Bound Amino Acids of Ryegrass: The Isolation of Amphoteric Peptide-like Substances of Low Molecular Weight

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Although grass is the most important foodstuff for ruminant and other herbivorous livestock, remarkably little is known about the nature of its non-protein nitrogenous compounds, other than the free amino acids. Although peptides of low molecular weight account for only a small percentage of the non-protein nitrogen, they may be important in the nutrition of rumen micro-organisms and of micro-organisms involved in the making of silage. More information on these compounds is desirable for a better understanding of plant physiology in general and for the mechanism of protein synthesis in particular. There have been few studies on the isolation of plant peptides but many qualitative observations on the differences between the amino acid pattern on chromatograms of plant extracts, before and after hydrolysis. This chromatographic evidence was usually unsupported by other observations and on its own was insufficient to justify the claim that the unidentified substance was a peptide. The aim of the present work was to isolate peptides of low molecular weight from the non-protein nitrogenous fraction of ryegrass, in other words, those amphoteric compounds with a molecular weight under 10 000 composed only of amino acids linked together by peptide bonds. In the course of their studies on neutral and acidic substances containing bound amino acids in ryegrass Syngé & Wood (1958) isolated a fraction (*PWPC*) which could have contained genuine peptides.

There are several major difficulties in the isolation of such peptides. They are present in such low concentrations that large amounts of grass must be fractionated in order to get a final yield of a few milligrams. Since oligopeptides are similar in their physical properties to their component amino acids and also because of their low concentration relative to the free amino acids, it is difficult to achieve a separation of the peptides from the amino acids by the usual chromatographic and electrophoretic procedures. The fractionation scheme used in the present work (see Fig. 1) was designed to collect as large a proportion as possible

of any neutral oligopeptides free from amino acids, strongly acidic and basic peptides and other non-protein nitrogenous compounds.

In the first stage the cell membranes were plasmolysed by treatment with ether (Chibnall, 1939) and the juice was squeezed out. The expressed juice contained little protein, since the cell wall acted as a filter. The cation-exchange resin not only rejected uncharged compounds and acids, but also by its molecular-sieve action rejected proteins and peptides of high molecular weight (cf. Waley, 1957). The amino acids, peptides and strong bases were retained on the resin, but only the amino acids and peptides were displaced by ammonia. Peptides have been shown to have a partition coefficient generally in favour of the phenol phase of a phenol-water mixture (Knight, 1951; Grassmann & Deffner, 1953). Countercurrent distribution with this system removed most of the dicarboxylic acids and partially concentrated the peptides relative to the free amino acids. The separation of the remaining free amino acids from the peptides was achieved by the technique described in a preceding paper (Carnegie, 1961). Peptides in acetic acid solution will migrate cationically, unless sulphonic or other strongly acid groups are present. The three-compartment cell removed uncharged substances, and also passage through the cellophan membrane into the cathode compartment ensured that substances collected in the cathode compartment had a molecular weight under 10 000. As the isolation of neutral peptides was the primary object of this investigation it was necessary, as a further precaution, to remove any acidic peptides, basic peptides and the free glutamic acid and aspartic acid which had been retained on the Dowex-1 (X8) resin and had migrated or diffused into the catholyte of the three-compartment cell. This separation was achieved by zone electrophoresis at pH 4. The mixture of supposed neutral peptides was then investigated with partition-chromatographic techniques.

Syngé & Youngson (1961) have used L-[¹⁴C]-valine to study the metabolic incorporation of this amino acid into a fraction similar to that studied in the present work and into fractions such as those previously studied by Syngé & Wood (1958).

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MATERIALS AND METHODS

For the large-scale experiment described here, grass (approx. 6 in. long) was cut by scythe from a sward, grown from a seed mixture of 7 parts of Italian ryegrass to 10 parts of perennial ryegrass, on 21 May 1958. Three cwt. of sulphate of ammonia/acre had been applied in April and 2 cwt. of Nitrochalk (Imperial Chemical Industries Ltd.)/acre had been applied at the beginning of May. Grass (21.6 kg.) was subjected to the ether treatment of Synge (1951; cf. Chibnall, 1939) and the juice was expressed with a pressure of 2000 lb./in.² The juice (volume 10.2 l.) was filtered through a pad of Celite 545 (Johns-Manville Co. Ltd., London). The general fractionation procedure is outlined in Fig. 1.

Chromatography on a cation-exchange resin (stage I)

This technique for preparing amino acids from plant extracts was first used by Westall (1950). The juice was processed in two equal batches. The filtered juice was slowly run (200 ml./hr.) through a column (12 cm. diam. $\times$ 17.5 cm. high) of Amberlite IR-120 A.G. resin (British Drug Houses Ltd., Poole, Dorset) in the H⁺ form. After the juice was run in, the column was washed with water until the effluent was free from dry matter. A solution of NH₃ (sp.gr. 0.88, 175 ml./l.) was used as the displacing agent. The elution with NH₃ solution was continued until the

effluent was shown, by evaporating and testing with ninhydrin, to be almost free from amino acids. The NH₃ eluate (6 l.) was concentrated by evaporation *in vacuo* at 35° to a small volume. A few drops of octan-2-ol (British Drug Houses Ltd.) were added to prevent frothing.

