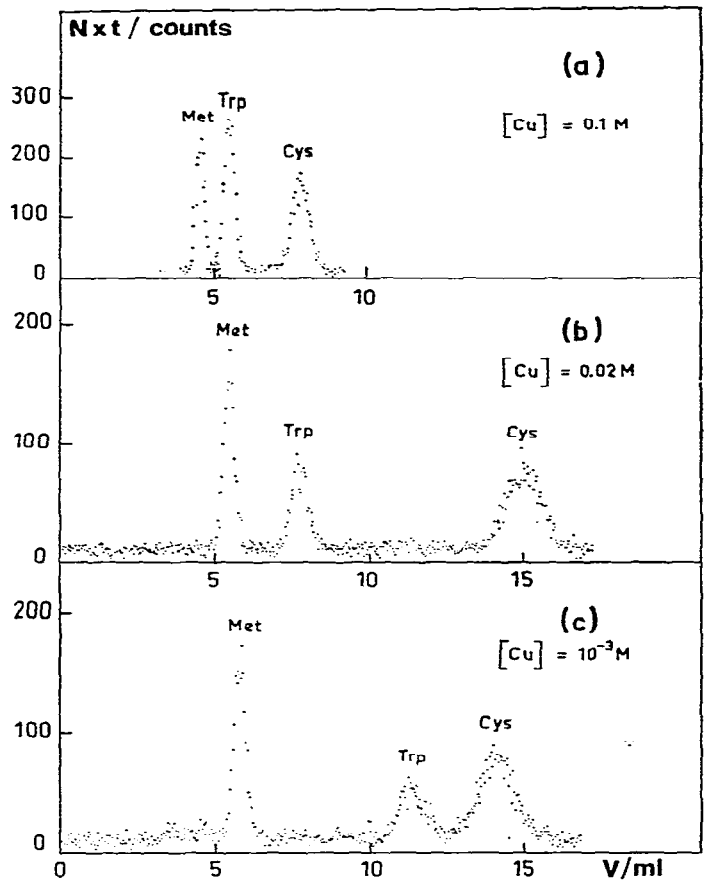

Fig. 4. Evolution of the separation of methionine–tryptophan–cystine mixture with the copper(II) concentration in solution. Resin and column, see Fig. 1; $[NH_3]$ 0.3 *M*; flow-rates (a) 15.8 ml/h, (b) 15.5 ml/h, (c) 15.6 ml/h; counting time 10 sec.

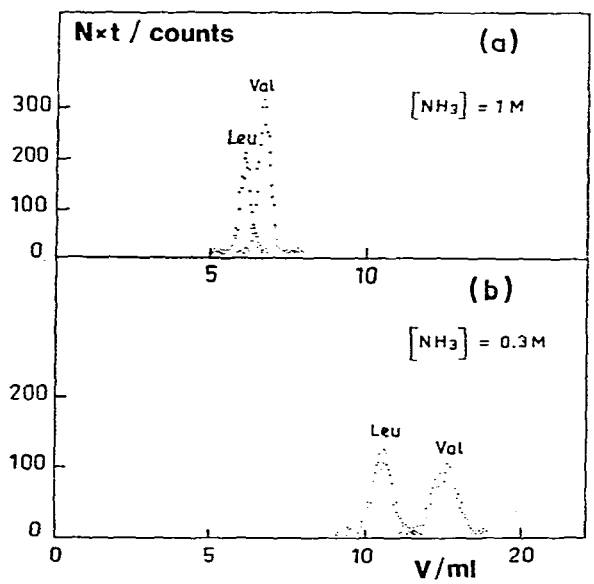

Fig. 5. Evolution of the separation of leucine–valine mixture with the ammonia concentration in solution (superposition of the individual chromatograms). Resin and column, see Fig. 1. [Cu] 10^{-3} *M*; flow-rates (a) 16.3 ml/h, (b) 18.2 ml/h; counting time 10 sec.

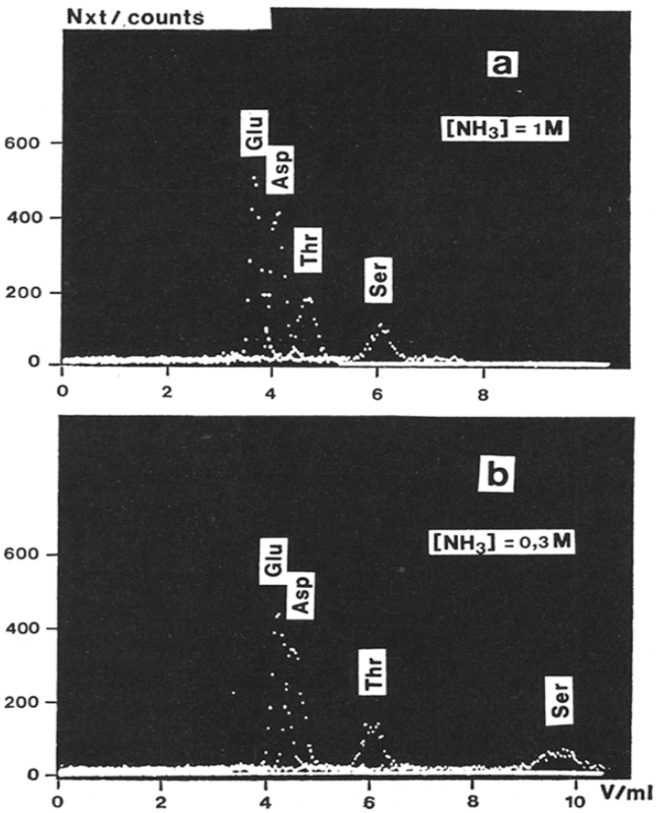

Fig. 6. Evolution of the separation of glutamic acid–aspartic acid–threonine–serine mixture with the ammonia concentration in solution (superposition of the individual chromatograms). Resin and column, see Fig. 1; [Cu] 10^{-3} *M*; flow-rates (a) 16.9 ml/h, (b) 16.1 ml/h; counting time 10 sec.

tion of acid and neutral amino acids into four main groups can be realized by means of a 1 *M* ammonia and dilute copper(II) solution. Dilute ammonia and concentrated copper solutions are more appropriate for the separation from each other and from the rest of the amino acids contained in protein hydrolysates of molecules such as cystine, histidine, lysine and arginine. The threonine–serine pair can also easily be isolated from the glutamic acid–aspartic acid group by means of 0.3 *M* ammonia and sufficiently dilute copper solutions.

