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A Method for the Separation of Peptides and α-Amino Acids

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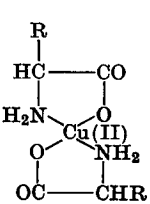
1. Peptides and α-amino acids, occurring in mixtures from various sources, can be separated into one fraction containing the amino acids and several peptide fractions. This is achieved by chelation of the mixture with Cu^{2+} ions and subsequent chromatography of these chelates over the acetate form of diethylaminoethylcellulose or triethylaminoethylcellulose. 2. The amino acid fraction is obtained by elution with 0·01 M-collidine–acetate buffer, pH 8·0. 3. Peptide fractions are eluted with 0·01 M-collidine–acetate buffer, pH 4·5, 0·17 N-acetic acid and 0·1 N-hydrochloric acid respectively. 4. With the exception of aspartic acid and glutamic acid, which are partly found in the acidic peptide fraction, the amino acids are completely separated from the peptides. 5. Contamination of the acidic peptide fraction with glutamic acid and aspartic acid can be largely avoided by previous addition of an excess of arginine. 6. Copper is removed from the eluates by extraction with 8-hydroxyquinoline in chloroform.

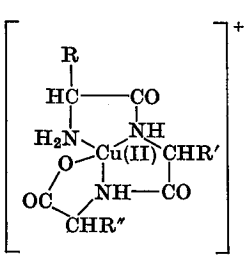
The isolation and purification of polypeptides of natural origin is often complicated by the presence of amino acids and amines. In particular the separation of small peptides from a large excess of amino acids is a difficult problem. The methods proposed in the literature generally have a limited applicability (Carnegie, 1961*a*,*b*; Carnegie & Synge, 1961; Fromageot, Jutisz & Lederer, 1948; Lampson & Singher, 1960).

Fazakerley & Best (1965) achieved separation of amino acids and peptides by the use of a Sephadex column loaded with Cu (II) and equilibrated with borate buffer, pH 11·0. The peptides were eluted by the borate buffer; the amino acids were retained, but could be obtained by a further elution with 0·2 N-hydrochloric acid. Cu (II) was removed from the eluate fractions by extraction with sodium diethyldithiocarbamate in chloroform. This method has, however, some disadvantages: (*a*) pH 11·0 may be harmful for peptides, in particular for those containing thiol or disulphide groups; (*b*) the fractions must be desalted; (*c*) the capacity of the column is small; (*d*) the extraction with sodium diethyldithiocarbamate in chloroform is not the most suitable method for removing Cu (II).

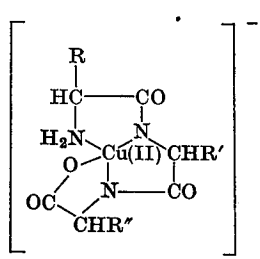
With the aim of avoiding as far as possible the disadvantages mentioned above, we developed a method for the separation of amino acids from peptides, based on the fact that at pH 8 the Cu (II) complexes of all amino acids except glutamic acid and aspartic acid are present as positive ions or as neutral molecules (I), whereas the Cu (II) complexes of peptides (II*a*) at this pH are at least partly in the anionic form (II*b*), as are the complexes of glutamic acid and aspartic acid (I:R contains CO_2^- group). Thus at pH 8 a separation of non-acidic



(I)



(II*a*)



(II*b*)

amino acids from peptides and acidic amino acids should be possible by chromatography over an anion-exchange column.

A preliminary account of this work has been given (Tommel, Vliegenthart, Penders & Arens, 1966).

MATERIALS AND METHODS

Anion-exchangers. Whatman DE11 DEAE-cellulose (capacity 1·0m-equiv./g.) was obtained from W. and R. Balston Ltd. (Maidstone, Kent). TEAE-cellulose* was prepared from DEAE-cellulose by treatment with ethyl bromide in ethanol (Porath, 1957). These anion-exchangers were purified by threefold recycling with 0·5N-HCl and 0·5N-NaOH, and converted into the acetate form with M-sodium acetate as described by Witter, Vliegenthart & Arens (1964). The columns were equilibrated with 0·01M-collidine–acetate buffer, pH 8·0.

