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A Method for Separating Neutral Amino Acids from Neutral Oligopeptides

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One of the main problems in the isolation of naturally occurring peptides from biological fluids is their separation from the free amino acids which are usually present in relatively large amounts. The separation of amino acids and peptides into groups of basic, acidic, neutral and aromatic compounds (Fromageot, Jutisz & Lederer, 1948) is of some use and Waley (1956), in a study of the acidic peptides of the lens, showed how it was possible to fractionate a complex mixture of acidic peptides once they had been separated from the neutral amino acids. The acidic peptides could readily be separated from the few acidic amino acids by partition chromatography. However, neutral peptides often have such similar chromatographic properties to neutral amino acids that unless they are first concentrated relative to the free amino acids then the latter would swamp them under the usual conditions of partition and ion-exchange chromatography. Precipitation methods with ethanol are not suitable in the isolation of peptides of low molecular weight. Moreover, the grass extracts, for whose fractionation the present study was initiated, contained polyphenolic material, which, if it were precipitated with ethanol, might absorb small peptides. One recent method uses ninhydrin to destroy the free amino acids and leave the unchanged peptide (Markovitz & Steinberg, 1957). The vigorous conditions necessary for this reaction could not be used with confidence in the isolation of naturally occurring peptides of unknown structure. Consden, Gordon & Martin (1946) (cf. Biserte, 1950) separated glycylglycine from glycine by zone electrophoresis at pH 9·2, at which pH the anionic mobility of the peptide exceeds that of the amino acid. This is a useful technique when the proportions of amino acids and peptides are similar but it could not conveniently handle a mixture in which the amino acids were present in many times the concentration of the peptides. For the study of the occurrence of oligopeptides in ryegrass juice it was essential to find a method which would fractionate a few grams of a mixture containing only a small proportion of peptides.

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Synge & Wood (1958) in their studies on bound amino acids in ryegrass obtained a partial separation of peptide-like compounds from amino acids on a column of anion-exchange resin. Their fraction *PVPC* contained practically no bound amino acids, whereas the slightly slower-moving fraction *PWPC* contained a mixture of bound amino acids and free amino acids. They used the resin Dowex-2 (X10) with 0·2 M-sodium acetate. It seemed that this separation might in part be due to the difference in the pK values of the amino groups, causing the peptides to be slightly retarded relative to the free amino acids. Dr S. G. Waley (personal communication) suggested that this effect might be enhanced by using a buffer of higher pH. The pK values for the amino group of the neutral amino acids are all greater than 9·13, and many of the dipeptides and tripeptides of neutral amino acids have pK values less than 8·3 (Cohn & Edsall, 1943). The optimum pH for separation can be calculated from the expression $\frac{1}{2}(pK_{\text{amino acid}} + pK_{\text{peptide}})$ (Davies, 1949). Accordingly, pH 8·65 was chosen, at which value the peptides are predominantly anionic and should be held on an anion-exchange resin, whereas the amino acids are on balance cationic and should pass through the column.

In preliminary experiments at the Rowett Research Institute, such as those described below with ryegrass extracts, such columns were found to pass free amino acids while retaining peptide-like material, and were therefore used as a step in the fractionation schemes of Carnegie (1961) and Synge & Youngson (1961). Later, at Ruakura Animal Research Station, the capabilities of the procedure have been tested with authentic neutral amino acids and dipeptides.

MATERIALS

Anion-exchange resin Dowex-1 (X8) 200–400 mesh/in. (from BioRad Laboratories, 800 Delaware, Berkeley, Calif., U.S.A.), received in the Cl^- form (1·5 kg.), was stirred with 3 l. of 2 N-NaOH for 4 hr. The 'fines' were removed and the resin was washed with water until neutral. It was converted into the Cl^- form by washing

with 4N-HCl, washed with water until neutral and then washed in a column with 4M-ammonium acetate until the effluent was Cl^--free. Before use this resin was equilibrated with the buffer to be used in the experiment.

The amino acids were from California Corp. for Biochemical Research, Los Angeles, U.S.A., and the peptides were from L. Light and Co. Ltd., Colnbrook, Bucks. They were checked for purity by two-dimensional chromatography and ionophoresis (see below). The solutions (1 mg./ml.) were prepared in CO_2-free water.

