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SEPARATION OF NEUTRAL AMINO ACIDS, DIPEPTIDES AND OLIGOPEPTIDES INTO CLASSES

APPLICATION TO URINE*

P. GILIBERTI AND A. NIEDERWIESER
Chemisches Labor der Universitäts-Kinderklinik, CH-8032 Zürich (Switzerland)

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SUMMARY

The behaviour of the copper complexes of amino acids and peptides was investigated on TEAE-cellulose, DEAE-Sephadex A-25 and DEAE-Sephadex A-50 at pH 8.0.

A method is described for the separation of the classes of compounds, neutral amino acids, neutral dipeptides and neutral oligopeptides, from each other via their copper complexes in two chromatographic steps at pH 8.0: DEAE-Sephadex A-25 separates amino acids from peptides, and DEAE-Sephadex A-50 separates dipeptides from oligopeptides. By a slight modification of the present procedure is also seems possible to separate tripeptides + tetrapeptides from pentapeptides + higher peptides.

The procedure is believed to be the first chemical technique to be described for the fractionation of peptide mixtures into classes of peptides. The method was applied to the fractionation of peptides from human urine on analytical and preparative scales.

INTRODUCTION

In a previous communication[1], a method for the separation of neutral oligopeptides from an excess of neutral amino acids in urine was described, based on the procedure of TOMMEL *et al.*[2]. After the formation of copper complexes at pH 8.0, the negatively charged copper–peptide complexes are separated from the electro-neutral copper–amino acid complexes by ion-exchange chromatography on TEAE-cellulose at pH 8.0. From theoretical considerations, however (see under DISCUSSION), one can expect dipeptides to be lost into the amino acid fraction. In order to clarify this subject and to eliminate this possible disadvantage, we investigated the behaviour of synthetic peptides as their copper complexes on TEAE-cellulose, DEAE-Sephadex A-25 and DEAE-Sephadex A-50 at pH 8.0. As a result, we propose a method for the separation of dipeptides from oligopeptides and excess of amino acids.

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EXPERIMENTAL AND RESULTS

Materials and preparation of the columns

Amino acids and peptides. These were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A., and from Fluka AG, CH-9470 Buchs, Switzerland.

Borate buffer, 50 mM solution, pH 8.0. Boric acid, 15.5 g, was dissolved in 20.5 ml of 1 *N* sodium hydroxide solution and diluted to 5 l with distilled water. Using a glass electrode, the pH was adjusted by adding sodium hydroxide or acetic acid.

Sodium bicarbonate buffer, 5 mM or 50 mM solution, pH 8.0 or 9.0. Sodium bicarbonate, 2.1 g or 21 g, was dissolved in 5 l of distilled water and the pH was adjusted by using a glass electrode by adding 0.1 *N* or 1 *N* sodium hydroxide solution or by bubbling carbon dioxide through the solution. The 5-m*M* buffer was separated from the air by a layer of toluene. The pH of the buffer of pH 8.0 increased slowly on standing and was re-adjusted with carbon dioxide.

Basic copper(II) carbonate, $CuCO_3 \cdot Cu(OH)_2$. This was obtained from E. Merck AG, Darmstadt, G.F.R.

Ion exchangers. TEAE-cellulose (Serva Entwicklungslabor, Heidelberg, G.F.R.) and DEAE-Sephadex A-25 and A-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were used. The ion exchanger was swelled and sedimented in distilled water and the fines were removed by suction. Prior to use, the residue was washed thoroughly on a sintered glass funnel, provided with a filter-paper, successively with 0.5 *N* sodium hydroxide solution, distilled water, 0.1 *N* hydrochloric acid, distilled water, 0.5 *N* sodium hydroxide solution, distilled water, 50 m*M* sodium bicarbonate (or borate) buffer and, for column chromatographic experiments, finally with 5 m*M* sodium bicarbonate buffer (see below). This washing procedure with the chemicals (but not with the distilled water) was continued until the pH of the eluate was identical with that of the washing fluid. The equilibrated resin was suspended in buffer and rapidly de-gassed *in vacuo*.

Preparation of the column

The rapidly de-gassed suspension of ion-exchange resin was loaded into a column* to give a gel bed of dimensions 2.5 × 35 cm after further equilibration overnight with 5 m*M* sodium bicarbonate buffer (pH 8.0) at a flow-rate of 120 ml/h. over the recin bed there should be free (buffer-filled) space to allow for the expansion of the gel during equilibration. Prior to using the column, the pH of the column effluent must be 8.0–8.1 (glass electrode).

Thin-layer chromatography

The behaviour of copper–peptide complexes on the weakly basic ion-exchangers TEAE-cellulose and DEAE-Sephadex A-25 and A-50 was investigated at pH 8.0 by thin-layer chromatography (TLC). Layers 1-mm thick (20 × 20 cm) were prepared from a slurry of the washed and equilibrated ion exchangers in 50 m*M* borate buffer (pH 8.0) by using a Desaga spreader**. When still wet, the plates were immediately

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* Type K 25/45 with flow adapter, Pharmacia Fine Chemicals AB, Uppsala, Sweden.
** C. Desaga GmbH, Heidelberg, G.F.R.

put in a BN-chamber* and further equilibrated for at least 4 h with 50 mM borate buffer (pH 8.0) by the flow-through technique[3].

For formation of copper complexes, 1 mg of peptide was dissolved in 1 ml of 50 mM borate buffer (pH 8.0) and 50 mg of basic copper(II) carbonate were added. After shaking for 30 min at room temperature, the mixture was centrifuged, the clear liquid containing the copper complex was aspirated off and the pH was re-adjusted to 8.0 by using a glass electrode and sodium hydroxide solution or acetic acid in a microburette**. This solution (10–20 μl) was applied on to the thin-layer. A solution containing 2.5 μmole/ml of each amino acid was treated in a similar manner and applied as a reference.

