*Journal of Chromatography*, 131 (1977) 73–90


CHROM. 9464

# LIGAND-EXCHANGE SEPARATIONS OF AMINO ACIDS

## I. DISTRIBUTION EQUILIBRIA OF SOME AMINO ACIDS BETWEEN AMMONIACAL COPPER(II) NITRATE SOLUTIONS AND PHOSPHONIC, CARBOXYLIC AND IMINODIACETIC ION EXCHANGERS IN THE COPPER(II) FORM

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(First received March 29th, 1976; revised manuscript received June 18th, 1976)

## SUMMARY

The ligand-exchange process for the distribution of glycine, alanine and leucine between a copper(II) phosphonate resin and ammoniacal solutions of copper nitrate has been studied. The formation in both resin and solution phases of mixed $CuA(NH_3)_i^+$ complexes ($i = 1$–$3$) was demonstrated. A theoretical expression for the distribution coefficient, taking into account the various parameters upon which it depends, has been established and verified experimentally. Some experiments carried out with carboxylic and iminodiacetic resin in the Cu(II) form confirmed the validity of the theoretical model.

The distribution of an amino acid by ligand exchange occurs as a complex phenomenon that brings many equilibria into play. However, by simplifying the theory with some approximations, it is possible to describe the distribution by means of two equilibria, one of ion exchange and the other of the formation in solution of mixed complexes. This simplified theory clearly displays how the ammonia and copper(II) concentrations affect the amino acid fixation. Some experimental graphs illustrate the variation of the distribution coefficients for different amino acids with each of these parameters.

Finally, some observations on the selectivity that may be expected from this process are made. In particular, it is shown how the optimal conditions for the separation of two amino acids can be determined.

## INTRODUCTION

During recent years, problems arising from the separation of molecular species, mainly organic, have been given new solutions due to a technique first introduced by Helfferich[1] and called "ligand exchange". Reviews have recently been published[2–5] that show the great number of studies carried out on this aspect. The many results obtained with nitrogenous bases, notably amines, encouraged us to carry out a theoretical study of this phenomenon on compounds closely related to them, *viz.*, amino acids.

Like amines, amino acids can participate through both functional groups to form complexes with cations such as $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Co^{2+}$ and $Hg^{2+}$, previously fixed on a solid support. Some examples of chromatographic applications of this technique have already been published; separation of amino acids[6–11]; separation of amino acids from peptides[12–15], from amino sugars[16,17] or from a medium containing mineral ions[18,19]; concentration of amino acids dissolved in sea water[20]; and resolution of racemates on a sorbent containing an optically active copper complex[21–23]. Elution is then performed either by ligand-exchange chromatography with a solution of another ligand, usually ammonia[14–19,21,23] and sometimes pyridine[24], or by destruction of the bound complex with hydrochloric acid solutions[12] or with acetate[6–9,11] or borate[13] buffers, sometimes including small amounts of the metal ion involved. Displacement chromatography using ethylenediamine as displacing ligand has also been applied[22]. It is interesting to note, however, that the separation of amino acids by means of a solution of an exchangeable ligand has never been thoroughly investigated.

The first studies were conducted on chromatographic columns packed with Sephadex saturated with copper(II)[12]. At present, the solid supports most frequently used are resins with sulphonate, carboxylate and iminodiacetate functional groups. A sulphonic acid resin has the drawback of exchanging too easily the metallic element it contains with other elements in solution, thus losing part of its ligand-binding capacity. Carboxylate[6–8,16,17] and iminodiacetate[13–15,18–20] resins are often preferred because of the complexing properties of their functional groups, which are able to bind the metal cation strongly.

Although it offers the same advantages as carboxylate and iminodiacetate resins, phosphonic acid ion exchangers have never been used in separations involving amino acids. Only the fixation of ammonia and amines in ammoniacal solution by such a support in the Cu(II) or Zn(II) form has been studied up to now[25–27].

In particular, these studies led to the determination of the stability constants of ammine–copper(II) and ammine–zinc(II) complexes within this support. In addition, it is the only resin that has so far been the object of such determinations. As the values of these constants appear in the theoretical study of the fixation of amino acids in ammoniacal medium, we had to choose the phosphonate resin as a support. As the complexes formed by ammonia with copper(II) are more stable than those with zinc(II), we preferred to use the resin in the copper(II) form. Further, variable amounts of copper nitrate were introduced in the solution. Preliminary tests showed that copper(II) has a notable influence on the fixation of amino acids within the resin.

Some experiments on carboxylate and iminodiacetate ion exchangers loaded with copper(II) were also performed in the same cupriammine medium as a means of comparison with the phosphonate resin.

We chose to study the behaviour of three monocarboxylic monoamine amino acids among the simplest of the aliphatic series: glycine, alanine and leucine. Some results obtained with valine and isoleucine are also reported.

## THEORETICAL

We have established a general mathematical expression for the distribution coefficient, displaying the main parameters on which it depends. Thus, it has been necessary to take into consideration all of the species that may exist within the resin and in solution. In particular, a detailed account of the different forms in which the amino acid can appear in both phases is drawn.

*Species in solution*

In the pH range 9–12 where the experiments were carried out, the free amino acid exists in solution only in the anionic and neutral forms, represented by $A^-$ and HA, respectively. Moreover, amino acid molceules are able to displace ammonia from cupriammine complexes in solution, to give mixed complexes such as $[CuA_j(NH_3)_i]^{(2-j)+}$. However, as the concentration of amino acid in solution is very low compared with that of the copper ion, $j$ cannot exceed 1; the value of $i$ then varies from 0 to 3, as the maximum conventional coordination number of copper(II) is 4. The formation of these complexes of general formula $CuA(NH_3)_i^+$ can be represented by equilibrium reactions of the type

$$Cu^{2+} + A^- + iNH_3 \rightleftharpoons CuA(NH_3)_i^+$$

with stability constants $\beta_i^1$:

$$\beta_i^1 = \frac{[CuA(NH_3)_i^+]}{[Cu^{2+}]\,[A^-]\,[NH_3]^i} \qquad (1)$$

*Species within the resin*

Studies concerning the binding of ammonia by copper(II) ions in phosphonate, carboxylate and iminodiacetate resins have shown that, in all instances, the cupriammine complexes of general formula $Cu(NH_3)_i^{2+}$ ($i = 0$–4) can be formed in the resin, their distribution depending on the concentration of ammonia in solution[25–29]. The same studies have established that chelation between the functional group R of the resin and the cupriammine complex undergoes cleavage as the number of ammonia molecules coordinated to the copper(II) ions carried by the resin increases.