Influence of the nature of the chromatographic support

Selectivity. Fig. 12 shows that the relative affinities of the molecules for the chromatographic support vary widely according to the nature of the latter. The retention of the amino acids on the Bio-Rex 70 resin varies inversely with the molecular size (Figs. 12c and 13), whereas the Chelex 100 and Bio-Rex 63 resins more strongly retain the largest species (Fig. 12a and b). This is illustrated by the fact that the chromatographic peak of leucine emerges after the alanine and glycine peaks on Chelex 100 and Bio-Rex 63, whereas this sequence is reversed with Bio-Rex 70. Moreover, this last support exhibits a resolution power that is far superior

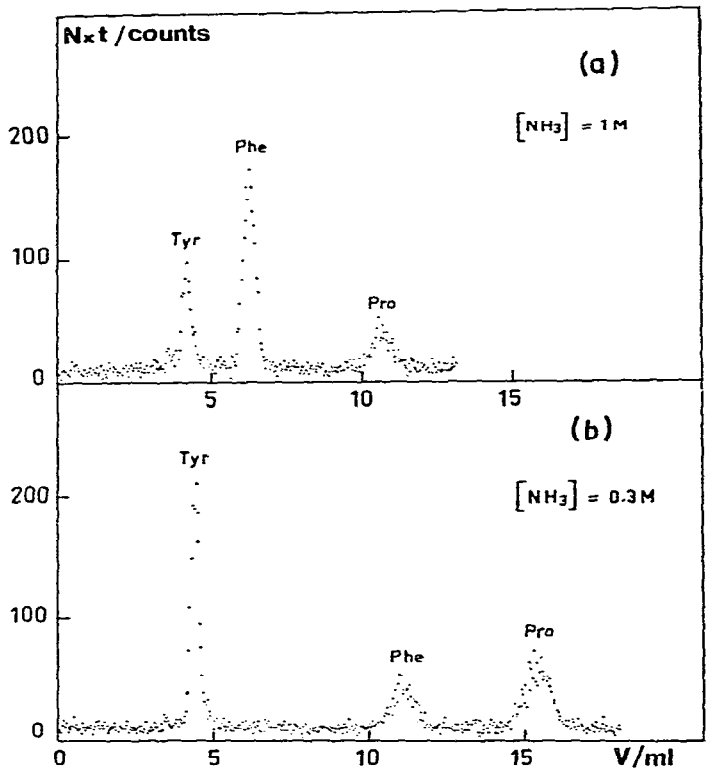

Fig. 7. Evolution of the separation of tyrosine–phenylalanine–proline mixture with the ammonia concentration in solution. Resin and column, see Fig. 1; [Cu] 10^{-3} *M*; flow-rates (a) 16.3 ml/h, (b) 15.8 ml/h, counting time 10 sec.

TABLE I

RETENTION VOLUMES OF GLYCINE, ALANINE AND LEUCINE OBTAINED WITH CHELEX 100, BIO REX 63 AND BIO REX 70

Composition of the eluent: $[NH_3] = 0.2$ *M*, [Cu] = 0.02 *M*.

Acid	*MW*	*Ion exchanger*	*Retention volume**
Glycine	75.05	Chelex 100	2.20
		Bio-Rex 63	2.40
		Bio-Rex 70	2.15
Alanine	89.07	Chelex 100	2.10
		Bio-Rex 63	2.10
		Bio-Rex 70	1.60
Leucine	131.18	Chelex 100	3.35
		Bio-Rex 63	2.70
		Bio-Rex 70	1.06

* Volumes expressed as multiples of the total volume of the column.

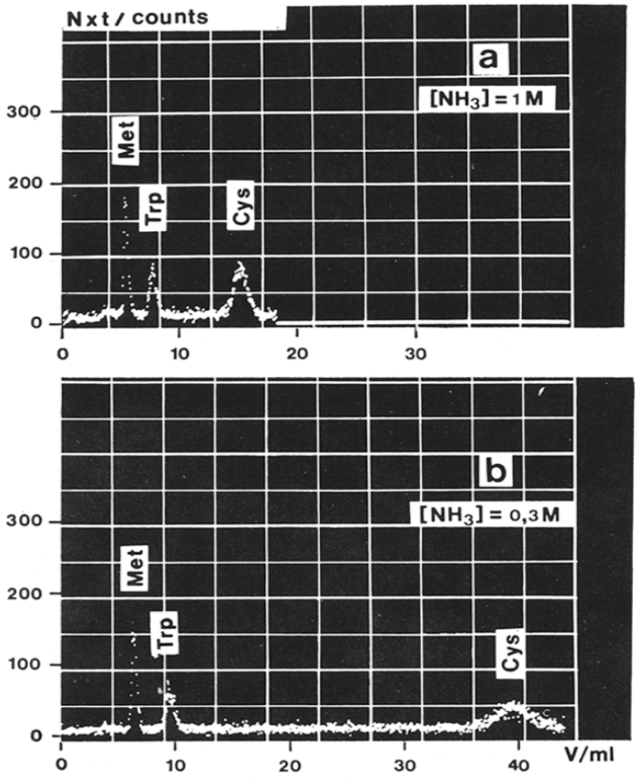

Fig. 8. Evolution of the separation of methionine–tryptophan–cystine mixture with the ammonia concentration in solution. Resin and column, see Fig. 1; [Cu] 0.02 *M*; flow-rates (a) 15.6 ml/h, (b) 15.8 ml/h; counting time 10 sec.

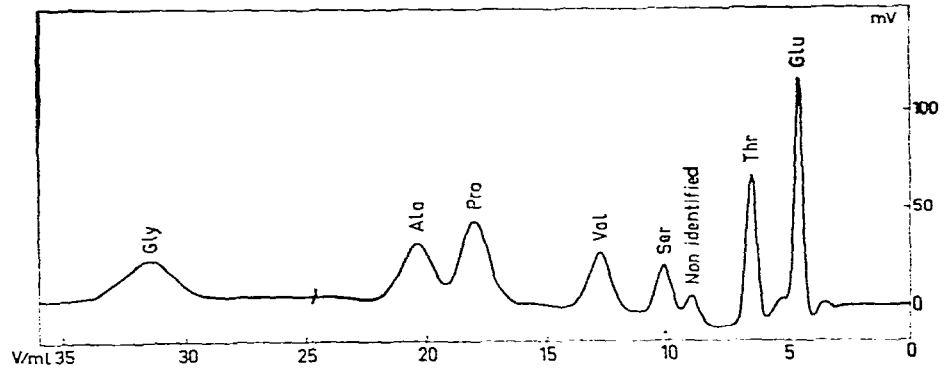

Fig. 9. Separation of some amino acids by simultaneous elution. Refractometric detection (R 404 type Waters Assoc. refractometer, volume of cells 70 μl); conditions: Resin and column, see Fig. 1; eluent, [NH_3] 0.3 *M*, [Cu] 10^{-3} *M*; flow-rate, 16 ml/h; temperature, 20°; injected volume, 100 μl; amount injected, 200 nmoles per amino acid.

