Filter-Paper Ionophoresis of Cupric Complexes of Neutral Amino Acids and Oligopeptides

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We did these experiments in the hope of developing a general procedure for separating dipeptides from higher oligopeptides. Dobbie & Kermack (1955*a, b*; cf. Dobbie, Kermack & Lees, 1955) have given evidence that tripeptides form different types of complex with cupric ions from those formed by dipeptides. This could be the basis for an ionophoretic group separation. Our preliminary experiments were encouraging and we have now made a detailed comparison of the ionophoretic behaviour of the cupric complexes of glycine, glycyglycine and diglycyglycine with that predicted from the equilibrium constants given by Kermack and colleagues. The agreement is good. Triglycyglycine (whose equilibrium constants are not available in the literature) behaved similarly to the tripeptide under our conditions, so the method has possibilities for group separations of naturally occurring mixtures.

Kermack and colleagues used filter-paper ionophoresis for testing their theory of complex formation with glycyglycine, but not with diglycyglycine. Our results with the latter compound provide an independent line of evidence on the reaction of cupric ions with the tripeptide. Moreover, whereas their experiments were done with a limited amount of cupric ion migrating in a buffer containing the ligand at fixed concentration, we have studied the migration of a limited amount of ligand in a buffer solution saturated with cupric ion.

We gave a preliminary account of this work to the New Zealand Institute of Chemistry (Carnegie & Syngé, 1959) and at the Parkville Symposium on Proteins (cf. Thompson, 1959).

MATERIALS

Glycine and glycyglycine were obtained from British Drug Houses Ltd., Poole, Dorset. Diglycyglycine was from L. Light and Co. Ltd., Colnbrook, Bucks. Triglycyglycine was synthesized from diglycyglycine with *p*-nitrophenyl bromoacetate on the suggestion of Dr D. W.

Russell (see Addendum). The resulting bromoacetyl derivative was not characterized but aminated as for the corresponding chloro compound (Fischer, 1904). *N*-2:4-Dinitrophenylethanolamine was prepared according to James & Syngé (1951).

METHODS

The paper-electrophoresis apparatus of Kunkel & Tiselius (1951) was used. The glass plates (45 cm. × 20 cm.) were smeared lightly with Silicone Fluid 550 (Dow Corning Corp., Midland, Mich., U.S.A.). A solution of 0.026*M*-sodium tetraborate (10 g. of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}/\text{l.}$) and 0.0046*M*-sodium phosphate (1.7 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}/\text{l.}$) was used as the buffer (pH 9.2). A suspension of copper phosphate in 2% (w/v) sodium tetraborate was prepared according to Martin & Mittelmann (1948). Whatman 3MM paper was dipped into the buffer, which had been saturated with copper phosphate by the addition of copper phosphate suspension (4 ml./l.). After being lightly blotted, the paper was left for a few hours in the apparatus to equilibrate with buffer. No copper phosphate suspension was added to the buffer used for the runs with the free amino acids and peptides. A potential gradient of 7.5 v/cm. was applied for 4½ hr. The runs were made at room temperature with no special arrangements for temperature control. Solutions of the amino acids and peptides used were 0.05*M* in the buffer. Copper phosphate suspension (0.2 ml.) was added to 1 ml. of the above-described solutions to form the copper complexes. The excess of copper phosphate was allowed to settle. Each solution (20 µl.) was applied to the starting line after the equilibration period. A saturated solution of *N*-2:4-dinitrophenylethanolamine was used as a marker of electroendosmosis; this was not appreciably adsorbed on the paper and did not seem to form a complex with borate. After the run the papers were dried at 105°.

Spray reagents. A 0.2% solution of ninhydrin in water-saturated butanol, to which was added about 2% (v/v) of acetic acid, was used to show the amino acids and peptides. A 0.1% solution of rubeanic acid (dithio-oxamide, British Drug Houses Ltd.) in aqueous 90% (v/v) acetone was used to show the position of copper.