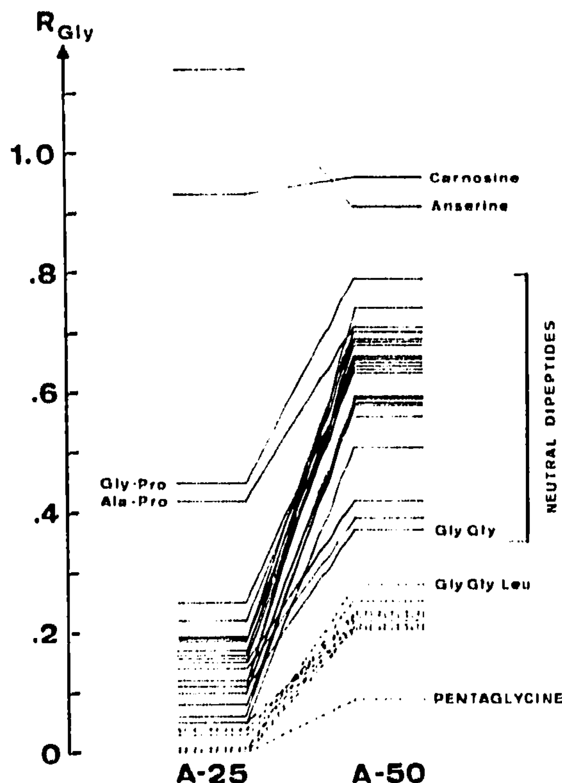


Fig. 1. Chromatographic behaviour of copper complexes of neutral peptides on DEAE-Sephadex A-25 and A-50 in 50 mM sodium borate buffer (pH 8.0) (data from Table I). On DEAE-Sephadex A-25, separation of peptides from amino acids occurs. On DEAE-Sephadex A-50, fractionation into classes of neutral dipeptides and neutral oligopeptides is possible. R_{Gly} = relative mobility of Cu (glycine)$_2$.

TLC data for the copper–peptide complexes, relative to the migration velocity of the neutral copper–amino acid complexes, are compiled in Table I and Fig. 1. These data should be regarded as indicative values only. However, the possibility of separating compounds into classes of neutral amino acids, dipeptides and oligopeptides by two-step chromatography is obvious from Fig. 1: all the neutral peptides investigated were retained as copper complexes on DEAE-Sephadex A-25 and were separated from the neutral (and basic) amino acids which ran through. The peptide fraction obtained in this way can be separated further on DEAE-Sephadex A-50

* C. Desaga GmbH, Heidelberg, G.F.R.
** Type E 457, Metrohm AG, Herisau, Switzerland.

TABLE I

hR_F VALUES OF COPPER–PEPTIDE COMPLEXES RELATED TO NEUTRAL COPPER–AMINO ACID COMPLEXES ON WEAKLY BASIC ION-EXCHANGERS IN 50 mM SODIUM BORATE BUFFER (pH 8.0)

Flow-through TLC technique[3] in BN-chamber at room temperature. hR_F values are means of three determinations.

Compound	*TEAE-Cellulose*	*DEAE-Sephadex A-25*	*DEAE-Sephadex A-50*
Amino acids, neutral, aliphatic	100	100	100
Dipeptides, acidic			
Ala–Asp	13	3	16
Gly–Asp	10	6	18
Gly–Glu	9	3	10
Dipeptides, basic			
γ-Abu–His (homocarnosine)		95	76
Ala–His	37	11	74
β-Ala–His (carnosine)	90	93	96
β-Ala–1-Mehis (anserine)		114	91
Gly–His	56	25	64
Gly–Lys		150	108
Dipeptides, neutral			
Ala–Gly	41	12	68
Ala–Ile	61	16	69
Ala–Pro	87	42	71
Ala–Ser	46	15	64
Ala–Thr	53	17	63
Ala–Tyr	21	8	56
Gly–Gly	28	16	37
Gly–Pro	80	45	79
Gly–Ser	38	14	39
Gly–Trp	15	5	51
Gly–Val	32	15	42
4-Hyp–Gly	45	19	70
Leu–Tyr	20	6	58
Met–Gly	43	14	58
Phe–Phe	22	10	59
Pro–Ala	56	22	65
Pro–Gly	54	19	65
Pro–Leu	50	19	63
Pro–Phe	36	17	68
Pro–Val	50	17	66
Tripeptides, neutral			
Gly–Gly–Gly	13	0	22
Gly–Gly–Leu	14	1	28
Gly–Gly–Pro	47	13	33
Gly–Leu–Tyr	9	0.5	25
Gly–Phe–Phe	11	4	21
Leu–Gly–Gly	13	4	23
Leu–Gly–Leu	18	5	22
Tyr–Gly–Gly	9	3	21
Val–Gly–Gly	11	4	23
Tetra- and pentapeptides			
Gly–Gly–Gly–Gly	9	0	21
Gly–Gly–Gly–Gly–Gly	3	0	9

into the neutral dipeptide fraction and the slow-moving oligopeptide fraction. The investigated neutral di- and tripeptides do not overlap.

Column chromatography

On the basis of the above results, we modified our procedure for the separation of peptides from amino acids[1] in order to isolate dipeptides from urine preparatively (Fig. 2). The replacement of the TEAE-cellulose used previously[1] by DEAE-Sephadex A-25 causes a long tailing of the amino acid peak. This was demonstrated particularly well when the absorption of the copper complexes was recorded in the UV region (250–280 nm), where it is much higher than in the visible region at 580 nm. It is therefore necessary to increase the rinsing volume by a factor of about 1.6 (from the start to the end of the amino acid fraction). On the other hand, the peptides are also bound more strongly. Many peptides cannot be eluted with 0.2 *M* acetic acid from DEAE-Sephadex A-25, an eluent which is adequate for TEAE-cellulose, and 2 *M* formic acid has to be used.