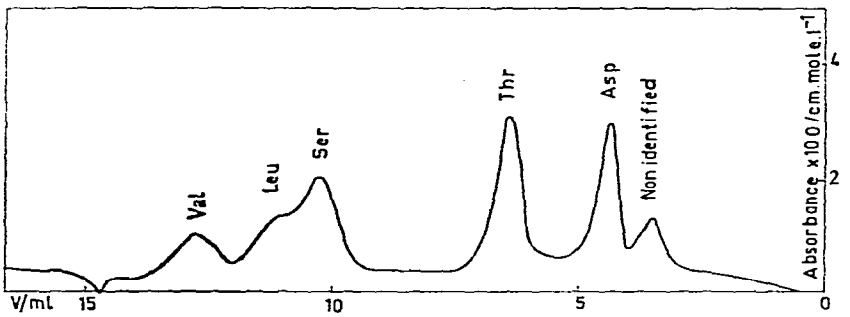

Fig. 10. Separation of some amino acids by simultaneous elution. Direct spectrophotometric detection at 260 nm (635 D Varian spectrophotometer, volume of cells 70 μl). Conditions: resin, column and eluent as in Fig. 9; flow-rate, 15.1 ml/h; temperature, 25°; injected volume, 100 μl; amount injected, 10 nmoles per amino acid.

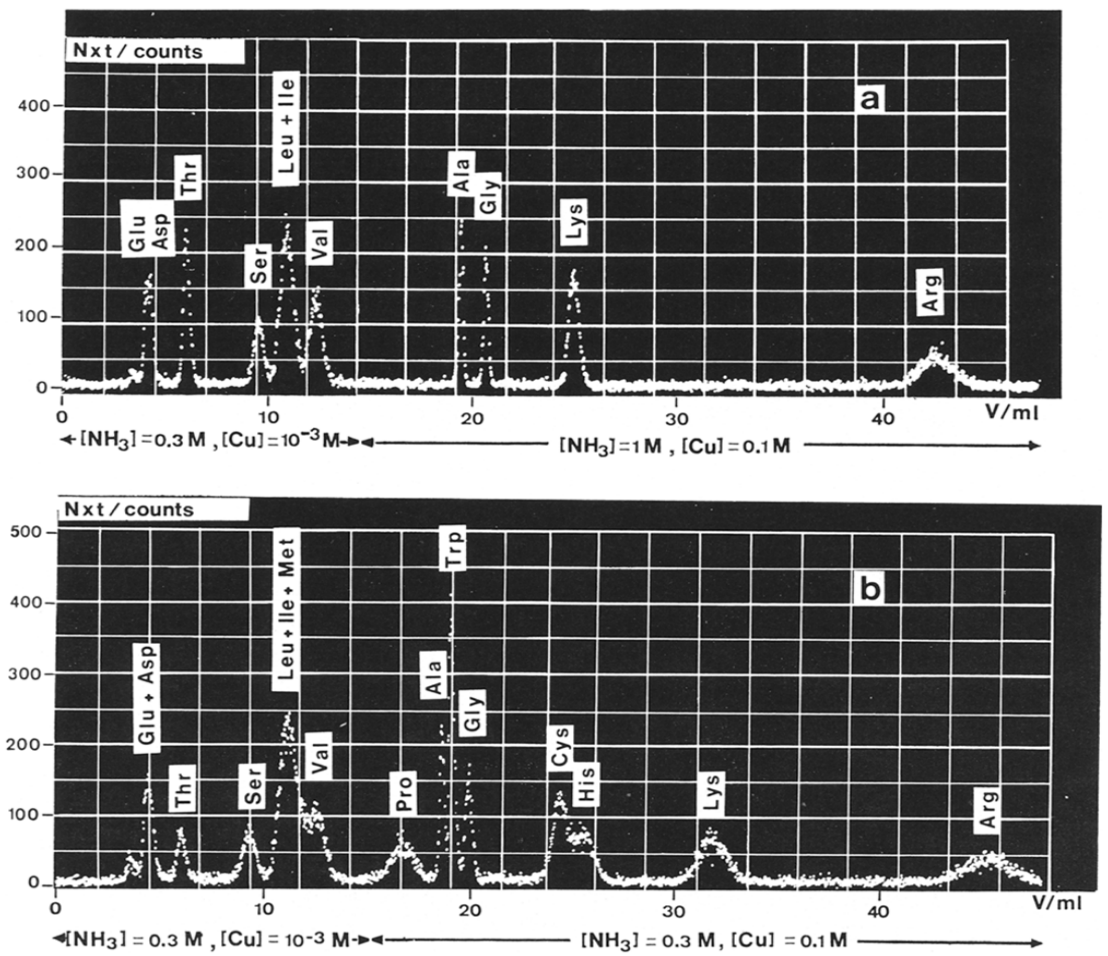

Fig. 11. Separation of a mixture of amino acids by selective elution. Scintillation detection. Conditions: resin and column as in Fig. 9; flow-rate, 8.8 ml/h; room temperature; counting time, 10 sec; amount injected, 4 nmoles per amino acid; injected activities between 0.02 and 0.07 μCi per amino acid.