Solubility product of copper phosphate. A value for the solubility product of copper phosphate could not be found in the literature. An approximate value was determined by estimating the concentration of copper in the supernatant of a saturated solution of copper phosphate in the buffer after prolonged centrifuging. Copper phosphate suspension (5 ml.) was mixed with 30 ml. of the above-described buffer and centrifuged for 30 min. at 2000 rev./min. (500 g). The

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supernatant was discarded and the precipitate stirred with 40 ml. of buffer and centrifuged for 8 hr. in a covered tube at 2400 rev./min. (720 g). The copper content of the supernatant, determined by the method of Clare, Cunningham & Perrin (1945), was found to be $10^{-5.5}M$ (g. atom/l.). This is compatible with values calculated from the blank determinations of Martin & Mittelmann (1948), Blackburn & Robson (1953) and Borchers (1959), who used slightly different supernatants. Albert (1951) gives evidence that there is no interaction of cupric ions with borate.

RESULTS

Table 1 shows the mobilities on paper of the cupric complexes of glycine and its peptides and of the free ligands after correcting for electroendosmosis, which was in the direction of the cathode. No correction was made for any adsorption on the paper. Glycine had a slight anionic mobility at pH 9.2 but in the form of its cupric complex it had a slight cationic mobility. The copper-glycine spot had a sharp leading edge. The mobilities of the free tripeptide and the copper complex of the tripeptide are similar, but the mobility of the copper complex of the dipeptide is much less than the mobility of the free dipeptide. The copper complexes of glycine and glycylglycine were blue in colour and that of diglycylglycine was violet. The 0.05M-solution of triglycylglycine gave a blue copper complex. When this solution was diluted with buffer on the paper it gradually changed to a violet colour similar to that of the tripeptide. The blue complex moved anionically at a slower rate than the violet complex, which thus appeared first as a streak on the anode side of the blue spot. As the run proceeded all the blue complex changed to the violet form, which had a mobility similar to that of the tripeptide-copper complex. The blue and violet tetrapeptide complexes changed to a rose colour when the pH was raised by spraying with aqueous NaOH. A mixture of the copper complexes of these peptides and glycine separated into three groups. The diglycylglycine-copper and triglycylglycine-copper complexes did not separate.

From the dissociation constants given by Dobbie *et al.* (1955) and Dobbie & Kermack (1955*a, b*) the

proportions of the different molecular species expected to be present at pH 9.2 were calculated. The results are shown in Table 2. In other experiments, conducted with the same methods, similar behaviour was observed for the cupric complexes of leucine, leucylglycine and leucylglycylglycine, and for the cupric complexes of alanine and alanyl-alanine.

DISCUSSION

Our results for the mobilities of the cupric complexes of glycine, glycylglycine and diglycylglycine seem consistent with those that would be expected from the distribution of molecular species calculated from the equilibrium constants of Kermack and colleagues (Table 2), if one assumes instantaneous equilibration and the usual relationship between hydrodynamic drag and molecular bulk for each species of complex present. These constants are in agreement (so far as the present experiments are concerned) with the data of Datta & Rabin (1956*a*) (cf. Manyak, Murphy & Martell, 1955; Rabin, 1956; Datta & Rabin, 1956*b*; Weiss & Fallab, 1957; Murphy & Martell, 1957; Biester & Ruoff, 1959). Koltun & Gurd (1959) and Koltun, Fried & Gurd (1960) have also provided quantitative corroboration of the reaction scheme of Kermack and colleagues. Some of these workers doubted whether existing evidence justified Kermack and colleagues in postulating ionizing copper-(peptide)₂ complexes. However, the amounts of these formed under our conditions should in any case have been negligible.

As concerns the cupric (M) complexes of free glycine, calculations for increasing dilutions of ligand (L) show an increasing proportion of ML^+ which would migrate cationically; at still higher dilutions L^- and L^{+-} not in a complex, with anionic migration, would predominate. This serves to explain both the cationic migration of the spot and the sharpness of its leading edge. These changes in distribution of molecular species with decreasing concentration of total ligand also serve to explain divergences from stoichiometry when small amounts of free amino acids are determined under conditions resembling those of Pope & Stevens (1939). Mixtures of ML^+ , L^- and L^{+-} could here also bear a spurious resemblance to ML_2 in their stoichiometry. Divergences have been noted by a number of workers, especially at higher phosphate concentrations in the buffer used, which result in lower $[Cu^{2+} \text{ ion}]$ (Kober & Sugiura, 1913; Martin & Mittelmann, 1948; Woiwod, 1949; Schroeder, Kay & Mills, 1950; Blackburn & Robson, 1953; Boser, 1954; Spier & Pascher, 1954; Beauchene, Berneking, Schrenk, Mitchell & Silker, 1955; Baudet & Cherbuliez, 1955; Hagan, Ablondi & Hutchings, 1956; Sobel,

Table 1. *Ionophoretic mobilities on filter paper at pH 9.2*

For conditions see text. +, Anionic mobility; -, cationic mobility.