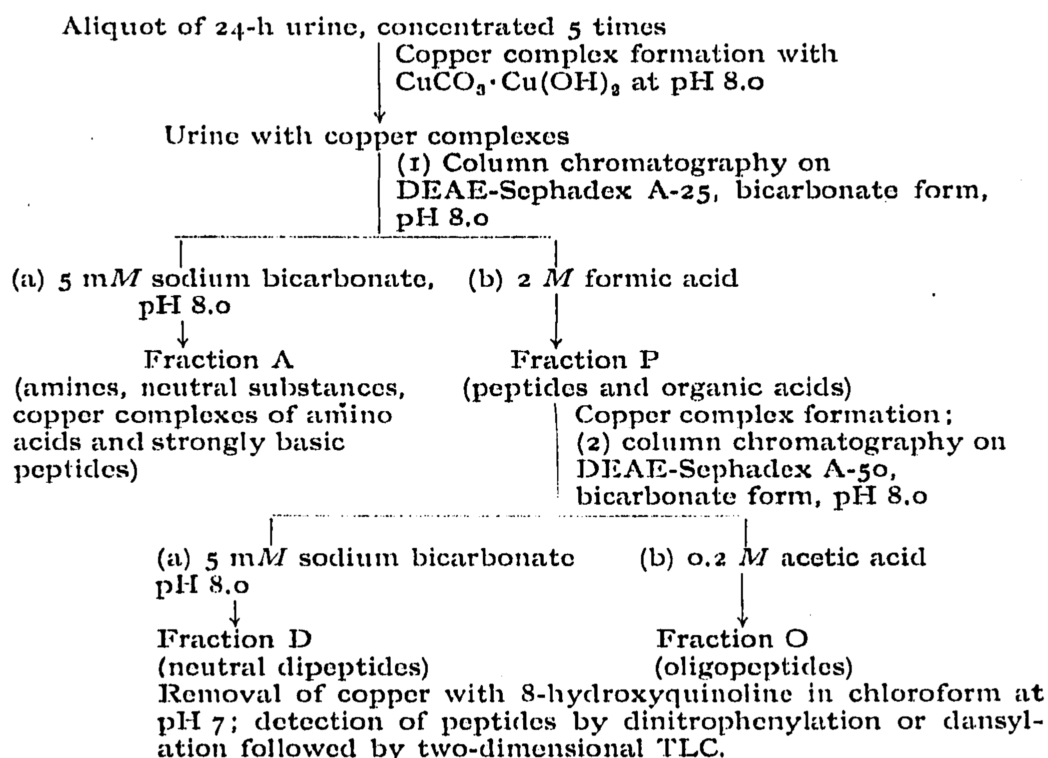


Fig. 2. Procedure for the fractionation of urine. For isolation of neutral dipeptides and oligopeptides the sample must first be separated by electrophoresis at pH 6 and the neutral fraction processed as described above.

To avoid excess salt in the peptide fraction, which would seriously disturb the second chromatographic step, the borate buffer was replaced by sodium bicarbonate buffer and the concentration was lowered to 5 m*M*. The ion exchanger in the bicarbonate–carbonate form naturally releases carbon dioxide gas when it is rinsed with 2 *M* formic acid. However, this does not affect the recovery of the peptides,

provided the column outlet is at the top. The elution is continued until all the gas is expelled from the column.

Because urine contains rather high and variable amounts of salt and urea, and in some diseases also high amino acid concentrations, it was necessary to study the effect of these substances on the new fractionation procedure. The test peptides for these studies were selected according to their behaviour in TLC (Table I).

Effect of salt

It can be expected that an increase of salt concentration in the sample solution will tend to increase the rate of migration of copper–peptide complexes on the ion exchanger, and will therefore cause a loss of peptides into the amino acid fraction A (Fig. 2). Therefore, the fastest moving dipeptides, glycylproline and glycylhistidine, were selected to check the influence of salt concentration on the separation of peptides from amino acids on DEAE-Sephadex A-25. Urea was included in the test solution at a concentration that simulates five-times concentrated urine. As shown in Table II, a concentration of sodium chloride of up to 6% in the sample solution does not

TABLE II

EFFECT OF SALT IN THE SAMPLE

DEAE-Sephadex A-25, bicarbonate form (pH 8.0), gel bed 2.5 × 35 cm. Sample: 10 ml of 5 m*M* sodium bicarbonate buffer (pH 8.0) containing copper complexes of 40 mg of glycine, 20 mg of Gly–His, 20 mg of Gly–Pro, 20 mg of Leu–Gly–Gly, 2 g of urea, and 0, 0.6 and 1 g of sodium chloride. Elution with 440 ml of 5 m*M* sodium bicarbonate buffer (pH 8.0) (fraction A), followed by 360 ml of 2 *M* formic acid (fraction P). Flow-rate 120 ml/h. Absorption recorded at 280 nm.

NaCl in sample (% w/v)	*Substance*	*Recovery (%)*[a]	
		In fraction A "amino acids"	*In fraction P "peptides"*
0	Gly	90	0.7
	Gly–His	0	74
	Gly–Pro	0	81
	Leu–Gly–Gly	0	61
6	Gly	81	0.7
	Gly–His	0	100
	Gly–Pro	0	100
	Leu–Gly–Gly	0	83
10	Gly	72	0.6
	Gly–His	72	18
	Gly–Pro	2	98
	Leu–Gly–Gly	0	77

[a] Analysis by automatic ion-exchange chromatography.

affect the separation. With higher salt concentrations (10%), the dipeptides run into the amino acid fraction to an extent depending on the nature of the dipeptide. It is noteworthy that glycylhistidine is displaced to a much higher extent than glycylproline, although on TLC (lower salt concentration, favourable adsorbent:adsorbate ratio) glycylhistidine moves more slowly than glycylproline. It must be emphasized that the loss of resolution is obviously caused by the amount and not by the concentration of salt: practically the same chromatographic picture (not

shown) was obtained when 10 ml of 6% or 100 ml of 0.6% sodium chloride solution were applied.

Effect of urea

Urea does not influence the chromatographic behaviour of copper–peptide complexes on DEAE-Sephadex A-25 at pH 8.0 at concentrations of up to 6 *M* in the elution buffer. However, a very high concentration (12 *M*) of urea is able to displace the peptides from the resin. As such high concentrations are not attained in our procedure, the influence of urea can be neglected. Urea is not retained on Sephadex A-25 and is found in the amino acid fraction A. Its influence on DEAE-Sephadex A-50 was therefore not tested.

The results outlined above were obtained by TLC experiments. Layers of DEAE-Sephadex A-25 were equilibrated with 50 m*M* sodium borate buffer (pH 8.0) containing urea at concentrations of 0, 18, 36 and 72% w/v. Copper complexes of glycine, triglycine, tetraglycine, pentaglycine, alanylaspartic acid, alanylglycine and alanylisoleucine were applied.