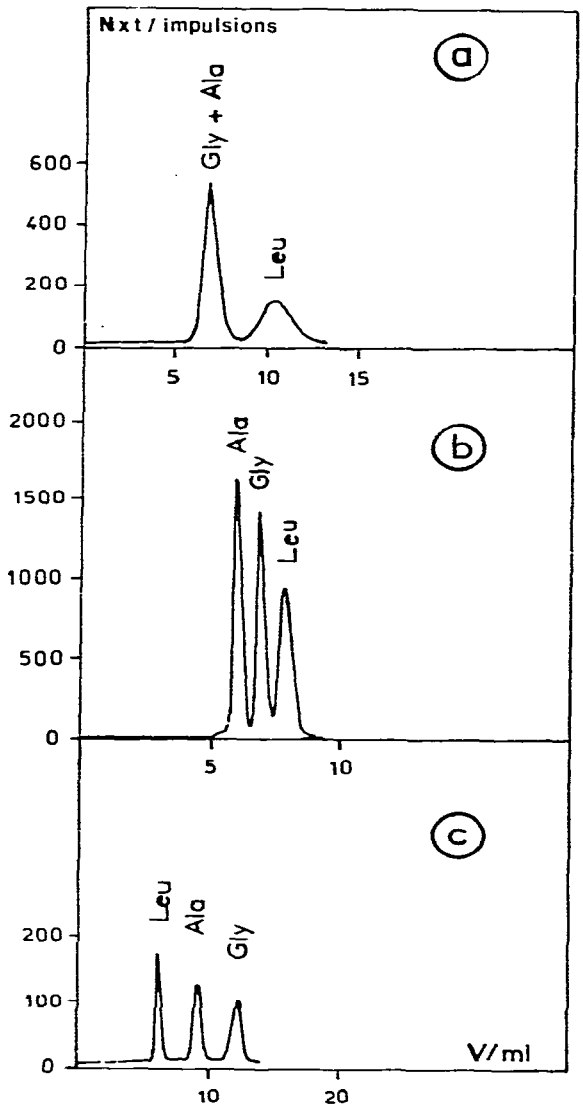

Fig. 12. Influence of the nature of the chromatographic support on the separation of glycine–alanine–leucine mixture. Eluent, [NH_3] 0.2 *M*, [Cu] 0.02 *M*; (a) Chelex 100 resin, copper(II) form, 50–65 μm, column 50 × 0.28 cm I.D.; flow-rate, 4.33 ml/h; counting time, 20 sec. (b) Bio-Rex 63 resin, copper(II) form, 50–65 μm, column 50 × 0.28 cm I.D.; flow-rate, 1.24 ml/h; counting time, 25 sec. (c) Bio-Rex 70 resin, copper(II) form, 50–65 μm, column 45 × 0.4 cm I.D.; flow-rate, 15.4 ml/h; counting time, 8 sec.

to that of the other two. The data listed in Table I show that the influence of the nature of the support becomes greater as the size of the molecules increases. While the fixation of glycine seems to be little affected by the nature of the resin, the retention of leucine varies considerably with the type of support. Indeed, leucine is eluted three times faster on Bio-Rex 70 than on the other stationary phases. Bio-Rex 63 and Chelex 100 generally have fairly analogous behaviour. The respective retention

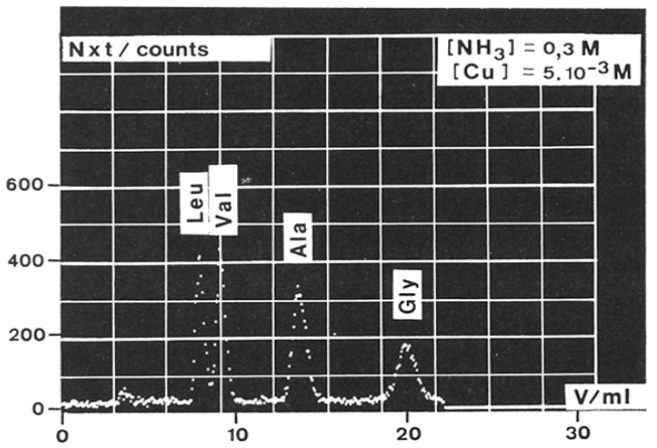

Fig. 13. Separation of leucine–valine–alanine–glycine mixture on the copper(II) Bio-Rex 70 resin, 50–65 μm, column 45 × 0.4 cm I.D.; eluent, [NH_3] 0.3 *M*, [Cu] $5 \cdot 10^{-3}$ *M*; flow-rate 16.4 ml/h; counting time 15 sec.

volumes of the various amino acids show relatively little variation between the two resins.

These observations indicate that selectivity due to the chromatographic support essentially depends on the chemical composition of the matrix. The physico-chemical phenomena that affect the fixation of the amino acids by the resin appear to be of a different character according to whether the support has an acrylic (Bio-Rex 70) or a polystyrene (Bio-Rex 63 and Chelex 100) matrix. It should be noted that similar reversals of affinity have also been reported for the separation of mono- and polyamines on supports of different chemical nature[15–18].

Two opposite types of phenomena seem to affect the affinity of the molecules for the support, some tending to weaken the fixation of the largest molecules, the others tending to favour their retention. This comparative study of different stationary phases was carried out with three amino acids having closely similar structures and chemical properties. As we have shown previously[1], the mixed complexes formed by these molecules in solution have very similar stabilities. The selectivity provided by ligand-exchange chromatography is therefore, in these cases, most probably due to only those parameters that differentiate the molecules, *i.e.* their polarity and the size of their hydrocarbon chain.

Phenomena of the first type may be attributed to variation with the size of the molecule of parameters such as the swelling pressure of the resin or electrostatic interactions between the functional group and the ionic complex. Such factors usually lead the resin to prefer the smallest molecules or ions. We should emphasize that these factors are correlated with the characteristics of the resin: cross-linking, mechanical and physico-chemical properties of the matrix, capacity, nature of the functional groups (particularly complexing properties and steric hindrance).

The second kind of phenomena can be ascribed to the existence in the resin of London or Van der Waals interactions between the matrix and the hydrocarbon backbone of the molecule. Indeed, it is known that the hydrocarbon character of supports with a polystyrene matrix, such as Bio-Rex 63 and Chelex 100, as well as the

most of resins containing sulphonate groups, gives them a retention power which is generally the greater the larger and less polar the hydrocarbon chain of the molecules[19,20]. The sorbent properties of the matrix are comparable to a solvation process and, in some cases, may play a decisive part in the elution sequence of molecular compounds which are chemically related and differ only by the size and polarity of their backbone. One of the clearest and most widely used examples is provided by the separation of amino acids by the conventional ion-exchange chromatography on sulphonated polystyrene resins[20,21]. It is clear that certain amino acids with very closely similar acid–base properties can be perfectly well separated, the retention volumes increasing with the size of the molecular chain. Analogous phenomena have been observed for the separation of aliphatic amines by ligand-exchange chromatography using Dowex 50, Chelex 100 and Bio-Rex 63[17,18,22]. Here too, the species appear in the effluent in the order corresponding to the increase in size of their hydrocarbon chain and to the weakening of their polar and hydrophilic character.

These observations overall predict that selectivity provided by the acrylic Bio-Rex 70 is almost entirely governed by the first kind of phenomena, polymer–solute interactions apparently being absent. The chromatographic behaviour of amino acids on the polystyrene Bio-Rex 63 and Chelex 100 more likely results from the superposition of the two phenomena. While the existence of linkings between the matrix and the solute may account for the high affinity of leucine for polystyrene-based resins, it appears that, in the case of glycine and alanine, these interactions are counterbalanced by opposite phenomena. The chromatographic peaks of these amino acids occur either simultaneously (Chelex 100) or in the reverse order of their molecular weights (Bio-Rex 63) (Fig. 12a and b). Therefore, it is not surprising that, for the three amino acids studied, Bio-Rex 70 provides a higher selectivity than Bio-Rex 63 or Chelex 100, which bring opposite effects into competition with consequent impairment of the selectivity.

The part played by the functional group is less obvious. Its action is probably partly masked by the matrix effects. Nevertheless, it is possible that the difference noted in the behaviour of the two polystyrene-based supports, *i.e.* Bio-Rex 63 and Chelex 100, is connected with the difference in character of their functional groups, phosphonate and iminodiacetate, respectively.