Buffers. Acetic acid was added to a 25% (w/v) solution of NH_3 to give pH 8·65 and then diluted with CO_2-free water to give a solution in which the acetate concentration was 0·2M. This solution (referred to as 0·2M-buffer) was diluted to give a solution in which acetate was 0·05M and adjusted to pH 8·65 with NH_3 (referred to as 0·05M-buffer). A solution of triethylamine acetate (0·05M-acetate) was prepared in the same way and adjusted to pH 8·5. A 0·25M-triethylamine solution was saturated with CO_2. It was diluted with water to give an approximately 0·05M-solution (pH 8·5). This is referred to as triethylamine carbonate buffer.

METHODS AND RESULTS

Preliminary experiment on separation of amino acids from peptide-like substances in ryegrass extract

The material used in these experiments was obtained by fractionating ryegrass juice according to the procedure of Synge & Wood (1958), to give fraction *PWPC*, which was the second fraction obtained from their Dowex-2 (X10) column with 0·2M-sodium acetate (Dowex-2 is no longer readily available; Dowex-1 is a similar quaternary-ammonium resin). A typical experiment is described below.

Fraction *PWPC* (85 mg. dry matter; N, 14% of dry matter; carboxyl N, 14% of total N before hydrolysis, 55% after hydrolysis) obtained from spring grass cut in 1956 was transferred in a small volume of 0·05M-buffer to a column of Dowex-1 (X8) (30 cm. × 1 cm.) that had been equilibrated with 0·05M-buffer. Elution was started with the same buffer (20–25 ml./hr.) and 35 g. of effluent was collected in fraction 1. Elution with 0·2M-buffer was then started; 35 g. of effluent was collected in fraction 2. 0·1N-HCl was then applied to the column and 22 g. of effluent collected in fraction 3 and 33 g. in fraction 4. (The acetic acid front emerged after 28 g. had been collected from beginning HCl elution.) The 0·1N-HCl was replaced with N-HCl and 27 g. collected in fraction 5. A dark band moved down just before the Cl^- front. After the Cl^- front emerged 15 g. was collected in fraction 6. Fractions were evaporated to dryness *in vacuo* below 40°. The fractions were analysed by the methods used by Carnegie (1961).

The results of this experiment are given in Table 1. Most of the free amino acids were recovered in fractions 1 and 2. Phenylalanine and tyrosine were mainly in fraction 2. The dicarboxylic amino acids were recovered in fraction 4. In fraction 1 there were bound amino acids which could have come from molecules too large to enter the resin particles and be retained. The material in fraction 4 contained a considerable proportion of the total bound amino acids.

Separation of neutral dipeptides from neutral amino acids

A mixture of 1 mg. each of glycine, L-alanine and L-leucine and 0·2 mg. each of glycylglycine, 'DL-alanylalanine' and 'DL-leucyl-leucine' was transferred in 0·05M-ammonium acetate buffer, pH 8·65, to a column of Dowex-1 (X8) (20 cm. × 1·1 cm.). The column was eluted (15 ml./hr.) with 0·05M-buffer and 50 ml. of effluent collected. It was then eluted with M-acetic acid and the effluent collected until the pH of the effluent fell to that of M-acetic acid (30 ml.). The fractions were evaporated to dryness *in vacuo* and the recoveries estimated by the technique described below (Table 2). Within the limits of the technique of

Table 1. *Separation of free from bound amino acids in a fraction from ryegrass on Dowex-1* (X8) *column at* pH 8·65

For details see text. For definitions of abbreviations of amino acids see *Biochem. J.* (1957), **66**, 6.

Fraction	Percentage of carboxyl N taken, recovered in fraction: Before hydrolysis	After hydrolysis†	Major amino acids and other ninhydrin-reacting substances*: Before hydrolysis	After hydrolysis†
Material applied to column‡	100	100	—	—
1. 0·05M-Buffer effluent	80·0	26·8	Gly, Ala, Val, Leu	Gly, Ala, Val, Leu, Phe, Glu
2. 0·2M-Buffer effluent	7·3	23·0	Phe, Tyr, spot near Glu	Gly, Ala, Val, Phe, Leu, Tyr
3. Same as 2	0·0	0·0	None	None
4. Acetic acid front	8·5	24·9	Glu, faint spot between Val and Phe	Gly, Ala, Val, Leu, Phe, Tyr, Asp, Glu
5. Pre-chloride front	10·3	9·2	Glu, Asp, faint spot ahead of Leu	Same as 4
6. Chloride front	0·0	0·5	None	Glu, Asp
Total	106·1	84·4		

* Chromatography in butanol–acetic acid–water (4:1:5, by vol.), Whatman no. 1 paper.
† Hydrolysis with 6N-HCl for 24 hr. at 105°.
‡ Contained (before hydrolysis) 1·65 mg. of carboxyl N, rising to 6·4 mg. after hydrolysis.

estimation all the peptides were recovered in the fraction displaced with acetic acid. The peptides were contaminated with a trace of amino acid. That this did not result from slight hydrolysis of the peptides was demonstrated by a control experiment, in which the peptides alone were run through the column and no free amino acids were detected.