Effect of amino acid concentration

The influence of increasing loads of amino acids on the separation of peptides from amino acids was studied on DEAE-Sephadex A-25. Unfortunately, these investigations were carried out at pH 9.0, at which pH the tailing of the amino acid peak is very pronounced and a few per cent of the amino acids run into the peptide fraction P (Table III). At pH 8.0, usually only traces of neutral amino acids are found in the peptide fraction (with the exception of tyrosine and tryptophan, see below). Nevertheless, the data in Table III demonstrate that the amino acids in concentrations to be expected in human urine do not influence the separation of peptides from amino acids.

TABLE III

EFFECT OF LOAD OF AMINO ACIDS

DEAE-Sephadex A-25, bicarbonate form (pH 9.0), gel bed 2.5 × 35 cm. Sample: 10 ml of 5.0 m*M* sodium bicarbonate buffer (pH 9.0) containing 20 mg of Gly–His, 20 mg of Gly–Pro, 20 mg of Gly–Gly–Leu, 0.6 g of sodium chloride, 2 g of urea and various amounts of amino acids. Elution with 440 ml of 5.0 m*M* sodium bicarbonate buffer (pH 9.0) (fraction A), followed by 360 ml of 2 *M* formic acid (fraction P). Flow-rate 120 ml/h. Extinction recorded at 280 nm. Analysis of fractions A and P by automatic ion-exchange chromatography.

Load of amino acids (mg)		*Peptides in fraction A (%)*	*Amino acids in fraction P*	
Ala	*Gly*		*%*	*mg*
0	50	0	2.3	1.15
0	100	0	2.9	2.9
100	100	0	0.6	1.2

Recovery and distribution of amino acids

Regarding the great differences in the chemical structures of the amino acids, one may expect that some of the amino acids will run partially into the "dipeptide" and "oligopeptide" fractions. Therefore, we checked the behaviour of the amino

TABLE IV

RECOVERY OF AMINO ACIDS IN THE FRACTIONS OBTAINED BY THE STANDARD PROCEDURE (FIG. 2) AFTER COPPER COMPLEX FORMATION, SEPARATION ON DEAE-SEPHADEX A-25 AND A-50 AT pH 8.0, AND REMOVAL OF COPPER

Sample: 10 ml, pH 8.0, containing 3.5 m*M* of each amino acid, 0.6 g of sodium chloride and 2 g of urea.

Amino acid	*Fraction*			*Total recovery (%)*
	A "*amino acids*"	*D* "*dipeptides*"	*O* "*oligopeptides*"	
Alanine	87	0.3	0.3	87.6
Arginine	97	< 0.3	< 0.3	97
Asparagine	86	< 0.3	< 0.3	86
Aspartic acid	7	0.5	43	50.5
Cystine	18	< 0.3	< 0.3	18
Glutamic acid	10	0.9	54	64.9
Glycine	96	0.9	0.8	97.7
Histidine	87	13	1.9	101.9
Hydroxyproline	80	< 0.5	< 0.5	80
Isoleucine	84	0.3	0.3	84.6
Leucine	90	< 0.2	< 0.2	90
Lysine	95	< 0.3	< 0.3	95
Methionine	84	< 0.2	< 0.2	84
1-Methylhistidine	77	3.0	0.9	80.9
Phenylalanine	92	0.4	0.3	92.7
Proline	92	< 0.5	< 0.5	92
Serine	85	0.9	1.0	87
Taurine	0	77	< 0.2	77
Threonine	86	0.3	0.2	86.5
Tyrosine	72	9.5	0.5	82
Valine	84	0.3	0.3	84.6

acids in the standard procedure by using a test mixture and analysing the different fractions by automatic ion-exchange chromatography. The results are shown in Table IV.

The dipeptide fraction D contains 13% of the applied amount of histidine, 3% of the methylhistidine and 9% of the tyrosine, presumably because of "aromatic adsorption" on DEAE-Sephadex A-25. Moreover, all the taurine is found in the "dipeptide" fraction. Taurine, bearing a sulphonic acid group instead of a carboxylic group, behaves here in an intermediate manner between a neutral and an acidic amino acid.

The "oligopeptide" fraction contains only traces of the neutral and basic amino acids, but a large proportion of the acidic amino acids. The total recovery is between 80 and 100% with the exception of cystine (18%) and the acidic amino acids (50–64%). Similar results were obtained by using our previous procedure[1]. The reason for the low recovery of cystine is still unknown.

Recovery and distribution of peptides

Similarly, the recovery of peptides was checked by fractionation of di- and tri-peptides by using the standard procedure and analysing the fractions by automatic ion-exchange chromatography (Table V). As the peptides were determined quantitatively with ninhydrin without prior hydrolysis, the rather low colour response affected the precision (see also Table II).

TABLE V

RECOVERY OF PEPTIDES IN THE FRACTIONS OBTAINED BY THE STANDARD PROCEDURE (FIG. 2) AFTER COPPER COMPLEX FORMATION, SEPARATION ON DEAE-SEPHADEX A-25 AND A-50 AT pH 8.0, AND REMOVAL OF COPPER

Sample: 10 ml, pH 8.0, containing 60 mg of glycine, 20 mg of Gly–Gly, 20 mg of Gly–Pro, 20 mg of Gly–Gly–Leu, 0.6 g of sodium chloride and 2 g of urea. Recoveries are means of four experiments in each instance.

Peptide	*Fraction*			*Total recovery (%)*
	A "*amino acids*"	*D* "*dipeptides*"	*O* "*oligopeptides*"	
Gly–Gly	0	96	4	100
Gly–Pro	0	90	< 0.6	90
Gly–Gly–Leu	0	0	87	87

The tailing of the dipeptide band on the DEAE-Sephadex A-50 column causes slow-moving dipeptides (*e.g.* glycylglycine, see Fig. 1) to run into the oligopeptide fraction to a small extent (4%). On the other hand, there was no trace of the fastest moving tripeptide detectable in the dipeptide fraction D.

The total recovery of the peptides was between 70 and 100%, which is satisfactory. By analogy with the results with amino acids (Table IV), however, one may expect high losses of cystinylpeptides (not tested).

PROCEDURE

This involves five-times concentration, ultrafiltration and copper complex formation of an aliquot of 24-h urine (see previous communication[1]). The following procedure can be scaled up proportionally for preparative purposes.