The influence of the nature of the metallic cation seems to be of only secondary importance. A transition from the copper(II) to the zinc(II) form of the resin does not change the elution sequence of the three amino acids investigated (Figs. 1 and 14). On the other hand, the retention volumes and the selectivity are notably less with the zinc(II) form, especially in dilute solutions of the metallic cation. The highest resolution is obtained with medium concentrations of the metallic cation (Figs. 14b and 15). Very dilute solutions cause a substantial drop in the retention volumes, which are then too close to the void volume of the column to enable the separation to be effected. The weakening of the retention volumes and of the selectivity that is observed by passing from the copper(II) to the zinc(II) form has also been reported by Walton[15,16] for the separation of aliphatic amines. The differences noted in the chromatographic behaviour of the two metallic forms of the carboxylate resin are probably due to differences in the stability of the mixed complexes formed by the amino acid with the two metallic cations. The complexes given by the zinc(II) cation with ammonia on the one hand and with the amino acids on the

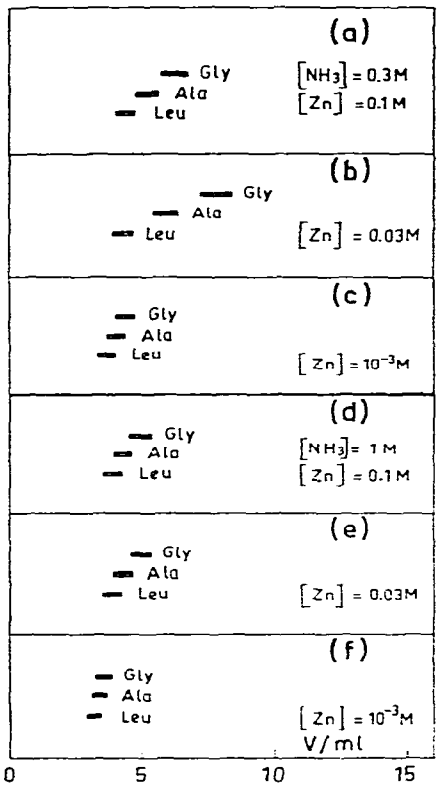

Fig. 14. Variation of the retention volumes of glycine, alanine and leucine with the composition of the eluent. Bio-Rex 70 resin, zinc(II) form, 50–65 μm, column 45 × 0.4 cm I.D; flow-rates, 14.6–15.4 ml/h; width of the peaks corresponding to 99.7% of injected substance.

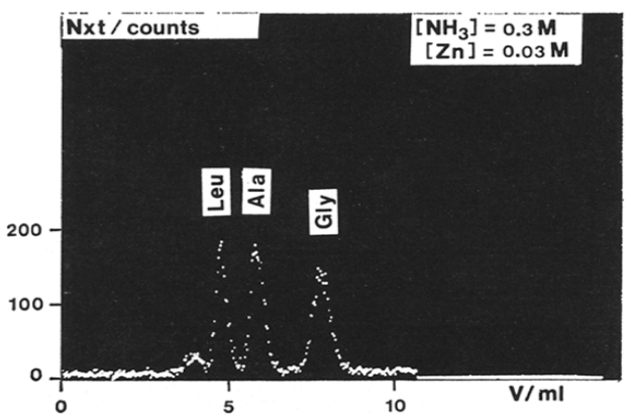

Fig. 15. Separation of leucine–alanine–glycine mixture on zinc(II) Bio-Rex 70 resin, 50–65 μm, column 45 × 0.4 cm I.D.; eluent, [NH_3] 0.3 *M*, [Zn] 0.03 *M*; flow-rate, 15.4 ml/h; counting time, 8 sec.

other are, as a general rule, less stable in aqueous solution than the copper(II) ones[23]. It can be assumed that the same holds true in respect of the mixed complexes.

Efficiency of the chromatographic columns. The influence of the eluent composition on the values of the height equivalent to a theoretical plate (HETP) was studied for each of the chromatographic supports (Table II). It is clear that the efficiencies of Bio-Rex 63 and Bio-Rex 70 do not depend on the concentrations of copper(II) and ammonia in solution. On the other hand, despite the small number of experimental determinations, it seems that the efficiency of Chelex 100 increases with higher ammonia concentrations and lower copper(II) concentrations.

TABLE II

INFLUENCE OF THE CONCENTRATIONS OF AMMONIA AND COPPER(II) IN SOLUTION ON THE HETP

Ion exchanger	$[NH_3]$ *mole/l*	$[Cu]$ *mole/l*	*Linear speed (cm/sec)*	*HETP (mm)*		
				Glycine	*Alanine*	*Leucine*
Chelex 100, 50–65 μm	1.0	$1.0 \cdot 10^{-3}$	$2.0 \cdot 10^{-2}$			1.00
Column 50 × 0.28 cm I.D.	1.0	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$			2.00
	0.2	$5.0 \cdot 10^{-3}$	$2.0 \cdot 10^{-2}$			2.30
	0.2	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$			3.00
Bio-Rex 63, 80–150 μm	1.0	$2.5 \cdot 10^{-3}$	$7.5 \cdot 10^{-3}$		0.70	1.10
Column 5.7 × 1 cm I.D.	1.0	$5.0 \cdot 10^{-3}$	$7.2 \cdot 10^{-3}$	0.60	0.70	1.10
	1.0	$7.5 \cdot 10^{-3}$	$7.2 \cdot 10^{-3}$	0.70	0.80	1.25
	1.0	$1.0 \cdot 10^{-2}$	$7.2 \cdot 10^{-3}$	0.70		1.25
	1.0	$1.5 \cdot 10^{-2}$	$7.8 \cdot 10^{-3}$	0.60	0.70	1.20
	0.5	$5.0 \cdot 10^{-3}$	$7.5 \cdot 10^{-3}$	0.65	0.60	1.10
	0.3	$5.0 \cdot 10^{-3}$	$7.5 \cdot 10^{-3}$	0.65	0.70	1.20
	0.2	$5.0 \cdot 10^{-3}$	$7.8 \cdot 10^{-3}$	0.65	0.75	1.10
Bio-Rex 70, 50–65 μm	1.0	$1.0 \cdot 10^{-3}$	$3.5 \cdot 10^{-2}$	0.30	0.30	0.35
Column 45 × 0.4 cm I.D.	1.0	$2.0 \cdot 10^{-2}$	$3.7 \cdot 10^{-2}$	0.30	0.30	0.40
	1.0	$1.0 \cdot 10^{-1}$	$3.6 \cdot 10^{-2}$	0.30	0.30	0.40
	0.3	$5.0 \cdot 10^{-3}$	$3.6 \cdot 10^{-2}$	0.30	0.30	0.35
	0.3	$2.0 \cdot 10^{-2}$	$3.6 \cdot 10^{-2}$	0.30	0.30	0.35
	0.3	$1.0 \cdot 10^{-1}$	$3.6 \cdot 10^{-2}$	0.30	0.35	0.40