Semi-quantitative technique for estimation of amino acids and dipeptides

A method combining filter-paper electrophoresis and chromatography was used (cf. Biserte, Plaquet-Schoonaërt, Boulanger & Paysant, 1960) and the ninhydrin colours produced were eluted (cf. Thompson & Morris, 1959).

The electrophoresis apparatus was on the principle of Kunkel & Tiselius (1951) with certain modifications to prevent losses and distortion. Two glass plates (43 cm. × 30 cm.) were lightly coated with silicone fluid M.S. 550 (Hopkin and Williams Ltd., London). A sheet of Whatman 3MM paper [washed with 10% (v/v) acetic acid, followed by water until neutral] (53 cm. × 29 cm.) was dipped in 0·026M-sodium tetraborate (10 g. of $Na_2B_4O_7,10H_2O$/l.) buffer, pH 9·2, and lightly blotted. It was placed between the glass plates with the region where the spots were to be applied prevented from touching the glass by Perspex spacers (cf. Boyd, 1954). The edges and ends were sealed with polythene film coated with grease. The paper was left to equilibrate for 1 hr. before application of the samples. Three samples (two standard mixtures and one unknown) could be run simultaneously on the same sheet if placed at suitable intervals along a line 2·5 cm. from, and parallel to, the longest edge of the paper. Best results were obtained with 50 μg. of each peptide and 25 μg. of each amino acid in a total volume of 50 μl. A potential gradient of 6 v/cm. was applied for 2·5 hr. The paper was then removed, the ends were torn off and it was dried at 70°. It was then suspended in a chromatography tank over both phases of a mixture made from phenol (redistilled) and the above-mentioned tetraborate solution (1:1, v/v). After a few hours the paper was lowered into the phenol phase and developed by ascending chromatography for 11 hr. It was then dried for 5 hr. at 30°. It was dipped briefly into the ninhydrin reagent [2 g. of ninhydrin, 10 ml. of acetic acid, 5 ml. of water, 0·2 ml. of pyridine (British Drug Houses Ltd., technical grade) and 85 ml. of acetone] and heated at 70° in an oven (atmosphere saturated with ethanol vapour) for 30 min. A typical chromatogram is shown in Fig. 1. The spots were cut out under subdued light and immediately placed in 5 ml. of a mixture of methanol (500 ml.) and saturated aqueous cupric nitrate (0·2 ml.) (Zaĭtseva & Tyuleneva, 1958) and shaken in the dark for 30 min. The copper changes the purple ninhydrin colour to a more stable red colour. Absorption was read at 540 mμ after decanting the solution into colorimeter tubes and allowing any lint to settle. Colours were stable for at least 2 hr. As with most estimations of this type, it is advisable to run standard samples on the same sheet to allow for any variation in colour production.

Control experiments indicated that standard quantities of amino acids (25 μg.) and peptides (50 μg.) estimated by this technique gave colours whose extinctions had the following coefficients of variation: leucine 7·5, alanine 1·3, glycine 2·5, leucyl-leucine 2·7, alanylalanine 0·9, glycylglycine 4·5.

Ammonium acetate is not a very volatile buffer. A column was run with the more volatile 0·05M-triethylamine acetate buffer, pH 8·5. Similar recoveries to those shown in Table 2 were obtained.

As an additional check, the recovery of glycylglycine from a column was estimated with the ninhydrin–CO_2 procedure of Van Slyke, Dillon, MacFadyen & Hamilton (1941) as modified by Synge (1951).

Glycylglycine (1 mg.) was transferred to a column of Dowex-1 (X8) (25 cm. × 1·1 cm.) in the acetate form with 0·05M-ammonium acetate buffer. The column was operated as already described. The fractions, after evaporation to dryness, were hydrolysed in 6N-HCl at 105° for 24 hr. and the carboxyl N was determined. Glycylglycine (1 mg.) was similarly hydrolysed and the carboxyl N estimated.