Exhibiting the same behaviour as in solution, amino acids entering the resin can displace ammonia molecules from cupriammine complexes. Mixed complexes so formed are, according to the value of $i$, in either the covalent $\overline{RCuA(NH_3)_i^-}$ or ionic $R^{2-}$, $\overline{CuA(NH_3)_i^+}$ form. No distinction will be made between these two forms in the following discussion, all species being represented by the formula $\overline{RCuA(NH_3)_i^-}$.

The fixation of the amino acid can then be described by the following equilibrium reactions:

$$\overline{RCu} + A^- + iNH_3 + NH_4^+ \rightleftharpoons \overline{RCuA(NH_3)_i^-} + \overline{NH_4^+}$$

whose equilibrium constants are

$$\overline{\beta_i^{\mathrm{I}}} = \frac{\overline{[\mathrm{RCuA(NH_3)_i^-}]}\,\overline{[\mathrm{NH_4^+}]}}{\overline{[\mathrm{RCu}]}\,[\mathrm{A^-}]\,[\mathrm{NH_3}]^i\,[\mathrm{NH_4^+}]} \tag{2}$$

The terms with a bar represent species within the resin; thus $\overline{[\mathrm{RCu}]}$ represents the copper(II) concentration within the resin, as the free $Cu^{2+}$ ion or chelated RCu that is being uncomplexed by ammonia or amino acid.

Attention is drawn to the fact that, according to the electroneutrality of the resin, the fixation of one amino acid molecule must correspond with that of one positive charge. In the present case, this can be achieved only by one ammonium ion.

Further, given the higher concentration of copper(II) within the resin compared with that of the amino acid, we have speculated the formation of mixed binuclear complexes of general formula $R_2Cu_2A(NH_3)_i^-$, in which the two copper ions are bridged by the amino acid molecule:

$$\overline{2\mathrm{RCu}} + \mathrm{A^-} + i\mathrm{NH_3} + \mathrm{NH_4^+} \rightleftharpoons \overline{\mathrm{R_2Cu_2A(NH_3)_i^-}} + \overline{\mathrm{NH_4^+}}$$

The equilibrium constants are

$$\overline{\beta_i^{\mathrm{II}}} = \frac{\overline{[\mathrm{R_2Cu_2A(NH_3)_i^-}]}\,\overline{[\mathrm{NH_4^+}]}}{\overline{[\mathrm{RCu}]}^2\,[\mathrm{A^-}]\,[\mathrm{NH_3}]^i\,[\mathrm{NH_4^+}]} \tag{3}$$

the number, $i$, of ammonia molecules necessary to satisfy the total coordination of both metal cations cannot, in this instance, exceed 6.

*General mathematical expression for the distribution coefficient*

The distribution coefficient, $D$, of the amino acid between the resin and the solution is given by the expression

$$D = \frac{\overline{[\mathrm{A}]}_{\mathrm{T}}}{[\mathrm{A}]_{\mathrm{T}}} = \frac{\sum_{i=0}^{i=3} \overline{[\mathrm{RCuA(NH_3)_i^-}]} + \sum_{i=0}^{i=6} \overline{[\mathrm{R_2Cu_2A(NH_3)_i^-}]}}{[\mathrm{A^-}] + [\mathrm{HA}] + \sum_{i=0}^{i=3} [\mathrm{CuA(NH_3)_i^+}]}$$

where $\overline{[\mathrm{A}]}_{\mathrm{T}}$ and $[\mathrm{A}]_{\mathrm{T}}$ represent the total concentration of amino acid within the resin and in solution, respectively.

From eqns. 1–3 the general mathematical expression of $D$ is obtained:

$$D = \sqrt{K_0 \cdot \frac{[\mathrm{Cu}]_{\mathrm{T}}\,\overline{[\mathrm{Cu}]_{\mathrm{T}}}}{\alpha\,\bar{\alpha}}} \cdot \frac{\sum_{i=0}^{i=3} \overline{\beta_i^{\mathrm{I}}}\,[\mathrm{NH_3}]^i + \frac{\overline{[\mathrm{Cu}]_{\mathrm{T}}}}{\bar{\alpha}} \sum_{i=0}^{i=6} \overline{\beta_i^{\mathrm{II}}}\,[\mathrm{NH_3}]^i}{1 + \frac{[\mathrm{H^+}]}{K_{\mathrm{A}}} + \frac{[\mathrm{Cu}]_{\mathrm{T}}}{\alpha} \sum_{i=0}^{i=3} \beta_i^{\mathrm{I}}\,[\mathrm{NH_3}]^i} \tag{4}$$

where $[\mathrm{NH_3}]$ is the concentration of free ammonia and $[\mathrm{Cu}]_{\mathrm{T}}$ and $\overline{[\mathrm{Cu}]}_{\mathrm{T}}$ are the total

concentrations of copper in solution and within the resin, respectively; detailed expressions of the last two parameters are:

$$[Cu]_T = [Cu^{2+}] + [Cu(NH_3)^{2+}] + \cdots + [Cu(NH_3)_4^{2+}]$$

$$[\overline{Cu}]_T = [\overline{RCu}] + [\overline{RCu(NH_3)}] + \ldots + [\overline{RCu(NH_3)_4}]$$

The low amino acid to ammonia concentration ratio led us to neglect in both phases the concentrations of mixed complexes compared with those of cupriammine complexes. $K_0$ is the ion-exchange constant $NH_4^+/Cu^{2+}$ as defined by the relationship

$$K_0 = \frac{[\overline{RCu}]\,[NH_4^+]^2}{[Cu^{2+}]\,[\overline{NH_4^+}]^2} \tag{5}$$

$K_A$ is the acidity constant of the amino acid:

$$K_A = \frac{[A^-]\,[H^+]}{[HA]}$$

$\alpha$ and $\bar{\alpha}$ are the ratios of the total copper concentration to that of free copper cation in solution and within the resin, respectively:

$$\alpha = \frac{[Cu]_T}{[Cu^{2+}]} = 1 + \sum_{i=0}^{i=4} \beta_i\,[NH_3]^i \tag{6}$$

$$\bar{\alpha} = \frac{[\overline{Cu}]_T}{[\overline{RCu}]} = 1 + \sum_{i=0}^{i=4} \bar{\beta}_i\,[NH_3]^i \tag{7}$$

with

$$\beta_i = \frac{[Cu(NH_3)_i^{2+}]}{[Cu^{2+}]\,[NH_3]^i}$$

and

$$\bar{\beta}_i = \frac{[\overline{RCu(NH_3)_i}]}{[\overline{RCu}]\,[NH_3]^i}$$

Eqn. 4, showing how $D$ varies with the free ammonia concentration in solution, is not simple and depends on the nature of the copper complexes which are predominant in the two phases. The value of $D$ also depends on the total concentration of copper(II) in solution and within the resin, as well as on the pH of the solution.