Data listed in Table III allow a comparison to be made of the column efficiencies obtained with the various supports under similar conditions of granulometry, temperature and linear flow-rate. For a given amino acid, appreciable variations in the HETP (h) are observed depending on the support. The carboxylate resin exhibits the best kinetic performances with, for glycine, h values of *ca.* 0.25 mm with a linear flow-rate of $1.9 \cdot 10^{-2}$ cm/sec. Efficiencies of the Bio-Rex 63 and Chelex 100 columns are much weaker, with h values of 0.55 and 1.2 mm, respectively, under identical conditions. It is interesting to note that the lowest efficiencies are obtained with Chelex 100 resin, though its spherical beads are more conducive to homogeneous column packing than the irregular particles of Bio-Rex 63 and Bio-Rex 70. All these results show that the chromatographic column efficiencies depend on the molecular weight of the species. This phenomenon is more pronounced with Bio-Rex 63 and Chelex 100 than with the Bio-Rex 70. However, Bio-Rex 70 reveals

TABLE III

INFLUENCE OF THE NATURE OF THE CHROMATOGRAPHIC SUPPORT AND OF ITS METALLIC FORM ON THE HETP

Composition of the eluent: $[NH_3] = 1\ M$, $[Cu] = 0.02\ M$.

Ion exchanger	*Linear speed (cm/sec)*	*HETP (mm)*		
		Glycine	*Alanine*	*Leucine*
Chelex 100, 50–65 μm, Cu(II) form; Column 50 × 0.28 cm I.D.	$2.0 \cdot 10^{-2}$	1.20	1.30	2.00
Bio-Rex 63, 50–65 μm, Cu(II) form; Column 50 × 0.28 cm I.D.	$1.9 \cdot 10^{-2}$	0.55	0.70	
Bio-Rex 70, 50–65 μm, Cu(II) form; Column 45 × 0.4 cm I.D.	$1.9 \cdot 10^{-2}$ $3.6 \cdot 10^{-2}$	0.25 0.30	0.25 0.30	0.30 0.40
Bio-Rex 70, 50–65 μm, Zn(II) form; Column 45 × 0.4 cm I.D.	$3.2 \cdot 10^{-2}$	0.30	0.30	0.35

a general weakening of efficiency for the largest molecules (Fig. 16). The nature of the metallic cation seems to play a less important part, the zinc(II) form of Bio-Rex 70 giving a kinetic performance comparable with that of the copper(II) one (Table III).

Because of the formation of complexes between the functional group and the exchangeable species, the speed of exchange is, as a general rule, much slower with

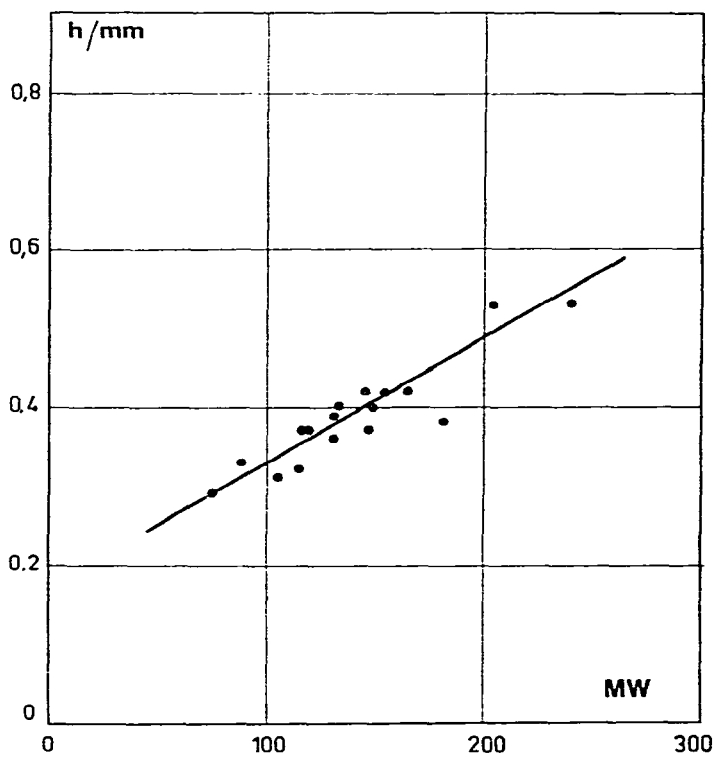

Fig. 16. Variation of the HETP with the molecular weight of the amino acid. Bio-Rex 70 resin, copper(II) form, 50–65 μm; linear flow-rate, $3.3 \cdot 10^{-2}$ cm/sec.

the complexing resins than with the fully ionized ion exchangers. However, it has been shown that, in the presence of sufficiently concentrated ammonia solutions (the media we used), the bonding between the functional group and the cupriammine complex is, for the three supports under consideration, largely ionic[24–26]. In these conditions, the overall speed with which the equilibria are established may be assumed to be limited mainly by diffusion phenomena as in the case of strongly acid resins. The low HETP values obtained with Bio-Rex 70 are closely related to those in classical inorganic ion-exchange chromatography using a strongly acid resin.

The difference in efficiency between the three supports calls for some comments. For instance, the performance of the acrylic-type resin is far superior to that of the polystyrene-based supports. This leads to the assumption that the species diffuse more slowly in the latter: the presence of polymer–solute interactions probably involves a slowing down of the species in the matricial network[27]. We can see that efficiency progressively decreases as the size of the hydrocarbon chain increases, hence as the strength of linkage between the molecule and the matrix increases. A similar phenomenon occurs with aromatic amines, whose strong affinity for polystyrene-based supports is well known[4,28]. The diffusion of the species in Bio-Rex 70 is certainly faster and less affected by the molecular weight of the compounds as interactions of this type do not occur. In addition, the weak cross-linking of the latter increases the mobility of the species.

Furthermore, although the bonds between the functional groups and the exchangeable species are much weakened, it seems that the complexing character and the steric hindrance of the functional groups may be factors that slow down the diffusion of the species in the support. This phenomenon suggests another explanation for the weak performance of Chelex 100, whose tridentate functional groups are particularly large and thus able to obstruct the exchangeable species.

Comparison with conventional ion-exchange chromatography using acidic or basic species

The comparison of the two techniques was carried out on the basis of two typical chromatograms. The ligand-exchange technique is illustrated by the chromatogram shown in Fig. 11b. For the ion-exchange process, we selected one of the chromatograms given in the Beckman technical literature[29] and obtained with the Beckman 119C automatic amino acid analyzer (Fig. 17).