Column chromatography on DEAE-Sephadex A-25

For preparation and equilibration of the column see EXPERIMENTAL and RESULTS. The clear copper complex solution (10 ml; pH 8.0) is applied by aspirating it through the flow adapter and the elution is started with 5 m*M* sodium bicarbonate buffer (pH 8.0). The flow-rate is 120 ml/h and the extinction is recorded at 280 nm. The first 90 ml of the effluent are discarded and the next 350 ml are collected (fraction A). With the column outlet at the top, the elution buffer is then changed to 2 *M* formic acid using the same flow-rate, and elution is continued until all the air is expelled from the column (360 ml, fraction P). The extinction can be recorded further using a de-bubbler at the flow-cell inlet. An aliquot of fraction A is adjusted to pH 7 for the extraction of copper (see below).

Copper-complex formation of fraction P

Fraction P is evaporated to remove the formic acid. The residue is dissolved in about 5 ml of distilled water, the pH of the solution is adjusted to 8.0 using 4 *N* sodium hydroxide solution in a microburette and a glass electrode, the volume is made up to 10 ml with water and the solution is transferred to a 20-ml ground-glass tube which fits into the centrifuge. About 400 mg of basic copper(II) carbonate is

added and the stoppered vessel is shaken at 40° for 20 min. After centrifugation at 4000 r.p.m. for 5 min, the liquid is ready for the second chromatography.

Column chromatography on DEAE-Sephadex A-50

The clear copper complex solution of fraction P (9 ml), re-adjusted to pH 8.0 if necessary, is aspirated through the flow adapter. Elution is started with 5 m*M* sodium bicarbonate buffer (pH 8.0) at a flow-rate of 120 ml/h. The extinction is recorded at 280 nm. The first 100 ml of the effluent are discarded and the next 380 ml are collected (fraction D) and the volume is reduced to about 20 ml by evaporation *in vacuo*. After collection of fraction D, the column content is transferred completely into a beaker and the suspension is acidified with 0.2 *M* acetic acid to pH 3–4. The elution of the oligopeptides is possible by either of the following procedures, which will give similar results; however, the second is faster.

(1) The suspension is loaded again into the chromatographic column and the effluent is collected quantitatively. The chromatographic bed is further rinsed with 340 ml of 0.2 *M* acetic acid (120 ml/h).

(2) The suspension is filtered under suction through a funnel equipped with a porous glass disk and further rinsed, with stirring, with several portions of a total of 340 ml of 0.2 *M* acetic acid.

The extracts (effluents or filtrates) are combined, evaporated to dryness *in vacuo*, and the residue is dissolved in about 20 ml of distilled water (fraction O).

Removal of copper

The pHs of the fractions are adjusted to 7 by adding sodium hydroxide solution or acetic acid, and the solutions are extracted with 20-ml portions of 0.6% w/v 8-hydroxyquinoline in chloroform until the yellow colour disappears. The 8-hydroxyquinoline dissolved in the aqueous phase is then extracted three times with pure chloroform.

Dinitrophenylation and two-dimensional TLC

Details have been given earlier[1]. An excess of 10% of solution is applied to the thin layer in order to compensate for the loss after the copper complex formation of fraction P (see above).

Example

An example of the TLC pattern of fractions D and O from urine (as the dinitrophenyl derivatives) is shown in Fig. 3. Using the same copper complex technique, but on a preparative scale, dipeptides have been isolated from fraction D and characterized by automatic ion-exchange chromatography after hydrolysis, and by mass spectrometry; these results will be described elsewhere.

DISCUSSION

The observed difference in the chromatographic behaviour of the copper complexes of amino acids, dipeptides and oligopeptides poses the question of the structures of the copper complexes at pH 8.0. Based on X-ray crystallographic analysis, excellent reviews on the structure of copper complexes have been published by FREEMAN[4,5].