In order to display how these factors influence the fixation process, the variation of the distribution coefficient of some amino acids was studied for several supports in terms of the different parameters that appear in its theoretical expression.

We ignored the formation of mixed complexes of the type $Cu(OH)_j(NH_3)_i^{(2-D)+}$ and $CuA(OH)_j(NH_3)_i^{(1-D)+}$ which may take place, as shown by Reeves and Bragg[30] and Fisher and Hall[31]. These approximations allow the theoretical expression of the distribution coefficient to be simplified considerably. On the other hand, the hypothesis

we made seems to be justified by the good agreement of the results to which the theoretical expression led us from experimental data; in particular, a satisfactory agreement was obtained between some values of stability constants we determined and those obtained by another method, as mentioned later.

## EXPERIMENTAL

Experiments were performed with Bio-Rex 63 (50–100 mesh) phosphonate resin, Bio-Rex 70 (50–100 mesh) carboxylate resin and Chelex 100 (100–200 mesh) iminodiacetate resin. All of these ion exchangers were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The three resins were treated several times in a wide column with hydrochloric acid and sodium hydroxide solutions, then converted into the metal forms by means of concentrated solutions of metal cation nitrate, washed with water and finally equilibrated with the ammoniacal metal ion solution.

Determinations of the distribution coefficients were carried out by both equilibrium shaking tests and column elution chromatography.

### *Equilibrium shaking tests*

The technique described by Tremillon[32] was employed. The concentration of copper(II) within the resin was modified by introducing appropriate amounts of ammonium nitrate in solution. A kinetic study showed that the distribution equilibrium of the amino acids is reached within a few minutes.

The amino acid concentrations in solution before and after equilibrium were determined by liquid scintillation counting of $^{14}C$-labelled amino acids: 50 $\mu$l of the aqueous solution under study were diluted into 10 ml of Instagel liquid scintillator Packard (Downers Grove, Ill., U.S.A.). Radioactivity measurements were performed on an Intertechnique SL 20 liquid scintillation spectrometer. No quenching was observed in the presence of ammonia and copper(II), even for concentrations as high as 1.5 *M* of ammonia and 0.1 *M* of copper(II).

### *Elution chromatography*

A few distribution coefficients were determined by elution chromatography[32]. In this instance, the $^{14}C$-labelled amino acids were detected with a continuous scintillation counting monitor system. Such an apparatus has been described elsewhere[33].

## RESULTS

Fig. 1 shows the influence of the concentration of ammonia on the fixation of glycine, alanine, valine, leucine and isoleucine by a copper(II) phosphonate resin, the concentration of metal ion in solution and within the resin being kept constant at 0.003 *M* and 2.5 mequiv./g, respectively. As with amines, *D* varies inversly as the concentration of ammonia in solution. Moreover, these five amino acids exhibit very similar behaviour.

Fig. 2 illustrates the influence of the copper(II) concentration in solution on the fixation of glycine, alanine and leucine by a copper(II) phosphonate resin, all the other parameters being given. A similar study of the binding of glycine by carboxylate and iminodiacetate resins loaded with copper(II) is represented in Figs. 3a and b. These

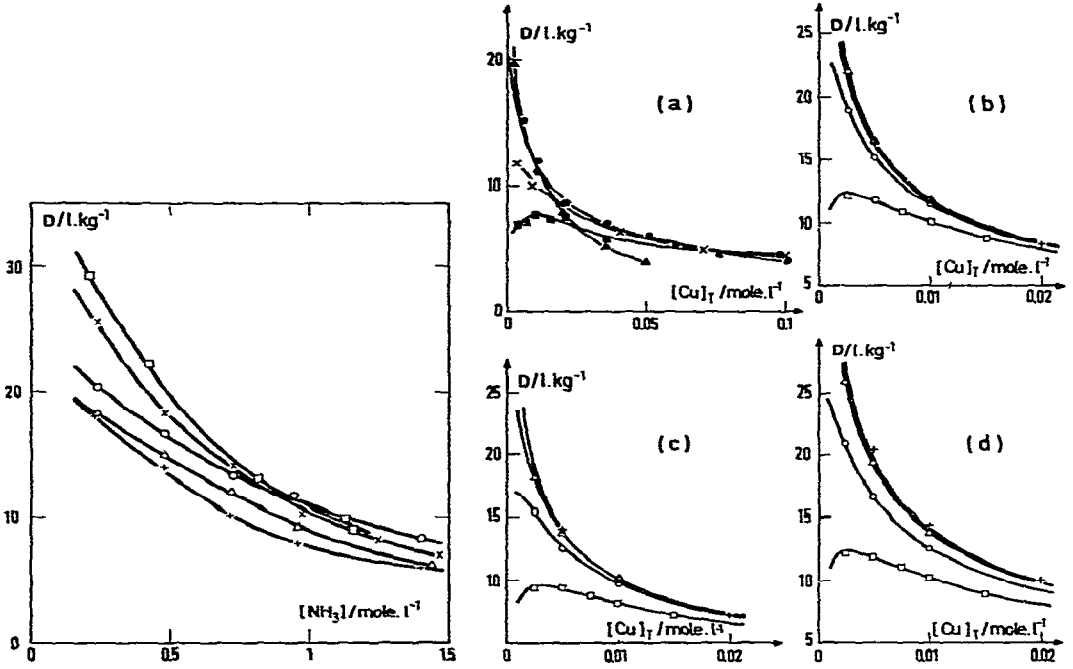


Fig. 1. Fixation of some amino acids in the copper(II) phosphonate resin. Variation of the distribution coefficient with the free ammonia concentration in solution [total copper(II) concentration $\approx 3 \cdot 10^{-3}$ *M*]. ○, Glycine; △, alanine; +, valine; □, leucine; ×, isoleucine.

Fig. 2. Fixation of three amino acids in the copper(II) phosphonate resin. Variation of the distribution coefficient with the total concentration of copper(II) in solution: (a) glycine (equilibrium shaking test); (b) glycine; (c) alanine; (d) leucine (elution chromatography, experimental points and curves displaying the theoretical variation of $D = f[Cu]_T$). Influence of ammonia concentration in solution: +, 0.2 *M*; △, 0.3 *M*; ○ and ●, 0.5 *M*; □, 1 *M*; ■, 1.5 *M* (free ammonia concentration); ▲, 0.29 *M*; ×, 1.015 *M* (total ammonia concentration).

results were obtained either by the equilibrium shaking test method (Figs. 2a, 3a and b) or by elution chromatography (Figs. 2b–d). The different graphs observed on each of these sets of curves correspond to different concentrations of ammonia in solution. These curves indicate a decrease in the distribution coefficient with increasing copper concentration, for ammonia concentration less than or equal to 0.5 *M*. However, when the ammonia concentration is greater than 0.5 *M*, the distribution reaches a maximum. Further, the values of *D* level out for high concentrations of copper(II). Similarly, the greater the concentration of ammonia, the less is the influence of the concentration of copper in solution on the distribution coefficient. The general trend of these sets of curves seems to be independent of the nature of the functional group of the resin.