Synthetic peptides. For model studies the synthetic L-peptides Val-Tyr-Pro ($ACTH^{22-24}$), Val-Lys-Val-Tyr-Pro ($ACTH^{20-24}$), Glu-His-Phe-Arg-Trp-Gly ($ACTH^{5-10}$), Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly ($ACTH^{1-10}$), and Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro ($ACTH^{11-24}$) were kindly provided by N. V. Organon, Oss, The Netherlands. Glutathione (GSH and GSSG) was purchased from F. Hoffmann–La Roche, Basle, Switzerland. Triglycine had been prepared in our Laboratory.

Natural amino acid–peptide mixtures. Two natural amino acid–peptide mixtures used in this study were obtained as follows.

(*a*) Posterior-pituitary extract. Acetone-dried pig posterior-pituitary lobes were extracted with 0·2M-pyridine–0·05N-acetic acid buffer. The low-molecular-weight fraction obtained after separation of the extract over Sephadex G-25 was percolated over Amberlite CG-50 (Vliegenthart, 1964). The part eluted with 0·25% acetic acid, combined with the part eluted with 0·1M-ammonium acetate, pH 5·0, was used in our experiments.

(*b*) Brain-stem extract. From acetone-dried calf brain stems an extract was prepared with 0·1N-HCl. After gel filtration over Sephadex G-25 a low-molecular-weight fraction was obtained and was used in our experiments.

Extinction measurements. These were performed with a Zeiss PMQ II spectrophotometer.

Paper chromatography. Paper chromatography was carried out at room temperature on 57cm. × 15cm. strips of Whatman 3MM paper by the descending method for 16–17hr. The solvent systems used were: I, butan-1-ol–acetic acid–pyridine–water (15:3:10:12, by vol.); II, butan-1-ol–acetic acid–water (4:1:5, by vol.).

Buffer. 2,4,6-Collidine (0·01M) was brought to the required pH with acetic acid.

Separation procedure. The amino acid–peptide mixture to be separated is dissolved in 0·01M-collidine and then adjusted with acetic acid to pH 8·0. If the solubility of the components is sufficient, a concentration of 10–15mg./ml. is used; otherwise, a more dilute solution can be applied. The constituents in the solution are converted into Cu(II) complexes by shaking this solution with an excess of $Cu_2(OH)_2CO_3$ at 45° for at least 15min., followed by filtration or centrifugation. [The $Cu_2(OH)_2CO_3$ was obtained from May & Baker Ltd., Dagenham, Essex; its purity was checked by X-ray fluorescence.] A clear faintly blue solution is obtained. If, owing to low solubility of the original non-chelated mixture, one had to start from a solution containing less than 10mg. of mixture/ml., the solution of the cupric chelates must be concentrated, e.g. by freeze-drying followed by redissolution in the proper amount of buffer at pH 8 (chelation generally enhances the solubility of amino acids and peptides).

The solution, which thus contains 10–15mg. of mixture/ml., is then applied to a 48cm. × 1·2cm. column of the acetate form of DEAE-cellulose or TEAE-cellulose, which has been equilibrated with 0·01M-collidine–acetate buffer, pH 8·0. The elution is performed stepwise successively with: (*a*) 0·01M-collidine–acetate buffer, pH 8·0; (*b*) 0·01M-collidine acidified with acetic acid to pH 4·5; (*c*) 0·17N-acetic acid; (*d*) 0·1N-HCl; each change is made when no further blue material leaves the column.

The recommended elution rate is about 15ml./hr., and the volume of the fractions 3ml. Amines, if present, are accumulated in the amino acid fraction, which is the first peak to leave the column.

The concentration of cupric chelates in the fractions can be determined by measuring E_{620}. Alternatively the Cu(II) content of the fractions can be determined by extraction at pH 4–9 with 3ml. of 8-hydroxyquinoline in chloroform (6·5mg./ml.), which brings the Cu^{2+} ions into the chloroform phase, and measuring E_{396} of this layer. This method is much more sensitive. The aqueous fractions freed from Cu^{2+} ions can be examined with a ninhydrin reagent (Matheson, Tigane & Hanes, 1961) before and after alkaline hydrolysis, or by paper chromatography, or both.

If glutamic acid and aspartic acid are present in substantial amounts, they contaminate some of the peptide fractions. By previous addition of an equal weight of arginine it is possible, however, to force glutamic acid and part of aspartic acid into the amino acid fractions.