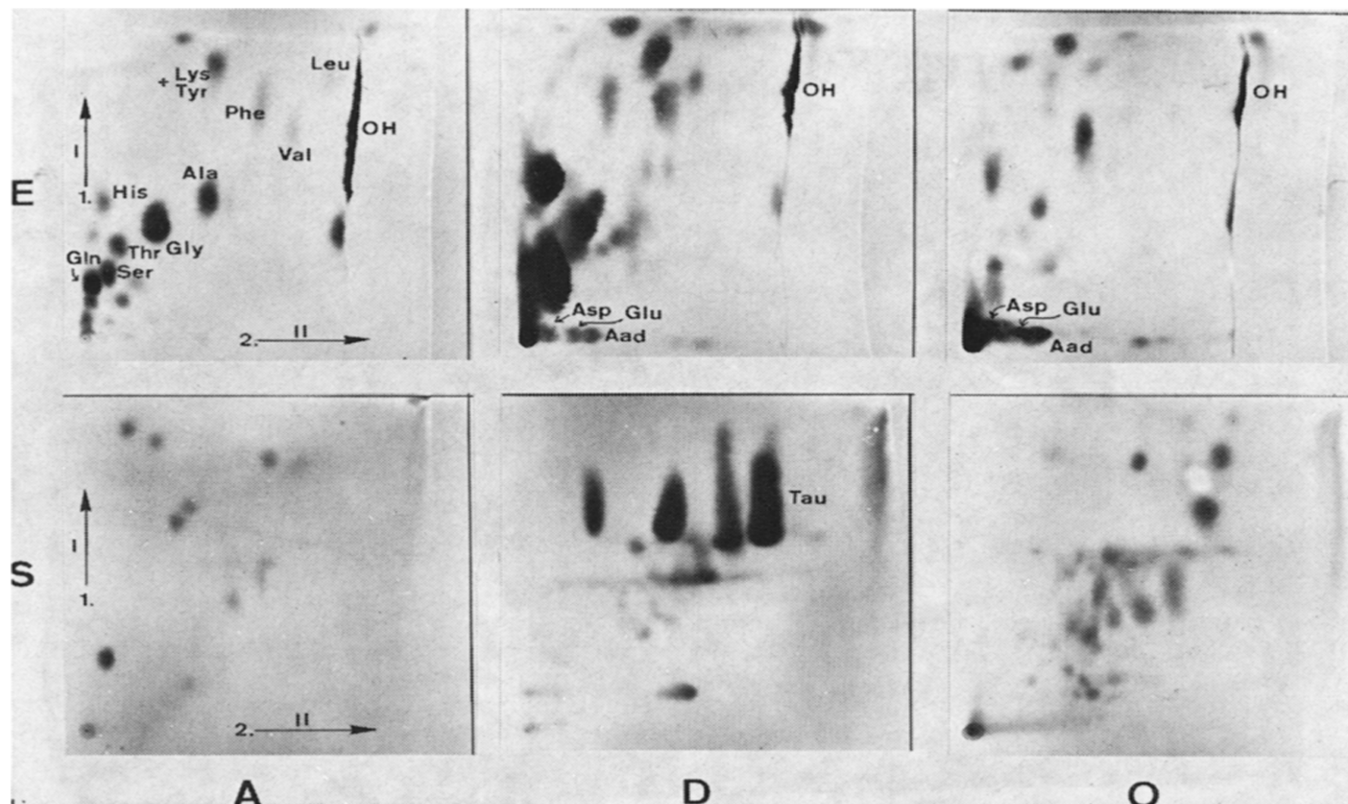


Fig. 3. Amino acid fraction (A), "dipeptides" (D) and "oligopeptides" (O) from normal human urine (11-year-old male). Ether-soluble (E) and acid-soluble (S) dinitrophenyl derivatives equivalent to 0.1% (fraction A) and 2.5% (fractions D and O), respectively, of 24-h urine separated by two-dimensional TLC[1]. The acidic amino acids aspartic acid, glutamic acid and α-aminoadipic acid (Aad) run into fraction O, but traces can also be detected in fraction D. Taurine is found in fraction D.

Simple amino acids are known to form electro-neutral 2:1 chelate complexes with copper(II) at about pH 7. Both ligands chelate strongly through the α-amino nitrogen atom and one oxygen atom of the carboxyl group to form an approximately planar square around the copper atom. Geometrical isomerism occurs (IR and Laser–Raman spectroscopy[6,7]) and it seems that the *cis*-isomer is the more stable species, at least in crystals. The environment of the copper atom is usually tetragonally distorted octahedral, with two oxygen atoms more weakly bound along the normal to the square of the four closest ligand atoms. In crystals, these oxygen atoms belong to a carboxyl group of the neighbouring complex and/or water (Fig. 4a); in solution these positions are filled by two water molecules.

In peptides, complex formation (in neutral and alkaline media) obviously starts with the binding of the N-terminal amino group. The next three peptide nitrogens can then be bound successively in the coordination square. Characteristically the protons from the peptide nitrogen atoms dissociate at higher pH values (see below), which leads to negatively charged complexes of tripeptides and higher peptides. The tendency of the copper to bind further ligands decreases: whereas the coordination number in the copper complex of the crystals of amino acids is six, it is only five in dipeptides[8] and four in the pentapeptide complex disodium pentaglycinatocuprate(II) 4,5-hydrate[9] (Fig. 4).

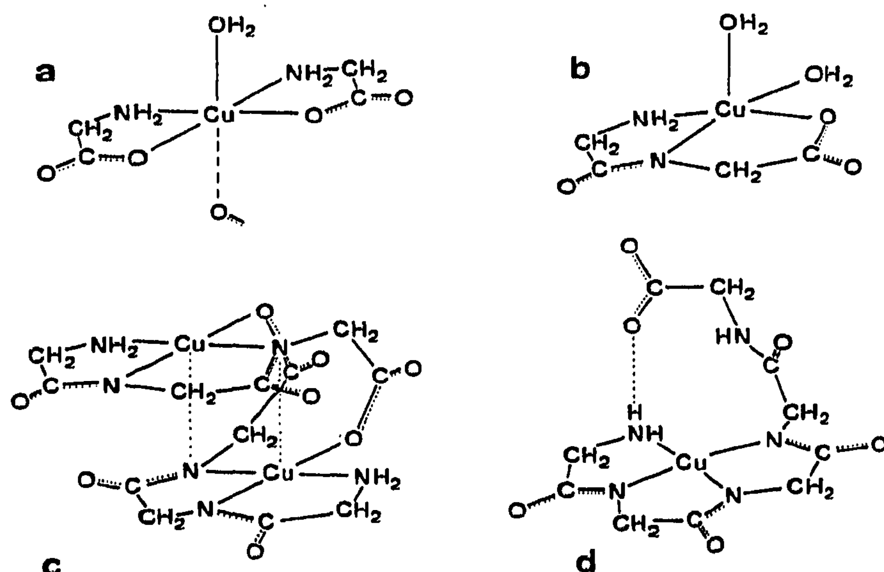


Fig. 4. Structures of copper complexes of amino acids and peptides, based on X-ray analyses[4]. a, Bis(glycinato)copper(II) hydrate, *cis*; b, glycylglycinatocopper(II) trihydrate[8]; c, sodium glycylglycylglycinatocuprate(II) hydrate[18], and d; disodium glycylglycylglycylglycylglycinatocuprate(II) 4.5-hydrate[9]. Sodium ions and hydrate molecules outside the copper coordination sphere have been omitted. Note the decreasing coordination number and the planar structure of the N-terminal peptide residue.

Histidine is able to chelate strongly with the imidazole-1 nitrogen[10] and, in dimers, with the imidazole-3 nitrogen[11,12]. For steric reasons, however, only peptide bonds on the side of its amino group can be involved in the complexes of histidyl-peptides[13]. The participation of the lysyl side-chain (vasopressin)[14] and the hydroxyl group in tyrosine[15,16] does not seem to occur in general; there is evidence, however, for the participation of tyrosine in transferrins[17]. Also, the hydroxyl group in threonine does not chelate[10].

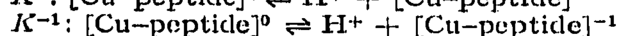
Unfortunately, however, the picture of the copper complexes is complicated by the possibility of the formation of polynuclear complexes. For example, dimers have been isolated of β-alanylhistidinatocopper(II) dihydrate[11] and sodium glycylglycylglycinatocuprate(II) hydrate[18] (Fig. 4c). Peptide–copper–peptide chains can be recognized in the crystals of bisglutamatocopper(II) dihydrate[19] and in glycylhistidinatocopper(II) sesquihydrate[20]. Cystinylglycylglycine binds two atoms of copper(II) per peptide molecule and it has been shown that a copper–copper interaction occurs in the binuclear complex[21]. It could be that the low recovery of aspartic acid, glutamic acid and cystine (Table IV) is due to the formation of polynuclear complexes of these amino acids.