A decrease in the value of the distribution coefficient can be achieved by decreasing the concentration of copper(II) within the resin, hence weakening its ligand-binding capacity towards amino acids (Fig. 4).

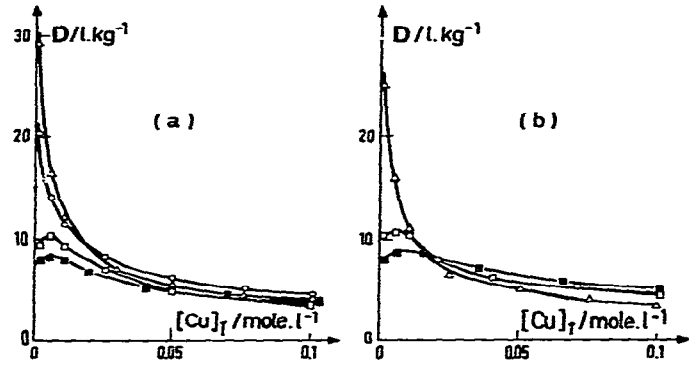


Fig. 3. Fixation of glycine in (a) copper(II) carboxylate and (b) copper(II) iminodiacetate resins. Variation of the distribution coefficient with total copper(II) concentration in solution (equilibrium shaking test). Influence of free ammonia concentration in solution: △, 0.3 *M*; ○, 0.5 *M*; □, 1 *M*; ■, 1.5 *M*.

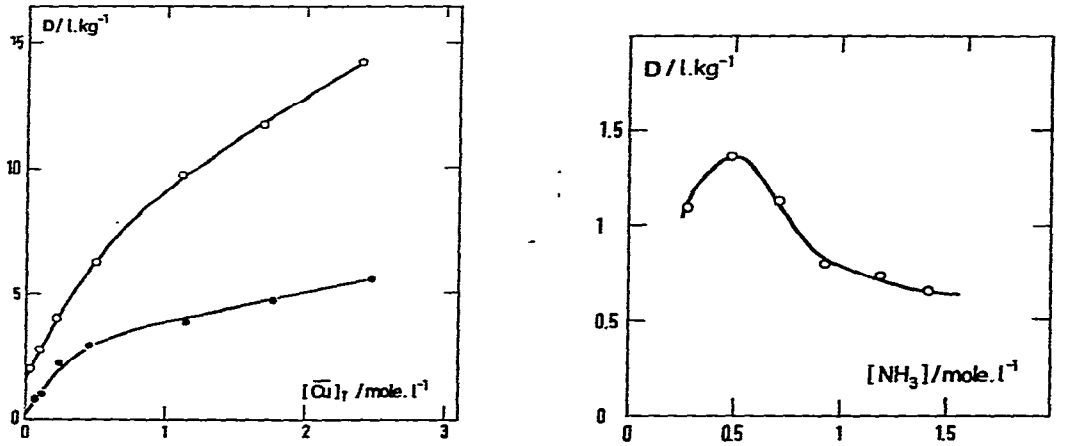


Fig. 4. Fixation of glycine in the copper(II) phosphonate resin. Variation of the distribution coefficient with the total copper(II) concentration within the resin. Composition of the solution: ○, $[NH_3] = 0.1\ M$, $[Cu]_T = 5 \cdot 10^{-3}\ M$; ●, $[NH_3] = 0.5\ M$, $[Cu]_T = 5 \cdot 10^{-2}\ M$.

Fig. 5. Fixation of glycine in the zinc(II) phosphonate resin. Variation of the distribution coefficient with ammonia concentration in solution ($[Zn]_T = 2 \cdot 10^{-3}\ M$).

A brief study of the distribution of glycine between a zinc(II) phosphonate resin and ammoniacal solutions containing zinc nitrate was carried out for comparison with the copper(II) phosphonate resin (Fig. 5). The distribution coefficient has low values and is slightly influenced by the concentration of ammonia in solution.

Finally, a few experiments in nickel(II)–ammonia medium were attempted. Owing to precipitation of nickel hydroxide, even at high concentrations of ammonia (1.5 *M*), we had to discontinue investigations of the fixation of amino acids in this medium.

## DISCUSSION

The theoretical expressions established above were applied to our results, with the aim of establishing the different forms in which the amino acid can exist in both phases.

### *Species in solution*

The study of the variation of $D$ in terms of the total copper(II) concentration, $[Cu]_T$, in solution, the other parameters, free ammonia concentration in solution and copper(II) concentration within the resin, being kept constant, provides a mean of determining the nature of the species that exist in solution. Under the above conditions, eqn. 4 becomes

$$D = \frac{a\sqrt{[Cu]_T}}{1 + \frac{[H^+]}{K_A} + b[Cu]_T} \tag{8}$$

where $a$ and $b$ are constants, as they depend only on the free ammonia concentration in solution and on the total copper(II) concentration, $\overline{[Cu]}_T$, within the resin, which equal half the ion-exchange capacity, $C_E$, of the resin. The terms 1 and $[H^+]/K_A$ represent the free amino acid forms $A^-$ and HA, respectively, and $b[Cu]_T$ the mixed complexes $CuA(NH_3)_i^+$.

Eqn. 8 yields

$$\log\left[\frac{D\left(1 + \frac{H^+}{K_A}\right)}{\sqrt{[Cu]_T}}\right] = \log a - \log\left(1 + \frac{b\,[Cu]_T}{1 + \frac{[H^+]}{K_A}}\right) \tag{9}$$

which can be written as

$$\log Y = \log a - \log(1 + bX)$$

The variation of $\log Y$ with $\log X$ for different free ammonia concentrations in solution is shown in Figs. 6a–c relative to the fixation of glycine, alanine and leucine, respectively, in the Cu(II) phosphonate resin. For low concentrations of ammonia and high concentrations of copper(II), the plots are straight lines with a slope of approximately $-1$. According to eqn. 9, this indicates that mixed complexes clearly predominate over the free amino acid forms in these media. When increasing the ammonia and decreasing the copper concentration, the graphs become curved. This phenomenon occurs much sooner at higher ammonia concentrations. This result can be explained by the presence of a mixture containing variable proportions of the complexed and free amino acid forms in these media.