The usefulness of the procedure is illustrated by an account of its application to an artificial mixture and to extracts from natural sources.

RESULTS

Separation of an artificial mixture of peptides and amino acids

No arginine added. A mixture containing 10μmoles of each of the amino acids aspartic acid, glutamic acid, arginine, histidine, lysine, ornithine, alanine, cystine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, and the peptides GSSG, GSH, $ACTH^{22-24}$, $ACTH^{20-24}$, $ACTH^{5-10}$, $ACTH^{1-10}$ and $ACTH^{11-24}$ was dissolved in 5ml. of 0·01M-collidine–acetate buffer, pH 8·0, and converted into the complexes by shaking with cupric carbonate. After centrifugation, 2ml. of the solution was applied to a 48cm. × 1·2cm. column of the acetate form of TEAE-cellulose, previously equilibrated with 0·01M-collidine–acetate buffer, pH 8·0. Elution was performed with this same buffer at a rate of 18ml./hr. followed by 0·17N-acetic acid and

* Abbreviations: TEAE-cellulose, triethylaminoethyl-cellulose; $ACTH^{x-y}$, peptide consisting of residues *x*–*y* of adrenocorticotrophic hormone.

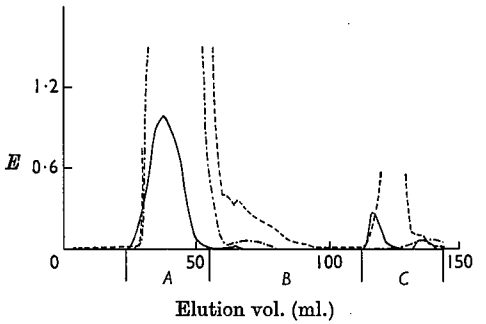


Fig. 1. Chromatography of a mixture of the cupric chelates of some amino acids and peptides on TEAE-cellulose. The place of the fractions *A*, *B* and *C* is indicated. ——, E_{620} of Cu^{2+} complexes; –·–·–, ninhydrin development (E_{570}); – – – –, E_{396} of the Cu^{2+}–8-hydroxyquinoline complex.

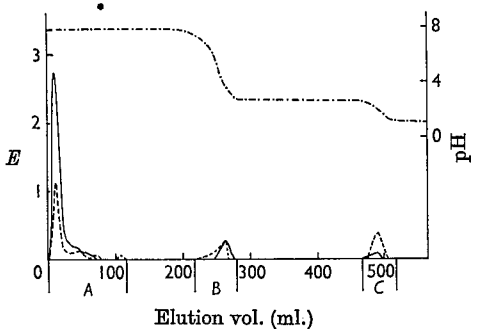


Fig. 2. Chromatography of a mixture of amino acids and peptides with added arginine (as the cupric chelates). The place of the fractions *A*, *B* and *C* is indicated. ——, Ninhydrin development (E_{570}); – – – –, $10 \times E_{620}$ of Cu^{2+} complexes; –·–·–, pH.

finally by 0·1 N-hydrochloric acid (90 ml./hr.). Details of the analysis are given in Fig. 1.

After removal of Cu(II) by extraction with 8-hydroxyquinoline the fractions constituting one peak were pooled and examined by paper chromatography. The amino acids, including part of aspartic acid and glutamic acid, were found in fraction *A* and no peptides could be detected there. Fraction *B* was free from amino acids and consisted of $ACTH^{5-10}$, $ACTH^{20-24}$ and $ACTH^{22-24}$. In fraction *C*, $ACTH^{1-10}$, $ACTH^{11-24}$ and GSSG were present, as well as part of the acidic amino acids glutamic acid and aspartic acid. Thus a good separation of neutral and basic amino acids from peptides was achieved, but glutamic acid and aspartic acid contaminated the peptides of fraction *C*.

The absence of GSH from the fractions indicated that this substance was oxidized by Cu^{2+} to GSSG.