Countercurrent distribution in phenol-water (stage II)

Essentially the same procedure as described by Synge & Wood (1958) was used. The concentrated eluate (SIB) was not, however, acidified before shaking with phenol. Synge & Wood experienced little difficulty in separating the two phases, possibly because of the acidity, the presence of polyphenols or high concentration of inorganic salts. In this experiment the phases separated very slowly and it was necessary to centrifuge the contents of the funnels at 1300 rev./min. for 10 min. to obtain a separation. The first eight charges (250 ml. each) of phenol-rich phase were pooled and concentrated *in vacuo*.

Chromatography on an anion-exchange resin (stage III)

The technique described by Carnegie (1961) was used. Fraction SII B (25.3 g. of dry matter, 3.2 g. of N) was brought to pH 8.6 with ammonia solution before it was transferred (125 ml.) to a column of Dowex-1 (X8) resin

Fig. 1. Fractionation procedure (for details see text).

(13 cm. high $\times$ 10.5 cm. diam.). The chromatogram was then developed with 0.05M-buffer. The effluent *SIIIA* (1750 ml.) was dark brown in colour. The column was then washed with 2260 ml. of 0.2M-buffer to give eluate *SIIIB*. Displacement-development with 0.1N-HCl followed and the third fraction was collected. The HCl caused two pigment fronts to move down the column: the faster, dark brown in colour, was followed by a greyish-green zone. The slower front was reddish brown in colour. When approx. 1 l. had been collected in *SIIIC* the faster front was about 6 cm. from the foot of the column and the pH of the effluent became more acid. When 2 l. had been collected in *SIIIC* the 0.1N-HCl was replaced by N-HCl. A total volume of 2690 ml. of golden-brown eluate was collected in *SIIIC*. A precipitate slowly settled out in this fraction. Collection of *SIID* was started when the pigment associated with the Cl^- front was approx. 3 cm. from the bottom of the column. Just before the Cl^- front emerged the effluent became dark brown in colour. The volume collected in *SIID* was 1360 ml. Collection of *SIIE* was started when Cl^- was first observed in the effluent and continued until all the dark-brown pigment was removed from the column (430 ml.). A pigment zone, which moved more slowly, was not collected. Samples were removed from the fractions for analysis. Fractions *SIIIA*, *SIIIB*, *SIIIC* and *SIID* were evaporated to dryness in a rotary evaporator *in vacuo* at under 40° . Fraction *SIIE* was evaporated *in vacuo* at under 20° in a rotary evaporator whose receiving chamber contained 5 ml. of 5N-NaOH.

Ionophoresis in the three-compartment cell (stage IV)

The apparatus described by Synge (1951) was used with two cellophan membranes. Because of the tendency for dark-brown material to precipitate and block the cathode-compartment membrane, the middle-compartment solution was placed inside a small sac made from cellophan dialysis tubing, which held back the insoluble material; the sac was immersed in the same solvent in the middle compartment.

The cathode-compartment membrane was renewed several times during the course of a run. Portions (approx. 800 mg. each) of *SIIIC* and *SIID* were taken up in aqueous 5% (v/v) acetic acid and placed in the middle compartment (insoluble material in *SIID* was first removed by filtering). The anolyte was 0.1N- H_2SO_4 and the catholyte aqueous 1.0% (w/v) NH_3 solution. The catholyte was removed at 6-hr. intervals and replaced with 1.0% (w/v) NH_3 solution. The run was continued for 48 hr. The pooled catholytes were combined and evaporated to dryness *in vacuo* at under 40° .

Preparative paper electrophoresis at pH 4.0 (stage V)

A portion of *SIVB* (400 mg.) from *SIID* was taken through the fractionation procedure illustrated in Fig. 1 as far as stage VII. The quantities of material in the final fractions were too small for detailed study. The remaining catholytes were pooled and taken through the same procedure as described here.