The same phenomenon is noted when copper(II) carboxylate and iminodiacetate resins are employed (Figs. 7a and b). All of our results agree with the theoretical expression of the distribution coefficient and confirm the assumption of the formation of mixed complexes in solution.

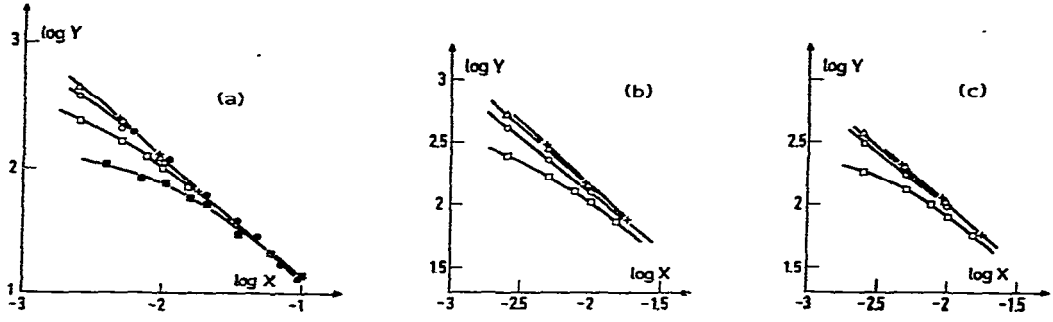


Fig. 6. Formation in solution of mixed complexes $CuA(NH_3)_i^+$ using the copper(II) phosphonate resin: (a) glycine, (b) alanine, (c) leucine. Influence of free ammonia concentration in solution: +, 0.2 *M*; △, 0.3 *M*; ○, 0.5 *M*; □, 1 *M* (elution chromatography); ●, 0.5 *M*; ■, 1.5 *M* (equilibrium shaking test).

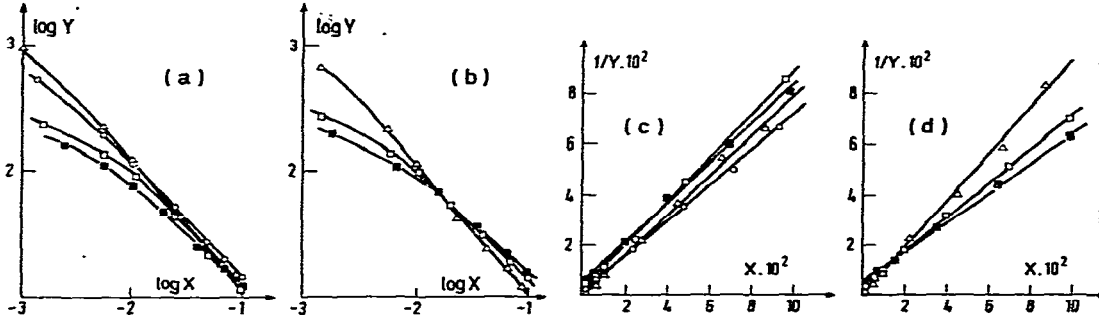


Fig. 7. Formation of mixed glycine complexes $CuA(NH_3)_i^+$ in solution using (a and c) copper(II) carboxylate and (b and d) copper(II) iminodiacetate resins. Influence of free ammonia concentration in solution: △, 0.3 *M*; ○, 0.5 *M*; □, 1 *M*; ■, 1.5 *M*.

The nature of the mixed complexes formed in solution can be determined according to the relationship

$$\frac{\sqrt{[Cu]_T}}{D\left(1+\frac{[H^+]}{K_A}\right)} = \frac{1}{a} + \frac{b}{a}\cdot\frac{[Cu]_T}{1+\frac{[H^+]}{K_A}}$$

derived from eqn. 8, and which can be written as

$$\frac{1}{Y} = \frac{1}{a} + \frac{b}{a}X$$

The plot of $1/Y$ against $X$ in Figs. 8a–d [copper(II) phosphonate resin] and Figs. 7c and d [copper(II) carboxylate and iminodiacetate resins] yields straight lines of slopes $b/a$ and intercepts at $1/a$. The value of $b$ can be calculated from these values and reported in terms of the free ammonia concentration:

$$b = \frac{1}{\alpha}\sum_{i=0}^{i=3}\beta_i'[NH_3]^i$$

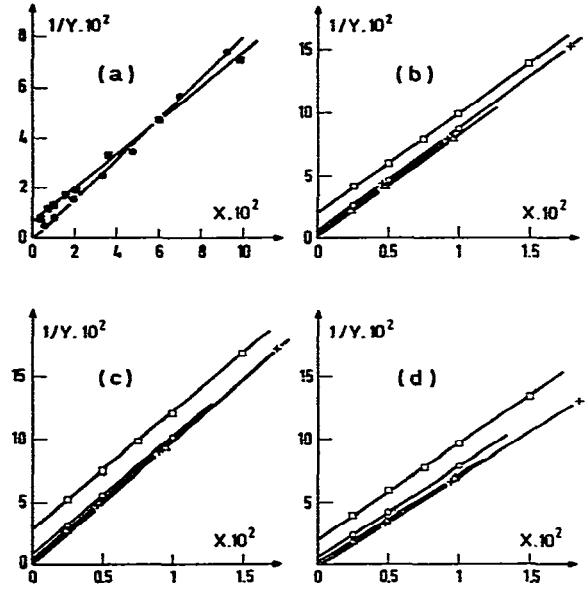


Fig. 8. Formation of mixed complexes $CuA(NH_3)_i^+$ in solution using the copper(II) phosphonate resin: (a and b) glycine; (c) alanine; (d) leucine. Influence of free ammonia concentration in solution: symbols as in Fig. 6.

Putting $b\alpha = F$, this equation becomes

$$F = \beta_0^l + \beta_1^l [NH_3] + \beta_2^l [NH_3]^2 + \beta_3^l [NH_3]^3$$

where $\beta_0^l$, $\beta_1^l$, $\beta_2^l$ and $\beta_3^l$ are the overall stability constants of the complexes $CuA^+$, $CuA(NH_3)^+$, $CuA(NH_3)_2^+$ and $CuA(NH_3)_3^+$, respectively, defined by eqn. 1.

The determination of $b$ for varying ammonia concentrations and the plot of log $F$ against log $[NH_3]$ (Fig. 9) provide a means of studying the $F$ function and thus allow the nature of the complexes to be determined. Attention is drawn, however, to the fact that only those results obtained by elution chromatography, that is concerning copper(II) phosphonate resin, have been used. Measurements by the equilibrium shaking test method are not accurate enough to allow such a treatment. Limiting slopes of the curves obtained with the three amino acids have values of 1.90 and 2.13 for glycine, 2.23 and 2.40 for alanine and 1.80 and 2.16 for leucine, thus indicating the probable existence of a mixture of complexes.