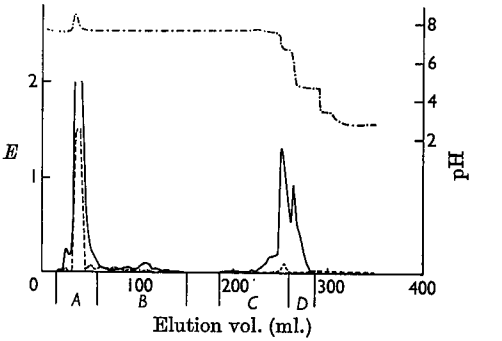


Fig. 3. Chromatography of a posterior-pituitary-lobe extract, after complex-formation with Cu^{2+}, on TEAE-cellulose. The place of the fractions *A*, *B*, *C* and *D* is indicated. ——, E_{396} of the Cu^{2+}–8-hydroxyquinoline complex; – – – –, ninhydrin development (E_{570}); –·–·–, pH.

Arginine added. The same mixture was used, but 205 μmoles of arginine were added. Further, the length of the DEAE-cellulose column was increased to 98 cm. and the elution rate diminished to 3 ml./hr. No other changes were made. The result of this experiment is given in Fig. 2.

After removal of Cu(II), fraction *A* consisted only of the amino acids and fraction *B* of the peptides $ACTH^{1-10}$, $ACTH^{5-10}$, $ACTH^{20-24}$ and $ACTH^{22-24}$, whereas fraction *C* contained $ACTH^{11-24}$, GSSG and the main part of aspartic acid, but no glutamic acid.

Comparison of this result with the previous one showed that addition of arginine resulted in a more complete separation of amino acids and peptides, although aspartic acid still contaminated the last peptide fraction.

Separation of amino acids and peptides present in extracts from natural sources

Pituitary extract. A 261 mg. portion of the pituitary extract was converted into the Cu(II) chelates by shaking with cupric carbonate at pH 8·0, and, after filtration, separated on a 48 cm. × 1·2 cm. column of the acetate form of DEAE-cellulose. Stepwise elution was carried out successively with 0·01 M buffer, pH 8·0, 0·01 M buffer, pH 7·5, 0·01 M buffer, pH 4·7, and 0·17 N-acetic acid, at a rate of 12 ml./hr. Details are given in Fig. 3.

It was obvious, from the ninhydrin development before and after alkaline hydrolysis, that the great bulk of amino acids were concentrated in fraction *A*, whereas peptide material was present in fractions *B*, *C* and *D*. By paper chromatography it was shown that the peptides were free from contaminating amino acids, except for the most acidic fraction, which contained aspartic acid and glutamic acid.

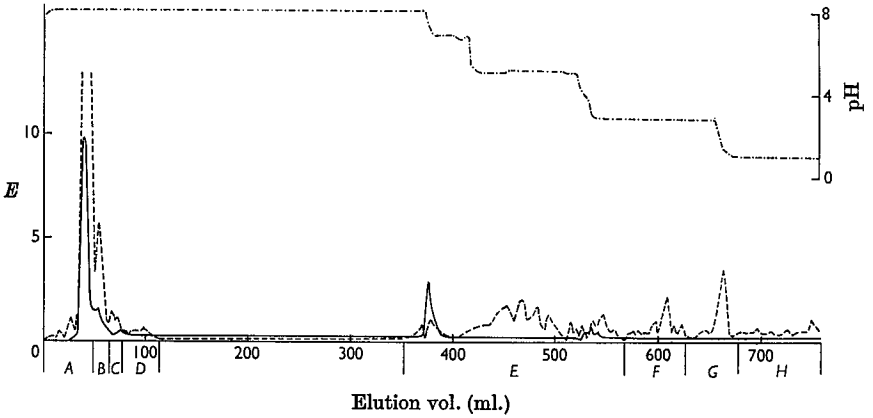


Fig. 4. Chromatography of a brain-stem extract, after complex-formation with Cu^{2+}, on DEAE-cellulose. The place of the fractions *A*–*H* is indicated. ——, $100 \times E_{620}$ of Cu^{2+}-complexes; –·–·–, pH; – – –, ninhydrin development after alkaline hydrolysis (E_{570}).

Brain-stem extract. A solution of 100mg. of the brain-stem extract in 7ml. of collidine–acetate buffer was chelated by shaking with cupric carbonate and after filtration applied to a 98cm. × 1·5cm. column of the acetate form of DEAE-cellulose. Elution was performed successively with 0·01M buffer, pH8·0, 0·01M buffer, pH5·0, and 0·1N-hydrochloric acid.

Again an excellent separation was observed into an amino acid fraction and several peptide fractions. Examination of the peptide fractions proved that generally no amino acids were present. Only the last fraction, eluted with 0·1N-hydrochloric acid, was contaminated with aspartic acid and glutamic acid.