Both techniques bring selective elution into play. Ion-exchange chromatography was performed in acid medium using three sodium citrate buffers with variable pH and sodium ion concentrations. Ligand-exchange chromatography was carried out in a basic medium using two cupriammoniacal solutions with different concentrations of copper(II). We would emphasize the wide differences that exist between the characteristics of the two techniques. The ion-exchange process has reached optimum performance (spherical AA-20 type polystyrene-based resin particles with a granulometry of 11 ± 1 μm, length of resin bed 32 cm, flow-rate $3.4 \cdot 10^{-2}$ cm/sec, temperature 50°, average number of theoretical plates 4500, average HETP 0.07 mm) whereas the ligand-exchange technique can probably be improved (pounded acrylic Bio-Rex 70, granulometry 57 ± 8 μm, length of resin bed 45 cm, flow-rate $1.9 \cdot 10^{-2}$ cm/sec, room temperature, average number of plates 2000, average HETP 0.25 mm).

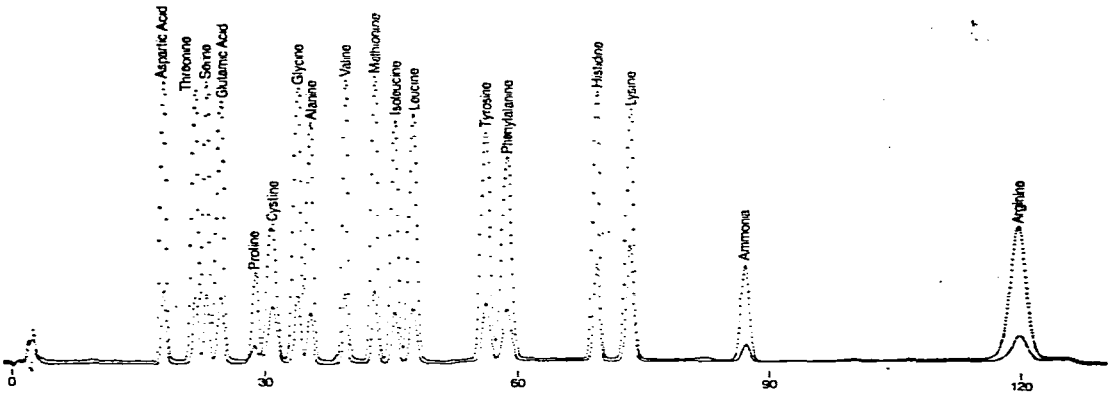

Fig. 17. Separation of the amino acids contained in a protein hydrolysate by ion-exchange chromatography using a Beckman 119C automatic analyzer. Beckmann AA-20 resin, 11 ± 1 μm, column 32 × 0.6 cm I.D.; flow-rate, 35 ml/h; temperature 50°; injected quantities 50 nmoles per amino acid. Successive buffer eluents: (1) pH 3.49, [Na^+] 0.2 *M*, [citrate] 0.2 *M*; (2) pH 4.12, [Na^+] 0.4 *M*, [citrate] 0.2 *M*; (3) pH 6.40, [Na^+] 1 *M*, [citrate] 0.2 *M*. (Reproduced by permission of Beckmann Instruments)

The overall elution sequence of the molecules is the same with both systems: the acid amino acids are less firmly retained than the neutral ones, the latter being more quickly eluted than the basic amino acids. However, notably differences in the retention order and in the chromatographic resolution appear within each group of amino acids. Fig. 18 allows a comparison of the retention volumes obtained with the two methods. For instance, given a series of amino acids with related properties such as the acid amino acids series (aspartic and glutamic acids) or the neutral amino acids group (glycine, alanine, valine, isoleucine and leucine), the elution sequences produced by the two techniques are reversed. Furthermore, it is clear that the aromatic amino acids (tyrosine and phenylalanine) are eluted much more quickly by ligand-exchange chromatography, particularly tyrosine which figures at the head of the chromatogram. Their resolution is also notably improved, although the two acids are eluted with aspartic and glutamic acids or with leucine. On the other hand, the chromatographic peaks of proline and cystine appear later than with ion-exchange chromatography, after the group comprising methionine, leucine, isoleucine, phenylalanine and valine. Unlike ion-exchange, ligand-exchange permits the rapid total separation of threonine and serine as well as their complete isolation from the glutamic acid–aspartic acid pair. Note also that the behaviour of the basic amino acids is similar in both systems, although the histidine–lysine separation is much better by ligand exchange. However, ion-exchange chromatography possesses a markedly superior resolution power for mixtures such as glutamic acid–tyrosine–aspartic acid and methionine–leucine–isoleucine–phenylalanine. In these two cases ligand exchange appears to be uncompetitive, at least at the present state of our research, as a correct resolution of these mixtures could not be achieved.

A few comments can also be made about the respective selectivities of the two methods. In both cases, the overall elution sequence depends on the acid–base properties of the molecules and particularly on the number of carboxyl and amino groups they contain. Let us take the example of three amino acids with different structures: a monoaminomonocarboxylic (neutral) amino acid such as glycine, a

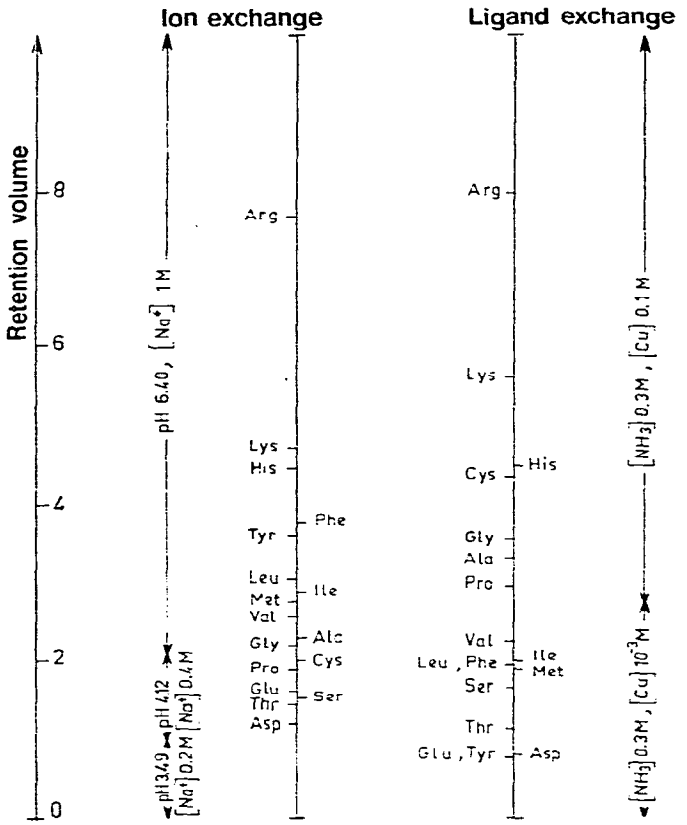

Fig. 18. Comparison between the retention volumes obtained with ligand-exchange chromatography and ion-exchange chromatography. Retention volumes expressed as multiples of the total volume of the column.