In spite of the few experimental points, it seemed interesting to analyze the $F$ function $F = f[NH_3]$ according to the De Ford and Hume method[34]. This study led us to accept the presence of the three complexes $CuA(NH_3)^+$, $CuA(NH_3)_2^+$ and $CuA(NH_3)_3^+$ as most likely in the case of glycine and leucine, while alanine seems to exist only in complexed forms containing two or three ammonia molecules. Approximate values of the stability constants are listed in Table I. Further experimental work is necessary if more accurate values of these constants are to be determined, but this was not the object of our study.

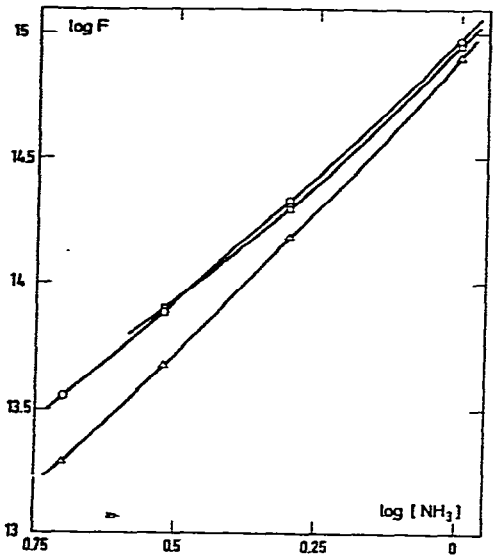


Fig. 9. Nature of mixed complexes formed by glycine (○), alanine (△) and leucine (□) in solution.

Stability constants of mixed complexes of a given amino acid are found to be very nearly the same and do not vary from one amino acid to another. Furthermore, with $CuA(NH_3)^+$ and $CuA(NH_3)_2^+$, our results are in good agreement with those reported by Bonnet *et al.*[35] using the potentiometric surface method. Data from the literature[35,36] concerning the stability of simple $CuA^+$ complexes in solution are also given in Table I. They show that simple complexes could rightly be neglected before the mixed complexes in the media we investigated.

TABLE I

STABILITY CONSTANTS OF MIXED COMPLEXES $CuA(NH_3)_i^+$ AND $RCuA(NH_3)_i^-$ FORMED BY GLYCINE, ALANINE AND LEUCINE IN SOLUTION AND WITHIN COPPER(II) PHOSPHONATE RESIN, RESPECTIVELY

| *Amino acid* | $Log\ \beta_0^I$ | $Log\ \beta_1^I$ | $Log\ \beta_2^I$ | $Log\ \beta_3^I$ | $Log\ \overline{\beta_1^I}$ | $Log\ \overline{\beta_2^I}$ | $Log\ \overline{\beta_3^I}$ |
|---|---|---|---|---|---|---|---|
| Glycine | | 13.6 | 14.8 | 14.4 | 9.7 | 10.4 | 10.1 |
| Glycine | 8.30* | 12.50* | 14.85* | | | | |
| Alanine | 8.18** | <12 | 14.6 | 14.6 | 8.9 | 10.3 | 10.1 |
| Leucine | 7.89** | 14.1 | 14.5 | 14.6 | 10.1 | 9.6 | 10.4 |

* Data from ref. 35.
** Data from ref. 36.

*Species within the resin*

Values of the stability constants of cupriammine complexes within the resin are needed in order to provide evidence of mixed complexes in that phase. Up to now, such determinations have been made only for the copper(II) phosphonate resin, and therefore only this support is considered further.

Binuclear complexes $R_2Cu_2A(NH_3)_i^-$ within the resin can be investigated by examining the distribution coefficient, as has already been done before. This time, the copper(II) concentration within the resin $[\overline{Cu}]_T$ is the variable parameter, the ammonia and copper(II) concentrations in solution being kept constant. Under these conditions, eqn. 4 becomes

$$\frac{D}{\sqrt{[\overline{Cu}]_T}}\left[1+\frac{[H^+]}{K_A}+\frac{[Cu]_T}{\alpha}\cdot\sum_{i=0}^{i=3}\beta_i[NH_3]^i\right]=c+d\,[\overline{Cu}]_T$$

or

$$Z=c+d\,[\overline{Cu}]_T$$

where $c$ and $d$ are constants and $d\,[\overline{Cu}]_T$ is a term relative to the formation of the mixed binuclear complexes.

If such binuclear complexes exist, then $Z$ must vary directly as $[\overline{Cu}]_T$. Results for glycine, plotted in Fig. 10, do not display such a variation. We can conclude that for glycine no such species appear within the resin. The same conclusions have been extended to alanine and leucine.

The nature of mixed mononuclear complexes that may exist within the resin can be examined in a simple manner. Indeed, the term $a$ in eqn. 8 can be expressed as

$$a=\sqrt{K_0\cdot\frac{[\overline{Cu}]_T}{\alpha\,\bar{\alpha}}\cdot\sum_{i=0}^{i=3}\overline{\beta_i}\,[NH_3]^i} \qquad (10)$$

where $K_0$, the equilibrium constant of the ionic exchange $NH_4^+/Cu^{2+}$, can be determined from experimental results according to the relationship

$$\log\left(\frac{\alpha\,[\overline{Cu}]_T}{\bar{\alpha}\,[Cu]_T\,[\overline{NH_4^+}]^2}\right)=\log K_0-2\log[NH_4^+]$$

derived from eqns. 5, 6 and 7.

According to eqn. 10, the $\overline{F}$ function representing the mixed complexes formation within the resin is easily established:

$$\overline{F}=\overline{\beta_0}+\overline{\beta_1}\,[NH_3]+\overline{\beta_2}\,[NH_3]^2+\overline{\beta_3}\,[NH_3]^3$$

where $\overline{\beta_0}$, $\overline{\beta_1}$, $\overline{\beta_2}$, $\overline{\beta_3}$ are the overall stability constants of complexes $RCuA^-$, $RCuA(NH_3)^-$, $RCuA(NH_3)_2^-$ and $RCuA(NH_3)_3^-$, respectively, within the resin.