Brain-stem extract (with an excess of arginine added). A solution of 30mg. of the brain-stem extract in 3ml. of 0·01M-collidine–acetate buffer, pH8·0, to which 15mg. of arginine had been added, was treated with cupric carbonate to form complexes; the pH was maintained at 8·0 during this process. The excess of cupric carbonate was filtered off, and the filtrate was evaporated to dryness in a film evaporator.

The residue was redissolved in 2ml. of 0·01M-collidine–acetate buffer, pH8·0, and this solution was applied to a DEAE-cellulose column (48cm. × 1·5cm.), which was equilibrated with the same buffer. Elution was performed successively with 0·01M - collidine – acetate buffer, pH8·0, 0·01M - collidine–acetate buffer, pH5·0, 0·17N-acetic acid and 0·1N-hydrochloric acid, at a rate of 6·7ml./hr. The fraction volume was 3ml. Details of the analysis are presented in Fig. 4.

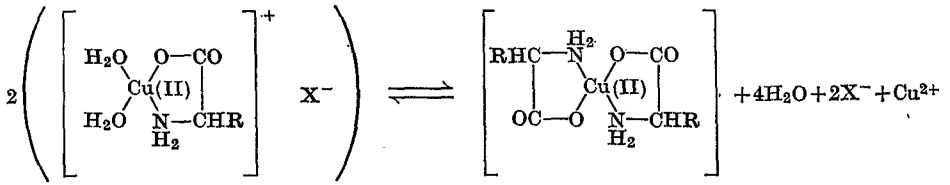
After removal of the Cu^{2+} by extraction with 8-hydroxyquinoline in chloroform (6mg./ml.), the fractions constituting each peak were pooled and examined by paper chromatography. In fraction *A* only amino acids could be detected, including the greater part of the acidic amino acids. Traces of the acidic amino acids were detected in the peptide fractions *G* and *H*. The other peptide fractions were completely free of amino acids.

DISCUSSION

There are two types of Cu(II) complexes of α-amino acids, both having the ligands in a square planar configuration (Gould & Vosburgh, 1942; Graddon & Munday, 1961–62). The first has 1 amino acid ion/Cu^{2+} ion, the other 2. Their relation is shown in Scheme 1.

The equilibrium constant [double complex]× $[Cu^{2+}]$/[single complex]^2 for the complexes of phenylalanine is 0·06 (Curchod, 1956), and may be of the same order for the other α-amino acids. Thus at low Cu^{2+} concentrations, the condition prevailing when the complexes are prepared by means of cupric carbonate, the amino acids are present almost entirely as double complexes. Generally the complexes are stable in neutral or alkaline medium; in acidic medium they decompose because protonation of the amino groups occurs.

At pH8·0 the double complexes of basic α-amino acids are positively charged, those of neutral α-amino acids are neutral and those of acidic α-amino acids are negative. The stability constants of the amino acid–cupric chelates are summarized in Table 1. Simple peptides form cupric complexes that contain 1mol. of peptide/Cu^{2+} ion; all four ligands belong to the same peptide (Breslow, 1961; Bryce & Gurd, 1966*a*; Bryce, Roeske & Gurd, 1965; Doran, Chaberek & Martell, 1964; Freeman & Szymanski, 1965; Freeman & Taylor, 1964; Gould



Single complex Double complex

Scheme 1. X^-, Anion in the solution, e.g. Cl^-.

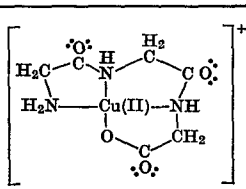
Table 1. *Stability constants, as log K_S, of the* Cu (*II*) *complexes of amino acids* [Cu (II)/*amino acid ratio* 1:2] *in water at* 25°

$K_S = \frac{[CuA_2]}{[Cu^{2+}][A^-]^2}$, where A represents amino acid (Greenstein & Winitz, 1961).

Amino acid	log K_S
Glycine	15·4
DL-Alanine	15·1
L-Proline	16·8
DL-Tryptophan	15·9
DL-Norleucine	15·5
DL-Valine	15·1
DL-Phenylalanine	14·9
DL-Serine	14·6
DL-Methionine	14·7
β-Alanine	12·9
Taurine	8
L-Tyrosine	15·0
DL-Ornithine	13·0
L-Lysine	13·7
L-Arginine	13·9
L-Histidine	—
L-Aspartic acid	15·9*
L-Glutamic acid	15·2*

*F. Jellinek (personal communication).