The apparatus of Kunkel & Tiselius (1951), in which filter paper is pressed between two sheets of plate glass, was used. Because filter paper contains considerable quantities of bound amino acids (Wynn, 1949) it was

necessary to wash the paper thoroughly before use. Whatman no. 17 paper (H. Reeve Angel and Co. Ltd., London) was washed by descending chromatography with 50% acetic acid for 24 hr., water for 12 hr., aqueous 0.25M-triethylamine (redistilled) for 24 hr. and finally with water for 24 hr. The paper was dried and two sheets (53 cm. $\times$ 19 cm.) were dipped into buffer, pH 4 (Grassmann, Hannig & Plöckl, 1955; pyridine 15 ml., acetic acid 50 ml., 2.5 l. of water), blotted and placed between two sheets of glass (43 cm. $\times$ 20 cm.), which had been smeared with silicone fluid M.S. 550 (Hopkin and Williams Ltd., London). The lower glass plate was clamped to a brass plate which was soldered to a cooling coil through which water at $+2.5^\circ$ flowed. The edges of the plates were sealed with polythene film smeared with silicone grease. The apparatus was set up and the paper left to equilibrate with buffer for 3 hr. before *SIVB* (400 mg.) was applied to a line 15 cm. from the anode end of the papers, approx. 2 ml. of buffer being used. A potential gradient of 11 v/cm. was applied for 6 hr. The pigment in *SIVB* moved slightly toward the cathode. At the completion of the run two prints were made on Whatman no. 1 paper by placing a sheet of no. 1 on top of the no. 17 paper and squeezing with a glass plate. The sheets were carefully removed, dried over a gentle heat, examined for fluorescence and the zones were marked. One of the prints was sprayed with ninhydrin and heated for 10 min. The other was treated by the chlorine-starch-KI method of Rydon & Smith (1952). From an examination of the prints and fluorescent areas zones were cut out for elution by descending chromatography, aqueous 50% (v/v) acetic acid being used in an apparatus similar to that of Dent (1947). Elution was continued for 12 hr., then the eluates were evaporated to dryness *in vacuo* below 40° .

Partition chromatography in butanol-acetic acid-water (stage VI)

SVD (166 mg.) was fractionated by chromatography in a column of kieselguhr with butanol-acetic acid-water (4:1:5, by vol.). Kieselguhr (Hyflo Supercel, Johns-Manville Co. Ltd., London) was washed with the phenol-rich phase of a phenol-water mixture until all the yellow colour was removed, then with water to remove the phenol. It was dried in the oven at 105° and spread on a tray to equilibrate with atmospheric moisture. Kieselguhr (22 g.) was mixed thoroughly with 14.7 ml. of aqueous phase of the freshly prepared solvent and made into a slurry with a few millilitres of the top phase and poured into a 2 cm. $\times$ 55 cm. column. The kieselguhr was carefully packed with a stainless-steel packing tool (Howard & Martin, 1950) to a height of 18 cm. The temperature was maintained throughout the run at $21.5 \pm 1^\circ$. *SVD* was quantitatively transferred to the top of the column with small volumes of top phase. Successive effluent fractions were collected, weighed and evaporated in tared flasks to dryness *in vacuo* below 40° . From an examination of the graph and from the appearance of the fractions it was decided which fractions to combine.

Preparative paper chromatography (stage VII)

The fractions obtained from *SVI* were fractionated with Whatman no. 3MM paper (washed with 50% acetic acid, then with water) and pyridine-isoamyl alcohol-water

(1:1:2, by vol.) (Edman, 1945). The material (approx. 20 mg./sheet) was transferred with a few μ l. of water to a line 7 cm. from one end of a sheet of paper (53 cm. $\times$ 22.5 cm.) and dried with a hair-drier at room temperature. Descending development was carried out for 12 hr. at 21°. The papers were dried at a gentle heat and then examined for fluorescence. Strips 0.5 cm. wide were cut from the edges of the paper and treated first with ninhydrin then by the chlorine-starch-KI procedure. Zones were cut out according to the results of these examinations and eluted by descending chromatography with aqueous 10% (v/v) acetic acid. Paper blanks contained very little dry matter and no amino acids on hydrolysis. The fractions from *SVI* which contained more than 100 mg. were fractionated on Whatman no. 17 paper by ascending chromatography.

Preparative paper electrophoresis at pH 9.07 (stage VIII)

Only fraction *SVIIA*₄ was subjected to this procedure. Redistilled triethylamine (0.25M) was converted into triethylamine carbonate with CO₂, 800 ml. of this solution was made to 2 l. and 400 ml. of 0.1M-triethylamine added to give pH 9.07 (cf. Porath, 1956). Whatman no. 3MM paper (53 cm. $\times$ 19 cm.), washed as previously described for *SV*, was used in the apparatus of Kunkel & Tiselius (1951). *SVIIA*₄ (13 mg.) was transferred in a few μ l. of buffer to a line 10 cm. from the cathode end of the paper and subjected to 6 v/cm. for 9 hr. The paper was removed, dried at a gentle heat and examined for fluorescence. A 0.5 cm.-wide strip was cut from one edge, sprayed with ninhydrin and heated for 10 min. Two zones, one staining yellow with ninhydrin and one staining purple, were cut out and eluted with 10% acetic acid.