monoaminodicarboxylic (acidic) one such as aspartic acid and a polyaminomonocarboxylic (basic) molecule such as arginine. These three acids are symbolized at their isoelectric point (pH at which the net charge is zero) by the formula HA for the basic and neutral amino acids and H_2A for the acid amino acid. Fig. 19 shows the different forms under which these three molecules can exist in aqueous solution according to the pH values (pK_a values from ref. 30). In the case of ion-exchange chromatography, where the fixation and separation take place in an acid medium ($3.5 < pH < 6.4$), the molecules are the more strongly held by the support the more they are protonized, hence the fewer carboxylic and the more amine functions they contain. The pK_a values may also play a part, the molecules being the more strongly held the less these functions are dissociated. Similar reasoning can be applied to ligand-exchange chromatography. Here the pH value of the eluent is basic ($10.5 < pH < 11.3$) and the free amino acid is present in solution as the respective A^{2-} and A^- forms for the acid and neutral species, and as the A^- and HA forms in varying proportions for the basic amino acid. Under these conditions, the mixed complexes present in solution are of the type $CuA(NH_3)_x$ for the acid amino acid and $CuA(NH_3)_x^+$ for the neutral one. In the case of the basic amino acid, the presence of a mixture of protonated $CuAH(NH_3)_x^{2+}$ and non-protonated $CuA(NH_3)_x^+$ com-

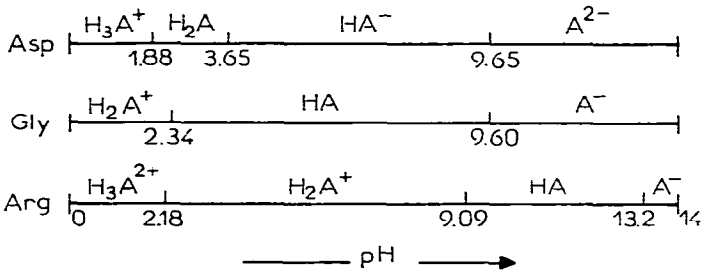

Fig. 19. Areas of predominance according to the pH value of the various forms of aspartic acid, glycine and arginine in solution.

plexes may be assumed. The retention therefore appears to be the greater the higher the positive charge of the complexes, hence the fewer acid carboxylic and the more basic amine functions the amino acid contains.

However, it is clear that the acid–base properties by themselves can explain neither the selectivity of each of these two processes nor the differences and reversals of affinity observed between the two methods. Other phenomena whose nature depends on the technique used must also play a part. For instance, the elution sequence observed with ion-exchange chromatography for neutral amino acids such as glycine, valine, methionine and isoleucine is the precise opposite of what would be expected from the dissociation constants of their carboxylic acid functions[30]. Furthermore, alanine and glycine are perfectly separated by this method although the dissociation constants of their acid functions are identical. Moreover, tyrosine and phenylalanine emerge much later than would be expected from their pK_a values. All these phenomena may be explained by the occurrence of interactions between the solute and the matrix of the polystyrene resin used.

The absence of selective interactions with the acrylic matrix of the resin used for ligand-exchange chromatography may provide a partial explanation of the completely different elution sequence obtained with the same acids, as well as the lack of selectivity observed for the leucine–isoleucine–phenylalanine–methionine group. The selectivity found with ligand-exchange chromatography seems to be mainly due to the formation within the resin and in solution of complexes whose stabilities and structures vary according to the nature of the molecule under consideration. It is clear, for example, that the separation of amino acids such as leucine, valine, alanine and glycine, whose structures, acid–base characteristics and complexing properties in solution are closely related, results from differences between the affinities of their complexes for the resin, hence from differences in the stabilities of complexes within the resin[14]. The strong retention of polyfunctional amino acids such as cystine, histidine and lysine, whose free forms in basic solutions are A^{2-} for cystine and A^- for lysine and histidine, and which are consequently able to give mononuclear complexes having a charge of 0 or $+1$, can be attributed to the formation, in varying proportions, of binuclear complexes $Cu_2A(NH_3)_y^{2+}$ or $Cu_2A(NH_3)_y^{3+}$ having a high positive charge[14]. On the contrary, the fairly weak retention of serine and threonine probably results from the coexistence of mononuclear complexes carrying 0 and $+1$ charges[14]. It must also be noted that the retention strength is a function of the molecular size but, contrary to ion-exchange, the retention obtained in ligand-exchange is higher the lower the molecular weight of the species.

CONCLUSIONS

These results demonstrate that, given suitable adjustment of the elution conditions, ligand-exchange chromatography can open up new possibilities for the separation of amino acids and may therefore be regarded as complementary to the classical ion-exchange technique.

One of the most important factors affecting the selectivity of the process is the choice of the chromatographic support. Chromatographic behaviour varies widely according to the resin matrix, the character of the functional groups and, to a lesser extent, the nature of the metallic cation. All our investigations on resins containing copper(II) phosphonate, copper(II) iminodiacetate and zinc(II) and copper(II) carboxylate groups led us to prefer the last stationary phase for the complete study of the chromatographic separation of the amino acids contained in protein hydrolysates. This support provides the best kinetic performances and appears to offer superior possibilities of separation. Furthermore, it should be possible to improve the chromatographic resolution by using resins of smaller particle size. The major problem here is that the acrylic network of the carboxylate resin is too soft to withstand the high pressures required by the finest particles. Other chromatographic supports of a less traditional nature can likewise be applied with advantage. Some recently reported examples of the synthesis of specific resins include optically active supports prepared by grafting in a polystyrene or acrylamide network of an α-amino acid group able to form optically active complexes with metallic cations[5–12]. At present, these stationary phases have a marked success in separating the optical isomers of amino acids. Another original example of the synthesis of supports suitable for use in ligand-exchange chromatography involves the preparation of an ion exchanger with a low cross-linking and containing iminodiacetate functional groups grafted to a polysaccharide network[31].

The study of the influence of the eluent composition (copper(II) and ammonia concentrations) on the selectivity demonstrates that it is often possible to determine an optimal eluent for a given separation. With regard to selectivity, dilute solutions of copper(II) and ammonia are often the most favourable. However, these media generally lead to a strong retention of the amino acids, particularly of polyaminated molecules. These can be eluted only by using media rich enough in copper(II) or ammonia.

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