For all concentrations of ammonia, the value of $\overline{F}$ can be calculated and the plots of log $\overline{F}$ against log $[NH_3]$ are shown in Fig. 11. The slopes of the curves obtained with the three amino acids are similar to those observed in solution. The values of limiting slopes of these curves, 1.69 and 2.05 for glycine, 2.00 and 2.26 for alanine and 1.50 and 1.98 for leucine, led us to assume the presence of a mixture of complexes $RCuA(NH_3)^-$, $RCuA(NH_3)_2^-$ and $RCuA(NH_3)_3^-$. Analysis of the function $\overline{F}=f[NH_3]$ according to De Ford and Hume method[34] seems to confirm this hypoth-

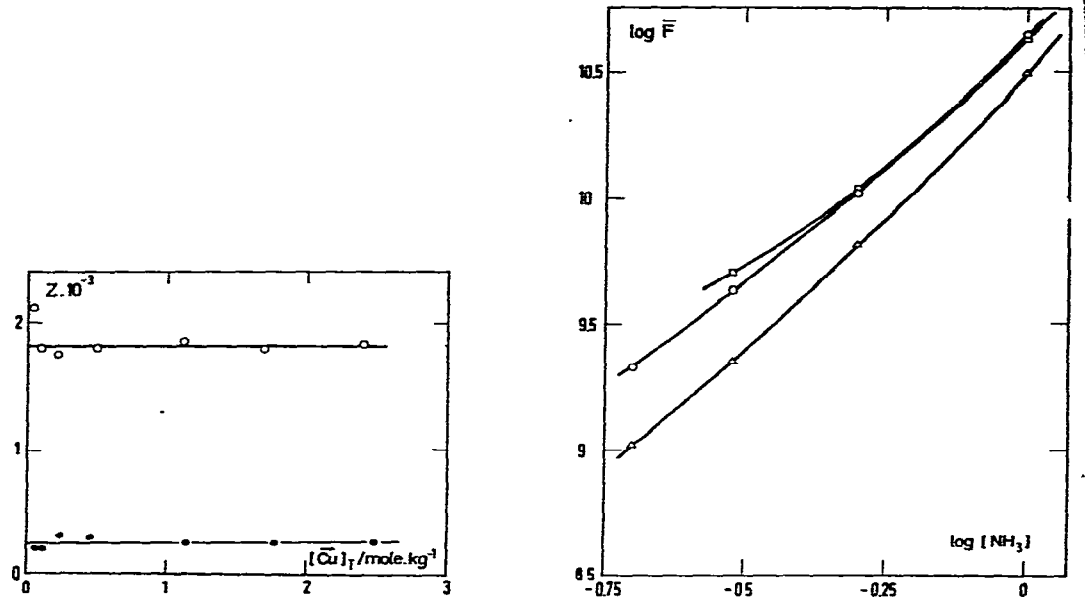


Fig. 10. Investigations on mixed binuclear $R_2Cu_2A(NH_3)_i^-$ complexes, formed by glycine within the copper(II) phosphonate resin. Composition of the solution: ○, $[NH_3] = 0.1\ M$, $[Cu]_T = 5 \cdot 10^{-3}\ M$; ●, $[NH_3] = 0.5\ M$, $[Cu]_T = 5 \cdot 10^{-2}\ M$.

Fig. 11. Nature of mixed complexes formed by glycine (○), alanine (△) and leucine (□) within the copper(II) phosphonate resin.

esis and permits the approximate determination of the stability constants $\overline{\beta_i^l}$ (Table I). We observe that the stabilities of the mixed complexes within the resin are, as in solution, nearly the same for a given amino acid and not very different from one amino acid to another.

*Comparison of the complexed forms in the solution and within the resin*

From our results, it is possible to calculate the value of the average numbers $i_m$ and $\overline{i_m}$ of ammonia molecules bound to copper(II) in the mixed complexes formed in both phases. The variation of $i_m$ and $\overline{i_m}$ for the three amino acids studied is plotted against the ammonia concentration in Fig. 12. The variations of $j_m$ and $\overline{j_m}$, the average numbers of ammonia molecules bound to copper(II) in the simple cupriammine complexes formed in the solution and within the resin, respectively, are also reported (Fig. 12a).

These results show that both the resin and solution media exhibit similar behaviour. For a given ammonia concentration, the nature of the simple and of the mixed complexes within the resin is nearly the same as in solution. The nature of the mixed complexes depends on the amino acid molecule and on the ammonia concentration. For glycine and alanine, the mixed complexes contain an average number of ammonia molecules close to two, for any ammonia concentration in solution. For leucine, the nature of the mixed complexes depends much more on the ammonia concentration, as the average number of ammonia molecules it contains varies from 1.3 to 2.7 in the concentration range considered.

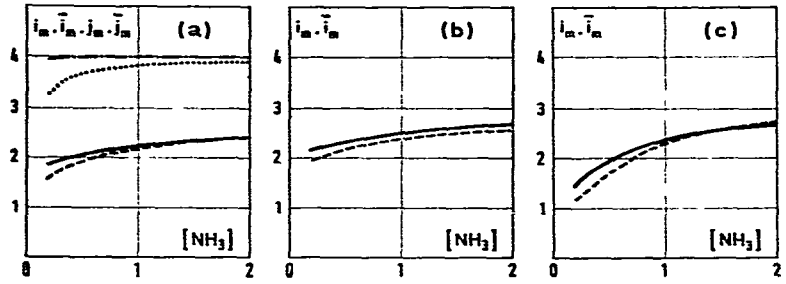


Fig. 12. Theoretical variation with the ammonia concentration of the average numbers of ammonia molecules bound to copper ion in the mixed and cupriammine complexes formed in the solution and within the copper(II) phosphonate resin: (a) glycine; (b) alanine; (c) leucine; ———, $i_m$ (mixed complexes in solution); — — —, $\overline{i_m}$ (mixed complexes within the resin); — · — ·, $j_m$ (cupriammine complexes in solution); ·····, $\overline{j_m}$ (cupriammine complexes within the resin).

*Simplified theory*

The results show that the general theory established above can be easily simplified. In the media we investigated, the species that predominate in solution are indeed restricted to $NH_3$, $Cu(NH_3)_4^{2+}$, $CuA(NH_3)_{i_m}^{+}$ and $A^-$ and within the resin to $Cu(NH_3)_{4-\varepsilon}^{2+}$ and $CuA(NH_3)_{\overline{i_m}}^{+}$, where, as pointed out before, $\varepsilon$ is less than unity and $i_m$ and $\overline{i_m}$ are nearly equal (thus let $i_m = \overline{i_m} = x$).