Other analytical procedures

The methods were similar to those used by Synge & Wood (1958), with the following differences. For one-dimensional chromatography butanol-acetic acid-water (4:1:5, by vol.) and Whatman no. 1 paper were used. When paper-ionophoresis diagrams containing sodium tetraborate buffer were sprayed with ninhydrin solution, it was necessary to add about 1% of acetic acid to the spray. To detect copper complexes a 1% (w/v) solution of dithio-

oxamide (rubeanic acid) in aqueous 90% (v/v) acetone was used.

End-group analysis

The dinitrophenylation method described by Waley (1956) was used. For the chromatography of the *N*-2,4-dinitrophenyl (DNP) amino acids an improved solvent system was introduced: *tert*-amyl alcohol (2-methylbutan-2-ol)-propan-2-ol (9:1, v/v) saturated with buffer, pH 7.15. Whatman no. 4 paper was saturated with this buffer made from 6.8 g. of KH₂PO₄, 10.65 g. of potassium hydrogen phthalate and 3.4 g. of NaOH/l. Sharp spots were obtained. The presence of phthalate ions probably reduced the tendency for the DNP amino acids to be adsorbed on to the paper with the production of tailing common with other solvent systems.

Quantitative amino acid analysis

A procedure similar to that of Moore, Spackman & Stein (1958) was used. The ninhydrin reagent was that of Connell, Dixon & Hanes (1955). For the basic amino acids a 20 cm. $\times$ 1 cm. column of Amberlite IR-120 resin (British Drug Houses Ltd.) was used.

RESULTS

Only the results relevant to the large-scale-fractionation procedure are given. The preliminary experiments on grass samples from previous years gave similar fractions, although the sequence of operations was not always the same as in the procedure described. Synge & Wood (1958) also observed that different batches of grass gave similar quantities of bound amino acids in their fractions.

From 21.6 kg. of spring grass (17.5% of dry matter, 4.0% of N in dry matter) 10.2 l. of juice was expressed. In Table 1 the values take into account the volume of juice which was not squeezed out of the grass and also allowance is made for any sample removed for analysis. When ammonia from the buffers contaminated the sample it was deter-

Table 1. Results of fractionation of juice expressed from ryegrass

Stage	Dry matter of fraction (% of dry matter of original grass)	N of fraction (% of N of juice)	N of fraction (% of dry matter of fraction)	Carboxyl nitrogen of fraction (% of N of fraction)	
				Before acid hydrolysis	After acid hydrolysis
Juice	29.0	100	2.3	40.0	60.0
<i>SIA</i>	21.5	25.0	0.8	20.0	37.0
<i>SIB</i>	3.5	59.0	11.3	52.0	77.0
<i>SIIA</i>	2.4	38.0	13.1	60.9	79.1
<i>SIIB</i>	1.1	20.0	12.6	22.8	55.8
<i>SIHA</i>	0.66	12.2	12.2	36.9	53.7
<i>SIHB</i>	0.07	1.1	11.3	16.3	53.6
<i>SIIC</i>	0.05	1.3	16.1	9.3	25.9
<i>SIID</i>	0.12	1.6	9.2	21.4	69.8
<i>SIIE</i>	0.07	0.9	8.8	9.5	119.6
<i>SIV B</i> from <i>SIIC</i>	0.02	0.85	16.7	—	—
<i>SIV B</i> from <i>SIID</i>	0.04	0.37	8.2	15.0	40.0

mined by the Conway procedure and the nitrogen value corrected accordingly. The grass juice was of similar composition to that studied by Synge & Wood (1958). About 20 % of the dry matter of the juice was not recovered from the Amberlite IR-120 resin. This material was probably composed of inorganic cations, organic bases stronger than ammonia and strongly adsorbed polyphenolic compounds. Both the ammonia eluate and aqueous washings were dark brown in colour. Since the chromatograms of samples from *SIA* failed to show any ninhydrin-positive substances it was probable that the CO_2 produced in the Van Slyke procedure was due to some artifact (cf. Synge, 1951). After hydrolysis a small amount of glutamic acid was present. Fraction *SIB* contained a complicated mixture of amino acids including γ -aminobutyric acid. The hydrolysate showed an increase in the proportion of the dicarboxylic acids and a spot with R_f (butanol-acetic acid-water; 4:1:5, by vol.) between those of valine and proline was no longer present. Most of the acidic bound amino acids studied by Synge & Wood would pass through the resin and be present in fraction *SIA*. The amount of dry matter in *SIB* was too great to be conveniently handled directly on the Dowex-1 (X8) column. Phenol-water countercurrent distribution concentrated certain peptides relative to the free amino acids and also removed most of the glutamic acid and aspartic acid. However, considerable quantities of the more acidic peptides, e.g. glutathione, would be retained in the aqueous phase. Several ninhydrin-positive spots on chromatograms of *SIIB* were no longer present after hydrolysis. In contrast *SIIA* showed little change in its amino acid pattern after hydrolysis.