Ligand	$cm^2.v^{-1}.hr.^{-1}$	
	Mobility of cupric complex	Mobility of free ligand
Glycine	-0.03	+0.10
Glycylglycine	+0.24	+0.44
Diglycylglycine	+0.39	+0.39
Triglycylglycine	+0.39	+0.34

Table 2. *Calculated percentage distribution of ligand between different molecular species present at pH 9.2*

In all cases the proportion of L^+ is negligible. For glycylglycine and diglycylglycine at 0.0005M the proportions present as different complexes are closely similar to those at 0.05M except that the complexes involving 2 molecules of ligand (marked*) are present in even smaller proportions.

Ligand	Total concn. of ligand (M)	In the presence of Cu ²⁺ ion (10 ^{-5.5} M)								In the absence of Cu ²⁺ ion	
		L ⁻	L ⁺ -	ML ⁺	ML	ML ⁻	ML(OH) ⁻	ML ₂	ML ₂ ³⁻	L ⁻	L ⁺ -
Glycine	0.05	0.003	0.015	6.1	—	—	—	93.9	—	18	82
Glycine	0.0005	0.034	0.155	37	—	—	—	63	—	18	82
Glycylglycine	0.05	0.0004	—	0.0008	73.8	—	26.2	—	0.03*	87	13
Diglycylglycine	0.05	0.0001	0.00001	0.00006	0.6	99.4	—	—	0.00012* 0.0009*†	91	9

* Complex involving 2 molecules of ligand.

† Aggregate of symmetrical and asymmetrical species.

Henry, Chiamori & Segalove, 1957; Borchers, 1959). A systematic study of the dependence of these divergences on concentration, both for free amino acids and for some oligopeptides, has been made by Rauen, Leonhardi & Buchka (1949).

Our calculations show that with glycylglycine and diglycylglycine only 1:1 cupric complexes are to be expected in appreciable quantity and the proportions of the main species present should not be much affected by dilution. The formation of only 1:1 complexes precludes the existence of mixed complexes of one atom of copper with two different ligands, which would interfere with separations of mixtures and does in fact interfere with separations of free amino acids. We have satisfied ourselves about the absence of interaction of the cupric complexes of the peptides and interaction of those of free amino acids (cf. Synge, 1956), with the continuous-electrophoresis procedure of Grassmann & Hübner (1953) (see also Pope & Stevens, 1939; Bailey, Chibnall, Rees & Williams, 1943; Schroeder *et al.* 1950; Bennett, 1959). Evidence for formation of 1:1 complexes of copper with peptides under a wide variety of conditions has been presented by Kober & Sugiura (1912, 1913), Kober & Haw (1916), Kerkkonen (1948), Rauen *et al.* (1949), Woiwod (1949), Spies & Chambers (1951), Baudet & Cherbuliez (1955), Buděšinský (1957) and Borchers (1959). Rising, Parker & Gaston (1934), Wenaas (1937), Manyak *et al.* (1955), Buděšinský (1957) and Rosenberg (1957) have isolated in crystalline form copper-peptide complexes possessing this stoichiometry. Li & Doody (1954), however, interpreted their results as implying that glycylglycine forms only a 1:2 complex with Cu^{2+} ion. Abderhalden & Fodor (1912), Abderhalden & Schnitzler (1927) and Feldman (1937) isolated cupric complexes of various peptides of both the above types. Sauerwein (1937) isolated complexes with more than 1 atom of copper to 1 molecule of peptide. Plekhan (1951*a, b*) has obtained spectrometric evidence for the formation of mixed cupric complexes of glycylglycine with diglycylglycine and triglycylglycine at high pH values. Our own observations on triglycylglycine suggest that at pH 9.2 it may exist largely as a complex with more than 1 molecule of peptide/copper atom (blue) passing over on dilution to a violet 1:1 complex of higher anionic mobility. The spectrometric studies of Kober & Haw (1916) on the cupric complexes of a number of tetrapeptides at different pH values support this view. The observations of Dobbie & Kermack (1955*b*) on the behaviour of the tripeptide complexes at different concentrations and pH values suggest similar behaviour.