(III)



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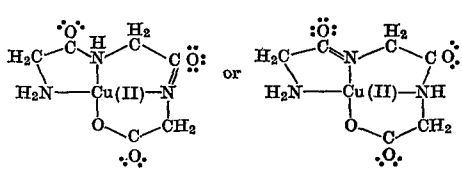

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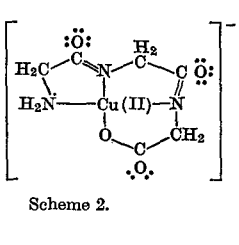

Scheme 2.

Table 2. *Complex and acidity constants of some peptide–cupric chelates*

$K_a = \frac{[CuP^+]}{[Cu^{2+}][P^-]}$; $K_c = \frac{[CuP][H^+]}{[CuP^+]}$; $K'_c = \frac{[CuP^-][H^+]}{[CuP]}$.

where P represents peptide.

Peptide	log K_a	log K_c	log K'_c
Gly-Gly	5·88	−4·25	−9·65
Gly-L-Tyr	5·90	−3·85	−9·25
Gly-L-Val	5·65	−4·85	—
Gly-L-Leu	6·10	−4·80	−9·65
Gly-L-His	—	−4·00	−4·50
L-Phe-Gly	4·66	−3·50	—
L-Ala-Gly	5·44	−4·16	—
L-His-Gly	—	−6·01	−10·6
Carnosine	9·72	−5·55	—
Gly-Gly-Gly	5·5	−5·4	−6·63
Gly-Gly-His	—	−4·75	−4·90
Ala-Ala-Ala	4·65	−4·47	−6·63
Ala-Gly-Gly	4·81	−4·98	−6·84
Gly-Gly-Gly-Gly	4·93	−5·45	−6·91
Gly-Gly-Gly-His	—	−6·35	−7·10
[8-Lysine]-vasopressin	5·31	—	—

& Mason, 1967; Peters & Blumenstock, 1967; Poddubnaya, el Naggar & Lyubinskaya, 1966).

The charge of the Cu (II) complex of a peptide is negative at alkaline pH. This feature is illustrated for triglycine (Scheme 2). In neutral medium form (III) is present, in alkaline medium form (V), and form (IV) may occur at intermediate pH.

The complexes of higher polypeptides may be more complicated, as these compounds may contain more than one Cu (II) ion/mol. (Jennings, 1963*a*,*b*; Jones & Perkins, 1965).

In general the stability of a peptide–cupric complex is lower than that of an α-amino acid–cupric complex. In Table 2 complex and acidity constants of some peptide chelates are summarized (Biester & Ruoff, 1959; Bryce, Pinkerton, Steinrauf & Gurd, 1965; Bryce & Gurd, 1966*b*; Campbell, Fun & Hubbard, 1963; Dobbie & Kermack, 1955*a*,*b*; Kim & Martell, 1966; Koltun, Roth & Gurd, 1963; Lenz & Martell, 1964).

The experiments mentioned above prove that the stability of the peptide complexes under the conditions employed is quite sufficient, and that

the difference in charge between the α-amino acid– and peptide–cupric complexes can be used for their separation.

The suitability of arginine addition for decreasing the contamination of peptide fractions with the acidic amino acids was deduced from the fact that at low Cu^{2+} concentration the amino acids are entirely or almost entirely present in double complexes, which do not necessarily consist of only one kind of amino acid. By adding a large excess of a suitable base to the mixtures before chelation, it could be hoped that during reaction with cupric carbonate (low Cu^{2+} concentration) mixed double complexes would be formed, consisting of 1 molecule of the added base and 1 molecule of an amino acid.

The electrical charge of such complexes derived from aspartic acid and glutamic acid would be zero or even positive, so that it could be expected that on anion-exchange chromatography at pH 8 these amino acids would not join the peptide fractions.

Of the several bases tested, arginine gave the best result, glutamic acid appearing entirely in the amino acid fraction. Aspartic acid, however, still partly remained in the peptide fraction and until now we have not been able to solve this problem.

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