The first fraction from the Dowex-1 (X8) column (*SIIIA*, 15 g. of dry matter) contained the free amino acids other than the dicarboxylic acids and a considerable amount of bound amino acids which were liberated on hydrolysis. An attempt was made to separate them from the free amino acids by precipitation with different concentrations of ethanol. Most of the brown pigment was precipitated by aqueous 85 % (v/v) ethanol. This precipitate on paper electrophoresis at pH 9.2 migrated anionically slightly slower than glutamic acid and gave no colour with ninhydrin. On hydrolysis, it yielded a mixture of amino acids. It was possible that these bound amino acids were similar to the high-molecular-weight acidic compounds studied by Synge & Wood (1958). They were probably held by adsorption on the cation-exchange resin. They were probably too large to enter the particles of anion-exchange resin and exchange with the acetate ions. Fraction *SIIB* (1.5 g.) was not studied in detail. Chromatograms

before hydrolysis showed with ninhydrin two faintly purple spots, one near the leucine position and the other near the valine position. There was also a spot with R_f 0.42 in butanol-acetic acid-water which gave no ninhydrin colour but a strongly positive reaction with chlorine-starch-KI. Hydrolysates of *SIIB* showed phenyl-alanine, leucine, valine, alanine, glycine, glutamic acid, aspartic acid and basic amino acids.

Fraction *SIIC* contained material associated with the breakthrough of acetic acid from the column. It contained free glutamic acid and several ninhydrin-positive substances which were not present after hydrolysis. On a two-dimensional chromatogram (phenol-ammonia/collidine) one of these ran near leucine, one near valine and the other near glycine. Hydrolysates of this fraction contained a mixture of amino acids, the main ones being alanine, leucine, valine, glycine, glutamic acid and aspartic acid. Adenine as the free base was also a major component in this fraction. Its isolation is discussed below. Fraction *SIID*, similar to *SIIC*, was taken for detailed study. The fraction associated with the breakthrough of the Cl^- front, *SIIE*, contained a faintly ninhydrin-positive substance which ran similarly to glutamic acid on a two-dimensional chromatogram. The hydrolysate of this fraction contained mainly glutamic acid and aspartic acid and only small amounts of the others. The high carboxyl-nitrogen value of the hydrolysates may be due to the aspartic acid content, since aspartic acid with ninhydrin liberates 2 mol.prop. of CO_2 (Van Slyke, Dillon, MacFadyen & Hamilton, 1941).

The results from the three-compartment-cell fractionation of *SIID* and *SIIC* are shown in Table 1. Separation of the cationic material was difficult because of the blocking of the cathode membrane by the high-molecular-weight pigmented material. A considerable proportion of the bound amino acids in fraction *SIID* remained in the middle compartment with the brown pigment.

Table 2 shows the results of the fractionation of the cathode-compartment material *SIVB*. Free glutamic acid and aspartic acid were removed into fractions *SVB* and *SVC* from the main peptide fraction *SVD*, which remained close to the origin. The fractions near the cathode contained a higher proportion of lysine and arginine. Adenine, as the free base, was found in fractions *SVH* and *SVI*. In the preliminary experiments, with material equivalent to *SIVB* prepared from other batches of grass, adenine was isolated by paper electrophoresis at pH 9.0, with triethylamine carbonate buffer, and also by electrophoresis in 5 % acetic acid. The isolated material was compared with adenine by paper chromatography in butanol-acetic acid-water (4:1:5, by vol.) and in propan-2-

ol-2N-HCl (65:33, v/v) (Wyatt, 1951). Its behaviour on paper electrophoresis at several pH values was also compared with that of adenine. It gave the same ultraviolet spectrophotometric curve as adenine. Its nitrogen content was 54.6% of the dry matter, compared with the theoretical 51.83% for adenine. In that experiment it accounted for 1% of the nitrogen of the expressed juice.

Several dinitrophenyl (DNP) amino acids were obtained on treating the *SV* fractions. DNP-aspartic acid and DNP-glutamic acid were observed in the anionic fractions. In the fraction *SVD* DNP-leucine and DNP-valine were the major products of dinitrophenylations with smaller proportions of DNP-glycine, DNP-alanine and DNP-glutamic acid. The yields of DNP amino acids and amino acids from the cationic fractions were much lower than would have been expected had all the dry matter of the fraction been composed of peptides.

The results of the fractionation of *SVD* on the butanol-acetic acid-water column are shown in Fig. 2. Chromatography of the pooled fractions in pyridine-isoamyl alcohol-water (1:1:2, by vol.) indicated that they were composed of a complex mixture of ninhydrin-positive substances and also substances which reacted strongly with chlorine-starch-KI. The relatively low yield of amino acids in the hydrolysates suggested that much of the dry matter of the fractions was not composed of bound amino acids. Fractions *E-H* gave only faint spots of amino acids and were not further investigated. Similar results were obtained on fractionating material from *SIHC*.