The recovery of glycylglycine in the fraction displaced by M-acetic acid was 101%. There was a slight evolution of CO_2 from the reaction with the hydrolysate of the 0·05M

Table 2. *Amino acids and peptides recovered in fractions from Dowex-1 (X8) with ammonium acetate buffer*

For details see text.

Amino acid or peptide	Percentage of amount taken in mixture: 0·05M-Buffer eluate	Percentage of amount taken in mixture: M-Acetic acid eluate
Leucine	94	<5*
Alanine	90	<5*
Glycine	99	<5*
Leucyl-leucine	0	103
Alanylalanine	0	101
Glycylglycine	0	101

* Quantities present were too small for accurate determination by ninhydrin technique.

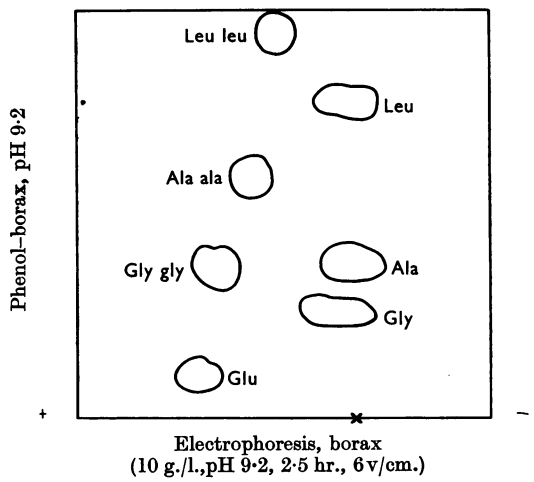

Fig. 1. Two-dimensional separation of amino acids and peptides on filter paper (see text). Ionophoresis horizontal (× indicates point of application of mixture), 2·5 hr., 0·026M-sodium tetraborate, pH 9·2, 6 v/cm. Chromatography upwards (phenol–0·026M-sodium tetraborate, pH 9·2) (× 0·23).

Table 3. *Recovery of glycine from Dowex-1 (X8) as percentage of glycine taken*

For details see text.

Fraction eluted with	Percentage recovery: Resin and buffer in acetate form	Percentage recovery: Resin and buffer in carbonate form
0·05 M-Buffer, pH 8·5	98	34·2
M-Acetic acid	2·2	63·3

fraction, possibly caused by the presence of a large amount of ammonium chloride.

Formation of carbamino acids

The presence of traces of free amino acids in the peptide fraction could not at first be explained. They were not removed by increasing the volume of effluent collected in the 0·05 M-fraction. Anion-exchange resins, especially in the higher pH regions, can exert a slight cation-exchange capacity (Partridge & Brimley, 1949), which might explain the slight retention of the free amino acid on the resin. Alternatively, and more likely, the retention might be due to traces of CO_2 converting the amino acids into the dicarboxylic carbamino acids, which would be held on the resin. As a check on this hypothesis, two columns were run under the same conditions except that in one the resin was in the acetate form and in the other it was in the carbonate form.

A column of Dowex-1 (X8) (20 cm. × 1·1 cm.) was equilibrated with 0·05 M-triethylamine acetate buffer, pH 8·5, with precautions to exclude CO_2. Glycine (10 mg.) in 1 ml. of CO_2-free water was applied to the column. The column was eluted with 50 ml. of buffer and the first fraction collected. Then it was washed with 30 ml. of M-acetic acid and the second fraction collected. The carboxyl N content of each fraction was determined after evaporation to dryness and leaving overnight in a vacuum desiccator.

A column of Dowex-1 (X8) in the acetate form (20 cm. × 1·1 cm.) was converted into the carbonate form by prolonged washing with approx. 0·25 M-triethylamine carbonate, pH 8·5. The column was then equilibrated with 0·05 M-triethylamine carbonate buffer. Glycine (10 mg.) in 1 ml. of CO_2-free water was applied to the column and eluted with 50 ml. of buffer, being collected in the first fraction. The resin was then transferred to a beaker and stirred with 30 ml. of M-acetic acid until evolution of CO_2 ceased. The slurry was returned to the column and washed with 35 ml. of M-acetic acid. The combined washings were the second fraction. After evaporation of the mixtures to dryness and leaving overnight in a vacuum desiccator the carboxyl N in each fraction was determined.

The results are given in Table 3.