At pH 9.2 our procedure should be generally useful for separating neutral dipeptides from tri-

peptides and perhaps from higher peptides. Experience with leucylglycine and leucylglycylglycine supports this view. The separation of oligopeptides from free amino acids is scarcely favoured by the use of cupric complexes. The differences in mobility of the free compounds at pH 9.2 due to differences in the pK values of their amino groups are equally great (Conden, Gordon & Martin, 1946), and separation on anion-exchange resin at pH 8.6 is also a useful procedure (Carnegie, 1961). The colours of the cupric complexes, which are easily seen, are useful for following the course of fractionations. There should be no difficulty in regenerating peptides from their cupric complexes with hydrogen sulphide; that encountered by Tomita, Hamamura, Tamiya, Takehara & Tomita (1953) is perhaps explained by the polymorphism of glycylglycine (Bernal, 1931).

SUMMARY

1. The ionophoretic behaviour of glycine, glycyglycine, diglycylglycine and triglycylglycine has been studied at pH 9.2 in the presence and absence of a fixed concentration of free cupric ion.

2. The behaviour of the cupric complexes of the first three compounds mentioned is consistent with that to be expected from the schemes of reaction and equilibrium constants given by Dobbie & Kermack (1955*a, b*) and by Dobbie *et al.* (1955).

3. The observations made are discussed in relation to the literature on the cupric complexes of amino acids and peptides. They suggest the basis for a group separation of dipeptides from higher oligopeptides.

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ADDENDUM

Acylation with Esters of *p*-Nitrophenol

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The purpose of this Addendum is to draw attention to the potentialities of *p*-nitrophenyl esters as acylating agents for amino acids in aqueous solution.

Satisfactory acylation of diglycylglycine with *p*-nitrophenyl bromoacetate is reported in the paper above, after poor results had been obtained with chloroacetyl chloride. The technique used was one developed for peptide synthesis, in which acylation of free amino acids with *p*-nitrophenyl esters of benzyloxycarbonyl amino acids is carried out in unbuffered solution with a high initial pH (Elliott & Russell, 1957; Russell, 1959). Reaction in neutral solutions is much slower, and low yields have been reported (Bodánszky, 1957; Farrington, Hextall, Kenner & Turner, 1957).

Yields of chloroacetyl amino acids obtained by Schotten-Baumann or direct acylation with chloroacetyl chloride (Ronwin, 1953) are usually only moderate and sometimes poor. In such cases better results may be obtainable with a *p*-nitrophenyl haloacetate, as in the present instance. Esters of *p*-nitrophenol are stable, crystalline solids which are easy to prepare (Elliott & Russell, 1957; Bodánszky & du Vigneaud, 1959). As such they offer advantages over the more reactive acyl halides and should be considered as alternative reagents in cases where the acyl halides prove unsatisfactory.

MATERIALS AND METHODS

p-Nitrophenyl bromoacetate prepared from *p*-nitrophenol and bromoacetyl bromide (cf. Speroni, Losco & Peri, 1951) and recrystallized from cyclohexane, had m.p. 83–84° (corr.) [Found: *p*-nitrophenol released on hydrolysis (Schwyzer & Gorup, 1958), 53.5; $C_8H_6BrNO_4$ requires: *p*-nitrophenol, 54.0%].

Technique of acylation. The amino acid or peptide (1 mole) and Na_2CO_3 ($\frac{1}{2}$ –1 mole) were dissolved in *n*-NaOH (1 equiv.) and a solution of a *p*-nitrophenyl ester (1 mole) in *NN*-dimethylformamide was added with vigorous shaking, which was continued for $\frac{1}{2}$ –1 hr. The reaction mixture was diluted with water and filtered, and the acylated product precipitated by acidification.

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