Table 3 shows the results of preparative paper

chromatography of *SVIA*. *SVIIA₂* was highly crystalline and had a lemon-yellow fluorescence in ultraviolet light. No change in *SVIIA₂* was observed after hydrolysis. The final yields of *SVIIA₂* and *SVIIA₄* obtained from pooling the products of both fractionations were 31 and 35 mg. respectively. The fractionation of *SVIB*, obtained from the second butanol-acetic acid-water column, is shown in Table 4. The yields of *SVIIB₂* and *SVIIB₃* were 37.6 and 67 mg. respectively. The major component of *SVD* was *SVIC*, which when fractionated by paper chromatography gave the results shown in Table 5. Most of the dry matter

Fig. 2. Fractionation of *SVD* (166 mg.) by partition chromatography in butanol-acetic acid-water (4:1:5, by vol.). Column, 18 cm. $\times$ 2.0 cm. (22 g. of kieselguhr). Vertical lines indicate effluent fractions pooled for further study.

Table 2. Results of preparative paper electrophoresis of *SIVB* from *SIHD*

Pyridine-acetic acid buffer, pH 4.0; Whatman no. 3MM paper; 400 mg. was applied; 11 v/cm.; 6 hr. For definitions of abbreviations used for amino acids (AA) see *Biochem. J.* (1957), 66, 6.

Fraction	Distance from origin* (cm.)	Strength of colour with ninhydrin	Percentage of dry matter of <i>SIVB</i> recovered	Amino acids in acid hydrolysate	
				Major	Minor
<i>SV A</i>	+15.0-+10.0	+	1.8	Cys, Glu, Asp, Gly	Val, Leu, Phe
<i>SV B</i>	+10.0-+6.0	++	5.0	Glu, Asp, Gly	Leu, Val, Ala
<i>SV C</i>	+6.0-+2.3	++	4.6	Gly, Glu, Ala, Val, Leu	Phe, Tyr, basic AA
<i>SVD</i>	+2.3--3.0	+++	60.0†	Gly, Glu, Ala, Val, Leu	Phe, Thre, Ser, Tyr, basic AA
<i>SV E</i>	-3.0--6.0	++	4.2	Gly, Glu, Ala, Leu	Phe, Thre, Ser, Tyr, Val, basic AA
<i>SV F</i>	-6.0--9.5	-	1.8	—	Gly, Glu, Ala, Leu, basic AA
<i>SV G</i>	-9.5--14.5	+	2.3	Gly	Basic AA, Ala, Val, Leu, Tyr
<i>SV H</i>	-14.5--18.5	(Cl ₂ -starch-KI ++)	1.7	Gly	Basic AA, β -Ala, γ -Aminobutyric
<i>SV I</i>	-18.5--21.0	+	0.6	Gly	—
<i>SV J</i>	-21.0--24.0	-	0.2	—	—
<i>SV K</i>	-24.0--27.0	+	0.6	Basic AA	Gly

* -, In direction of cathode; +, in direction of anode.

† Dry matter of *SVD* represented 0.025% of dry matter of grass.

Table 3. *Results of preparative paper chromatography of SVIA (0.003 % of dry matter of grass)*

Pyridine-isoamyl alcohol-water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour		Dry matter of fraction ($10^3 \times \%$ of dry matter of grass)	Major amino acids in acid hydrolysate
		Ninhydrin	Fluorescence		
SVIIA ₁	0.087-0.13	Purple	White	0.2	Gly, Glu, Asp, Ala, Ser, Val, Leu, Phe
SVIIA ₂	0.23-0.45	Strong purple	Faint white	1.1	Same as SVIIA ₁ , but Phe stronger
SVIIA ₃	0.50-0.54	Bright yellow	Strong lemon yellow	0.24	Same as SVIIA ₂ , plus Tyr
SVIIA ₄	0.55-0.70	Purple	Faint	0.66	Leu, Phe, Val, Tyr

Table 4. *Results of preparative paper chromatography of SVIB (0.0083 % of dry matter of grass)*

Pyridine-isoamyl alcohol-water (1:1:2); Whatman no. 17 paper. See also protocol to Table 2.

Fraction	R_F	Colour	Dry matter of fraction ($10^3 \times \%$ of dry matter of grass)	Major amino acids in acid hydrolysate
SVIIB ₁	0.1-0.25	Purple (ninhydrin)	0.32	—
SVIIB ₂	0.33-0.44	Purple (ninhydrin)	2.7	Asp, Glu, Ser, Gly, Val
SVIIB ₃	0.44-0.59	(Cl ₂ -starch-KI-positive)	5.4	Only Gly
SVIIB ₄	0.65-0.71	Blue (fluorescence)	0.1	—

Table 5. *Results of preparative paper chromatography of SVIC (0.006 % of dry matter of grass)*