DISCUSSION

Anion-exchange resins have been used in a similar manner to that described above for other separations. A difference between the pK values of the amino group of leucine and methionine was made use of by Davies, Hughes & Partridge (1950) to achieve a separation on a column of Dowex-2 by running an aqueous solution of the amino acids into the resin and then displacing them with hydrochloric acid. Similar procedures are coming into general use in work with nucleotides. Thus, for example, cytidine 2′:3′-phosphate was purified on a column of Dowex-1 with triethylamine acetate buffer, pH 7·4 (Crook, Mathias & Rabin, 1960). Synge & Youngson (1961) used the method described here, in their studies on the incorporation of L-[^{14}C]valine into non-protein nitrogenous fractions of ryegrass, to separate bound valine present in amphoteric peptide-like compounds from free amino acids. In their control experiments only 0·3% of free L-[^{14}C]valine applied to the column was retained on the resin. The properties of the bound amino acids retained on the resin are discussed by Carnegie (1961). It is possible that the presence of glutamic acid and aspartic acid residues with free carboxyl groups in some peptides aided in their retention on the resin.

Even with precautions to exclude carbon dioxide, small amounts of amino acids remained on the resin and were displaced with the peptides, which is a disadvantage if a complete separation were required. However, after removal of the majority of the amino acids the remainder can easily be removed by electrophoresis at pH 9·2 with a volatile-buffer system (cf. Carnegie, 1961). Care has to be taken that the flow rate of the buffer is not too fast and that the column is not overloaded. The exchange capacity of anion-exchange resins is low in the region pH 8–9 (Partridge & Brimley, 1949).

Formation of carbamino acids. This possible explanation for retention of free amino acids by the resin was suggested in a personal communication by Dr J. A. Mills (Adelaide). The results shown in Table 3 support the view that in the presence of carbonate there was a fairly rapid conversion of glycine into its carbamino derivative, which was retained on the resin. Leucine gave similar results.

There have been surprisingly few studies on this reaction of carbon dioxide with amino acids, which may be important in biological reactions, not only as a mechanism for carbon dioxide fixation but also in protein synthesis. The early work (for references see Siegfried, 1907) on the formation of carbamino acids was carried out in highly alkaline solutions and until recently it seems to have been assumed that the reaction takes place only at high pH. Recently, however, it has been shown that, in the presence of bicarbonate, carbamino acids can be formed at neutral and even slightly acid pH values (Neuberg, Grauer & Kreidl, 1955). Giustina & Temelcou (1955) have studied the formation of carbamino acids at different pH values and found

that the amount of carbon dioxide bound by glycine in aqueous solution at equilibrium reaches a maximum at pH 9·5, whereas glycylglycine and diglycylglycine bind the maximal amount at pH 8–9. Also these peptides have five times as great an affinity for carbon dioxide as has glycine.

It is interesting to speculate on the role that carbamino acids may play in amino acid activation and protein biosynthesis. There may be an enzyme system in the cell which would rapidly remove the carbamino acid driving the equilibrium reaction

$$\begin{array}{l} R{-}NH_2 + H{\cdot}CO_3^- \rightleftharpoons R{-}NH{\cdot}CO_2^- + H_2O \\ \quad | \qquad\qquad\qquad\quad | \\ \quad CO_2^- \qquad\qquad\quad CO_2^- \end{array}$$

from left to right in a manner analogous to the trapping of the dicarboxylic carbamino acid on the anion-exchange resin. The low-energy zwitterion is changed by the addition of carbon dioxide into a compound with higher energy. Wieland & Pfleiderer (1957) speculated on the role of carbamino acids in peptide synthesis. They suggested that carbon dioxide may also have a biological role in protecting the amino groups, analogous to the use of benzyl chloroformate in synthetic peptide chemistry.

SUMMARY

1. Monocarboxylic monoamino acids have been separated from their dipeptides on a column of anion-exchange resin eluted with ammonium acetate buffer, pH 8·65. The peptides are retained on the resin and later eluted with acetic acid.

2. A semi-quantitative technique with two-dimensional chromatography–ionophoresis on filter paper was devised for assessing the results of these separations.

3. The presence of carbon dioxide interfered with the separations and it is suggested that this was due to formation of carbamino acids. The possible role of such compounds in the biosynthesis of proteins is discussed.

4. With juice expressed from ryegrass the procedure was useful for removing free amino acids from peptide-like compounds of low molecular weight which it was desired to study in greater detail.

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