The net charge of the copper complexes at pH 8.0 is most important for the separation described in this paper. At this pH, the complexes of neutral amino acids are electro-neutral. The same is true also for most of the simple dipeptides. However, dissociation of a proton occurs from the water molecule positioned within the coordination square of dipeptides (Fig. 4b) at a higher pH. From the data in Table VI it can be seen that this occurs at pK 9.1–9.6. Hence, at a pH 8.0, about 5% of the molecules bear a negative charge. As the dissociation of the proton from the second peptide bond occurs at p$K \approx 7$, more than 90% of the molecules of copper complexes

TABLE VI

pK VALUES OF COPPER–PEPTIDE COMPLEXES

K^0: $[\text{Cu–peptide}]^+ \rightleftharpoons H^+ + [\text{Cu–peptide}]^0$


K^{-1}: $[\text{Cu–peptide}]^0 \rightleftharpoons H^+ + [\text{Cu–peptide}]^{-1}$

K^{-2}: $[\text{Cu–peptide}]^{-1} \rightleftharpoons H^+ + [\text{Cu–peptide}]^{-2}$

No differentiation was made according to the nature of the negative charge (OH^- or de-protonated peptide-bond nitrogen).

Peptide	*pK^0*	*pK^{-1}*	*pK^{-2}*	*Reference*
Ala–Gly	4.28			22
	4.16			22
Gly–Ala	4.03			22
Gly–dehydroAla	3.45	9.4		23
Gly–dehydroPhe	4.31	9.6		23
N,N-dimethylGly–dehydroPhe	3.14	9.3		23
Gly–Gly	3.90	9.37	12.2	24
	4.25			22
	4.79			25
Gly–Val	4.75	9.30	12.0	24
	4.85			22
Pro–Gly	3.95	9.34		24
Sar–dehydroAla	3.36	9.3		23
Sar–dehydroPhe	4.06	9.4		23
Sar–Gly	3.45	9.19	11.9	24
Val–Gly	3.85	9.13	11.8	24
Gly–Gly–Gly	5.10	6.89	11.9	26
	5.4	6.63	10.9	27
Gly–Gly–Sar	5.2	9.3	12	26
Ala–Gly–Gly–Gly	5.86	6.98	9.26	28
Gly–Gly–Gly–Gly	5.45	6.91	9.23	26
	5.85	6.95	9.30	28
	5.6	6.77	9.0	27
Ala–Gly–Gly–Gly–Gly	6.00	7.00	8.21	28
Gly–Gly–Gly–Gly–Ala	6.01	7.05	8.25	28
Gly–Gly–Gly–Gly–Gly	6.10	7.00	8.14	28
Val–Leu–Ser–Gln–Gly	5.98	6.98	8.13	28
Val–Leu–Ser–Glu–Gly	6.00	7.18	8.20	28

TABLE VII

PERCENTAGE OF COPPER–AMINO ACID AND COPPER–PEPTIDE COMPLEX MOLECULES BEARING AN ELECTRICAL CHARGE AT pH 8.0, ESTIMATED ACCORDING TO THE pK VALUES OF TABLE VI

The estimation is based on the following mean pK values: dipeptides: pK^{-1} = 9.3; tripeptides: pK^{-1} = 6.8; tetrapeptides: pK^{-1} = 6.95, pK^{-2} = 9.3; pentapeptides, pK^{-1} = 7.00, pK^{-2} = 8.2.

Type of compound	*Number of electrical charges*			*Mean of electrical charge*
	0	*−1*	*−2*	
Neutral amino acids	100	0	0	0
Neutral dipeptides	95	5	0	−0.05
Neutral tripeptides	6	94	0	−0.94
Neutral tetrapeptides	7	88	5	−0.98
Neutral pentapeptides	8	52	40	−1.32

of neutral tri- and tetra-peptides bear one negative charge at pH 8.0. In pentapeptides, the proton of the third peptide bond dissociates at p$K \approx 8.2$ (Table VI) and an appreciable percentage of the complex molecules bear two negative charges. From such estimations (Table VII), it can be concluded that a separation process according to the net electrical charge (ion-exchange chromatography, electrophoresis) separates the copper complexes of neutral amino acids and peptides into the three classes: amino acids + dipeptides; tri- and tetra-peptides; and pentapeptides (+ higher oligopeptides). Peptides containing one proline, hydroxyproline or N-methylamino acid (sarcosine) in positions 2–4 should be eluted in the next lower class as the peptide groups of secondary amines do not contain a dissociable proton. On the other hand, complexes of peptides containing an acidic amino acid bear an excess negative charge and should be eluted in the higher peptide fraction.

These estimations correspond qualitatively with the separations observed on the ion exchangers DEAE-Sephadex A-50 and TEAE-cellulose, as shown in Fig. 1 and Table I. Tripeptides are separated from dipeptides, and it seems to be possible that tetrapeptides run together with the tripeptides and are separated from pentapeptides. Unfortunately, only one example each of tetra- and penta-peptides could be included in these studies. However, it is difficult to understand why the dipeptides should be retained to such an extent, which is still more pronounced on DEAE-Sephadex A-25. At least glycylproline and alanylproline, which are unable to dissociate a peptide proton, should theoretically run into the amino acid fraction; in practice they run faster than the other dipeptides, but much slower than the amino acids on DEAE-Sephadex A-25 (Fig. 1).

An explanation of this unexpected behaviour of the dipeptides would be that free amino groups of the ion exchanger coordinate temporarily with the copper–dipeptide complexes and, in this way, bind the complexes to the resin as long as this

TABLE VIII

K_{av} VALUES OF COPPER COMPLEXES AT pH 8.0 AND ANION-EXCHANGE CAPACITY OF DEAE-SEPHADEX A-25 AND A-50

$K_{av} = \frac{V_e - V_0}{V_t - V_0}$, where V_e, V_0 and V_t are the elution volumes of the copper complex, starch (amylum solubile, Merck) and glucose, respectively.