Pyridine-isoamyl alcohol-water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour	Dry matter of fraction ($10^3 \times \%$ of dry matter of grass)	Major amino acids in acid hydrolysate
SVIIC ₁	0.0-0.09	Faint purple (ninhydrin), brown pigment	0.5	Lys, Gly, Glu, Asp, Ala, Ser, Pro, Val, Leu, Phe
SVIIC ₂	0.09-0.21	Strong purple (ninhydrin)	0.6	Same as SVIIC ₁ , plus Tyr
SVIIC ₃	0.28-0.34	Strong purple (ninhydrin)	0.3	Same as SVIIC ₂ , less Pro
SVIIC ₄	0.43-0.54	No ninhydrin colour; strong Cl ₂ -starch-KI reaction	3.4	Only faint Gly
SVIIC ₅	0.64-0.76	Blue fluorescence	0.15	Traces of Ala, Leu, Val

was recovered in fraction SVIIC₄. The material in this fraction gave no colour with ninhydrin but a strong chlorine-starch-KI reaction. However, there were no amino acids in the hydrolysate other than a small amount of glycine. Also there was only about 2 % of nitrogen in the dry matter. The substance was not investigated further. Fraction SVID obtained from the second column when fractionated by paper chromatography gave the results shown in Table 6. The yield of SVIID₁ was 10 mg.

Fractions SVIIA₄, SVIIB₂ and SVIID₁ were taken for further analysis. They gave single spots on a two-dimensional chromatogram when it was sprayed with ninhydrin (the pattern obtained is shown in Fig. 3). They were not crystalline and contained a greenish-brown pigment. Attempts to crystallize samples of these fractions with ethanol

and butanol were not successful. Table 7 shows the results of quantitative amino acid analysis of these fractions with their approximate molar ratios calculated relative to aspartic acid. The separation of the small amounts of basic amino acids was not good. The modified ninhydrin reagent described by Connell *et al.* (1955) was used; their values for the extinction coefficients were used in the calculations. Only about 35 % of the dry matter of each of the hydrolysates was composed of amino acid residues yet nitrogen accounted for 12.4, 11.8 and 8.3 % of the dry matter of fractions SVIIA₄, SVIIB₂ and SVIID₁ respectively. Fractions SVIIA₄ and SVIIB₂ when dissolved in water both showed a rather flat maximum of ultraviolet absorption at 255-265 m μ with a small peak at 260 m μ ($E_{1\text{cm}}^{0.001\%}$ 0.240).

It was obvious from these results that the

Table 6. *Results of preparative paper chromatography of SVID (0.0025% of dry matter of grass)*

Pyridine-isoamyl alcohol-water (1:1:2, by vol.); Whatman no. 3MM paper. See also protocol to Table 2.

Fraction	R_F	Colour with ninhydrin	Dry matter of fraction ($10^3 \times \%$ of dry matter of grass)	Major amino acids in acid hydrolysate
SVIID ₁	0.026-0.13	Pink	0.45	Asp, Glu, Ser, Thr, Gly, Ala, Val, Ileu,
SVIID ₂	0.13-0.26	Purple	0.29	Leu, Phe, basic AA
SVIID ₃	0.26-0.37	Yellow	0.1	Similar to SVIID ₁
SVIID ₄	0.49-0.60	No ninhydrin (Cl ₂ - starch-KI reaction)	—	Similar to SVIID ₁ —

Table 7. *Approximate molar ratios of amino acids in acid hydrolysates of fractions relative to aspartic acid*

Amino acid	Fraction			
	SVIIB ₂	SVIID ₁	SVIIA ₄	SVIIIA ₁
Aspartic acid	1.00	1.00	1.00	1.00
Threonine	0.79	0.32	1.80	1.70
Serine	2.00	0.41	2.40	2.40
Glutamic acid	3.70	1.20	1.40	1.40
Proline	0.62	0.23	0.00	0.00
Glycine	0.35	0.97	12.00	2.50
Alanine	0.43	0.56	1.40	1.40
Valine	1.10	0.54	7.60	7.70
Methionine	Trace	Trace	0.00	0.00
Isoleucine	0.75	0.24	6.80	7.70
Leucine	1.40	0.34	22.00	20.70
Tyrosine	3.20	0.17	12.70	4.70
Phenylalanine		0.34	22.00	21.30
Lysine	0.30	0.19	Trace	Trace
Histidine	Trace	0.17	Trace	Trace
Arginine	Trace	0.19	Trace	Trace
Ammonia	7.70	1.00	4.00	7.30

fractions were still very complex, being composed possibly of a mixture of peptides and purine or pyrimidine type of compounds. Samples from SVIIA₄, SVIIB₂ and SVIID₁ were examined by paper electrophoresis in sodium tetraborate (pH 9.2) and in 5% acetic acid. Spraying with ninhydrin revealed several purple-staining spots in all three, and treatment with chlorine-starch-KI showed some strongly positive spots which gave no reaction with ninhydrin. SVIIA₄ showed in acetic acid a purple spot which had moved slightly further towards the cathode than leucyl-leucine and a bright-yellow-staining spot which had moved slightly less towards the cathode than glutamic acid. In sodium tetraborate the purple spot had moved slightly further than leucyl-leucine and the yellow spot had moved about half as far as leucyl-leucine. There was also a very strong chlorine-starch-KI-positive, ninhydrin-negative spot which had moved slightly less than the purple spot in sodium tetraborate. This substance was present in SVIIB₂, which also contained several substances staining