Under these conditions, the distribution of the amino acid can be described in a simple manner, by means of an ion-exchange equilibrium:

$$2\,CuA(NH_3)_x^+ + \overline{Cu(NH_3)_{4-\varepsilon}^{2+}} + \varepsilon NH_3 \overset{K}{\rightleftarrows} 2\,\overline{CuA(NH_3)_x^+} + Cu(NH_3)_4^{2+} \quad (11)$$

and a complex formation equilibrium in solution:

$$Cu(NH_3)_4^{2+} + A^- \overset{k}{\rightleftarrows} CuA(NH_3)_x^+ + (4-x)NH_3 \quad (12)$$

The distribution coefficient of the amino acid is then

$$D = \frac{\overline{[CuA(NH_3)_x^+]}}{[CuA(NH_3)_x^+] + [A^-]}$$

thus, putting $\overline{[Cu(NH_3)_{4-\varepsilon}^{2+}]} = \overline{[Cu]}_T$ and $[Cu(NH_3)_4^{2+}] = [Cu]_T$, we obtain

$$D = \sqrt{\frac{K\,\overline{[Cu]}_T\,[NH_3]^{\varepsilon}}{[Cu]_T}} \cdot \frac{1}{1 + \dfrac{[NH_3]^{4-x}}{k\,[Cu]_T}}$$

This simplified expression clearly displays how the distribution coefficient varies with the ammonia and copper(II) concentrations in solution. Indeed, it is easy

to see that each of these two parameters produces two opposite effects upon the distribution equilibrium. In particular, an increase in the copper(II) concentration in solution favours the fixation by displacing equilibrium 12 towards the formation of the mixed complexes, but is unfavourable to the binding of the mixed complexes within the resin as the concentration of the exchangeable ion $Cu(NH_3)_4^{2+}$ increases in solution. On the contrary, increasing the ammonia concentration destroys the mixed complexes in solution, yielding the $A^-$ form, and causes a decrease of the distribution coefficient, but on the other hand displaces equilibrium 11 to the right, favouring the fixation of the mixed complexes. However, it must be noted that, given the weak values of the stoichiometric coefficient $\varepsilon$ of ammonia in equilibrium 11, this second effect of the variation of the ammonia concentration must be negligible in comparison with the first effect.

It can easily be deduced from these observations that the variation of $D$ with ammonia concentration is nearly always decreasing and that the function $D = f[Cu]_T$ necessarily exhibits a maximum when $[Cu]_T = [NH_3]^{4-x}/k$, that is when the concentration of the free $A^-$ form is equal to the total concentration of the mixed complexes. The free species predominate before the maximum point, that is for low concentrations of copper(II). In this instance, the expression for $D$ is

$$D = A \frac{k\sqrt{[Cu]_T}}{[NH_3]^{4-x}}$$

where $A = \sqrt{K[Cu]_T [NH_3]^{\varepsilon}}$, and $D$ must vary as the square root of the copper(II) concentration in solution. This effect is not observed on our curves, because this phenomenon would appear only in media in which the copper(II) concentrations are very low; such media were not investigated. However, it appears in the above expression that $D$ decreases when decreasing $[Cu]_T$. This result can explain why small elution volumes were observed for most of the amino acids (except histidine and lysine) on chromatograms obtained by Navratil *et al.*[17] using copper(II) carboxylate resin and an eluent containing 1 $M$ $[NH_3]$ and $10^{-4}$ $M$ $[Cu]_T$. On the other hand, in media rich in metal cation, the complex species are predominant, these complexes occurring for lower copper(II) concentrations the lower the ammonia concentration. Thus $D$ must vary with $[Cu]_T$ as $A/\sqrt{[Cu]_T}$, as shown in Figs. 2 and 3. The presence and position of the maximum point depend only on the nature of the amino acid (value of $k$) and the composition of the solution. This theoretical interpretation can explain why the general shape of the curves describing the binding of glycine by copper(II) phosphonate, carboxylate and iminodiacetate resins is not affected by the nature of the functional group of the support (Figs. 2a and b, 3a and b).

It is then possible to determine the optimal conditions for the fixation of one amino acid. It is in dilute ammoniacal media that the distribution coefficient reaches the highest values, the maximum fixation being obtained for a copper(II) concentration in solution equal to $[NH_3]^{4-x}/k$. Increasing copper(II) or ammonia concentrations in solution causes a decrease in the distribution coefficient, thus permitting the elution of the bound amino acid. Moreover, it must be noted that elution can be also achieved by decreasing the copper(II) concentration.

## CONCLUSION

The distribution of an amino acid by ligand exchange occurs as a complex phenomenon which brings many equilibria into play. However, it is possible, by simplifying the general theory with some approximations, to describe the distribution process by the means of two equilibria, one involving ion exchange and the other the formation of mixed complexes in solution. This simplified theory clearly displays how the two main parameters, ammonia and copper(II) concentrations in solution, affect the distribution coefficient. Thus it can be seen that the variation of each of these parameters produces two opposite effects acting simultaneously on the ion-exchange equilibrium and on the formation of mixed complexes in solution.

Some observations can then be made on the selectivity that can be expected from this process. The selectivity coefficient, which is described as the ratio $D_1/D_2$ of the distribution coefficients of two amino acids $D_1$ and $D_2$, is given by

$$\frac{D_1}{D_2} = \sqrt{\frac{K_1}{K_2}} \cdot \frac{1 + \dfrac{[NH_3]^{4-x_2}}{k_2\,[Cu]_T}}{1 + \dfrac{[NH_3]^{4-x_1}}{k_1\,[Cu]_T}}$$

and is easily deduced from the simplified theory.

This expression shows that, in media where complexed forms predominate, that is for high copper(II) concentrations in solution, the ratio $D_1/D_2$ must tend towards $\sqrt{K_1/K_2}$. The selectivity coefficient then depends only on the ratio of two ion-exchange constants and we can speculate that two amino acids with similar structures have approximately equal constants and a selectivity coefficient nearly equal to unity. However, we can assume that two amino acids that differ from one another in their molecular weights or structures will have more different constants.

In media where the free forms predominate, that is at low copper(II) concentrations, the limit of the selectivity coefficient is $\sqrt{\dfrac{K_1}{K_2}} \cdot \dfrac{k_1}{k_2}\,[NH_3]^{x_1-x_2}$. In this instance, the selectivity depends on, in addition to the ion-exchange constants $K$, the complex formation constants $k$ in solution and, when $x_1$ differs from $x_2$, the ammonia concentration. Considering complex formation generally as a much more selective phenomenon than simple ion exchange, the selectivity coefficient is probably more different from unity than in the first instance, even for amino acids that have similar structures.

The optimal conditions for the separation of two amino acids can therefore be easily estimated. The observations reported here allow us to assume that the maximal selectivity should be observed in media where $D_1/D_2$ depends on $k$ constants, that is at low copper(II) concentrations in solution. If $x_1$ is different from $x_2$, a complementary study of the variation of $D_1/D_2$ with ammonia concentration is necessary in order to determine accurately the solution composition that gives the optimal selectivity. Further, it must be borne in mind that the distribution coefficients are smaller the higher is the ammonia concentration.

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