Compound	*DEAE-Sephadex A-25, 5 mM sodium bicarbonate (pH 8.0)*	*DEAE-Sephadex A-50, 10 mM borate (pH 8.0)*
Triethylenetetramine	0.07	0.08
Alanine	1.5	0.9
Gly–Pro	12	1.6
Gly–Gly	18	2.1
Gly–Gly–Leu	70	6.1
Capacity (milliequiv. per ml of swelled resin)	3.1[a]	0.32[a]

[a] The chloride form of the anion exchanger was swelled in distilled water and loaded in a column to give a gel bed volume of 7.0 ml. After determination of V_t, the column was washed with excess of 0.1 N sodium hydroxide solution and an aliquot of the total effluent was titrated with 0.1 N hydrochloric acid. In distilled water, 1 g of DEAE-Sephadex A-25 and A-50 swelled to a volume of 7.0 and 105 ml, respectively.

complexing exists. It is well known that the apical position in octahedral or tetragonal pyramidal coordination can be exchanged rapidly. Partitipation of the exchanger amine in the apical position only would decrease the mobility of the copper–amino acid complexes also, and could not explain the observed difference in the chromatographic properties of amino acids and dipeptides. It may be noteworthy, in this context, that the copper–amino acid complexes move more slowly than expected on the DEAE-Sephadex ion exchangers (Table VIII), but this fact could also be caused by non-specific adsorption. In the dipeptide complexes, however, a free amino group within the coordination sphere of the copper would also be able to replace the strongly bound water molecule in the coordination square and would then be able to bind the complex to the resin for a much longer time.

The differences in chromatographic mobilities on the three ion exchangers (Table I) can be explained by their different exchange capacities (Table VIII). DEAE-Sephadex A-25 possesses a capacity per unit volume of the swollen exchanger about ten times greater than that of DEAE-Sephadex A-50. On TEAE-cellulose, a particularly high "aromatic adsorption" is observed, which slows down the mobility of peptides containing tyrosine, phenylalanine, histidine (and tryptophan), in comparison with DEAE-Sephadex A-50 (Table I).

In order to fully exploit the possibilities of the separation principle described here, the sample should be fractionated by electrophoresis at pH 6 prior to the formation of copper complexes, and only the neutral fraction thus obtained separated as copper complexes. As we were interested primarily in a screening procedure, we have omitted this electrophoretic step in our procedure.

It seems to be possible to modify the procedure described here by stepwise or gradient elution of the different substance classes from one single DEAE-Sephadex A-25 column with buffers of decreasing pH. We were anxious to do this because it is known that the stability of the copper–peptide complexes decreases remarkably at pH values below 7.

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REFERENCES

1 A. NIEDERWIESER AND H.-CH. CURTIUS, *J. Chromatogr.*, 51 (1970) 491.
2 D. K. J. TOMMEL, J. F. G. VLIEGENTHART, T. J. PENDERS AND J. F. ARENS, *Biochem. J.*, 107 (1968) 335; and 99 (1966) 48P.
3 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
4 H. C. FREEMAN, *Adv. Protein Chem.*, 22 (1967) 257.
5 H. C. FREEMAN, in P. AISEN, W. BLUMBERG AND J. PEISACH (Editors), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 77.
6 A. W. HERLINGER, S. L. WENHOLD AND TH. VEACH LONG II, *J. Amer. Chem. Soc.*, 92 (1970) 6474.
7 A. W. HERLINGER AND TH. VEACH LONG II, *J. Amer. Chem. Soc.*, 92 (1970) 6481.
8 B. STRANDBERG, I. LINDQVIST AND R. ROSENSTEIN, *Z. Kristallogr.*, 116 (1961) 266.
9 J. F. BLOUNT, H. C. FREEMAN, R. V. HOLLAND AND G. H. MILBURN, *J. Biol. Chem.*, 245 (1970) 5177.

10 H. C. FREEMAN, J. M. GUSS, M J. HEALY, R.-P. MARTIN AND C. E. NOCKOLDS, *Chem. Commun.* (1969) 225.
11 H. C. FREEMAN AND J. T. SZYMANSKI, *Chem. Commun.*, 23 (1965) 598.
12 M. IHNAT AND R. BERSOHN, *Biochemistry*, 9 (1970) 4555.
13 G. F. BRYCE, R. W. ROESKE AND F. R. GURD, *J. Biol. Chem.*, 240 (1965) 3837.
14 B. J. CAMPBELL, F. S. CHU AND S. HUBBARD, *Biochemistry*, 2 (1963) 764.
15 E. BRESLOW, *Biochim. Biophys. Acta*, 53 (1961) 606.
16 D. C. GOULD AND H. S. MASON, *Biochemistry*, 6 (1967) 801.
17 S. K. KOMATSU AND R. E. FEENEY, *Biochemistry*, 6 (1967) 1136.
18 H. C. FREEMAN, J. C. SCHOONE AND J. G. SIME, *Acta Crystallogr.*, 18 (1965) 381.
19 C. M. GRAMACIOLLI AND R. E. MARSH, *Acta Crystallogr.*, 21 (1966) 594.
20 J. F. BLOUNT, K. A. FRASER, H. C. FREEMAN, J. T. SZYMANSKI, C. H. WANG AND F. R. GURD, *Chem. Commun.*, (1966) 23; J. F. BLOUNT, K. A. FRASER, H. C. FREEMAN, J. T. SZYMANSKI AND C. H. WANG, *Acta Crystallogr.*, 22 (1967) 396.
21 A. ZUBERBÜHLER AND H. S. MASON, cited in Ref. 16.
22 G. F. BRYCE, J. M. PINKERTON, L. K. STEINRAUF AND F. R. GURD, *J. Biol. Chem.*, 240 (1965) 3829.
23 B. J. CAMPBELL, Y.-CH. LIN AND E. BALLEW, *J. Biol. Chem.*, 242 (1967) 930.
24 W. L. KOLTUN, M. FRIED AND F. R. GURD, *J. Amer. Chem. Soc.*, 82 (1960) 233.
25 M. A. DORAN, S. CHABEREK AND A. E. MARTELL, *J. Amer. Chem. Soc.*, 86 (1964) 2129.
26 W. L. KOLTUN, R. H. ROTH AND F. R. GURD, *J. Biol. Chem.*, 238 (1963) 124.
27 M. K. KIM AND A. E. MARTELL, *J. Amer. Chem. Soc.*, 88 (1966) 914.
28 CH. R. HARTZELL AND F. R. GURD, *J. Biol. Chem.*, 244 (1969) 147.