Fig. 3. Chromatography of fractions from stage VII in phenol-water-NH₃ (1%, w/v) and collidine-water; Munktel OB paper; sprayed with ninhydrin.

greyish purple with ninhydrin, with anionic mobilities in sodium tetraborate ranging from almost as fast as glutamic acid to only a slight anionic mobility. In acetic acid the bulk of the ninhydrin-positive material migrated cationically slightly faster than leucyl-leucine. The strongly chlorine-starch-KI-positive substance migrated cationically at the same rate as the main ninhydrin-positive material in acetic acid. Fraction SVIID₁ did not contain this chlorine-starch-KI-positive substance. It contained a mixture of ninhydrin-positive substances with the major proportion having an anionic mobility in sodium tetraborate approximately the same as that of glutamic acid. There was also some material with an anionic mobility slightly greater than that of glutamic acid. In acetic acid the major component had a cationic mobility between those of glutamic acid and leucyl-leucine.

The material in *SVIIA₄*, which gave a purple colour with ninhydrin, was isolated by preparative paper electrophoresis with triethylamine carbonate buffer, pH 9.07. This material *SVIIIA₁* represented approx. 0.00015 % of the dry matter in the grass. Most of the dry matter of fraction *SVIIA₄* was recovered from the chlorine-starch-KI-positive zone. The molar ratios of the amino acids in a hydrolysate of *SVIIIA₁* are shown in Table 7. *SVIIIA₁* is similar to *SVIIA₄* except that *SVIIIA₁* contains a lower proportion of glycine and tyrosine.

DISCUSSION

A detailed study of the nature of the peptide-like compounds in the final fractions was not possible because of the lack of material. Further work will be required to isolate substances of sufficient purity for a detailed study of their structure to be made. However, the material in several of the fractions isolated was similar to a mixture of oligopeptides. For instance, fraction *SVIIIA₁* appeared to contain a mixture of peptides similar to peptides of leucine. They had similar R_f values to those of leucyl-leucine in three different solvents and behaved similarly on electrophoresis at two different pH values. When the *SVI* fractions were treated with copper phosphate and then subjected to paper electrophoresis according to the procedure of Carnegie & Synge (1961), complexes were formed which migrated anionically as streaks. However, no colour was observed (possibly because traces of pigment masked it) and the yield of complex was low because of the non-peptide material present in the fractions. Pardee (1951) suggested that if the qualitative amino acid composition is determined for a peptide of known R_f , then the approximate number of amino acids in the molecule can be calculated. If the major peptides present in *SVIIIA₁* are assumed, from the amino acid analysis, to be composed of leucine, isoleucine, phenylalanine, valine and tyrosine, then with the formulae of Pardee and the determined R_f , the approximate number of amino acid residues per molecule can be calculated. The value obtained for n , the number of residues, from the R_f in pyridine-isoamyl alcohol-water was 2.5, and in phenol-water-ammonia was 4.5. DNP amino acid analysis on fractions from which *SVIIIA₁* was derived indicated that leucine was one of the major *N*-terminal amino acids. The properties of this group of compounds are all similar to those expected for a mixture of small peptides such as leucyl-leucine, leucylvaline, isoleucyl-leucine etc. However, all the dry matter in this fraction was not accounted for by amino acid residues. It is possibly contaminated by some of the strongly chlorine-starch-potassium iodide-positive material, which had a

slightly lower mobility at pH 9. The absorption in ultraviolet light at 260 m μ by fractions *SVIIA₄* and *SVIIB₂* would suggest the presence of purine or pyrimidine types of compound. These would also account for the non-amino acid nitrogen present in these fractions, which, when hydrolysed, were shown to contain only about 35 % of amino acids in the dry matter. Ferguson & Terry (1953, 1954) found that approximately 7 % of the non-protein nitrogen of grass was composed of purine-type compounds. Their identification of free adenine in grass (Ferguson & Terry, 1953) is confirmed.

Further work will be required to ascertain if these purines are merely contaminants of the peptide fractions or if they are in fact bound chemically to the peptides. This is the more important because of several recent reports of the isolation, with techniques similar to those used in the present work, of 'peptide-nucleotides' from plant material. Thus Hase, Mihara, Otsuka & Tamiya (1959) (cf. Hase, 1958) extracted water-soluble fractions from *Chlorella* and *Saccharomyces* similar in amino acid composition to those described here. Davies & Harris (1960) (cf. Harris, Davies & Parsons, 1958) have isolated similar substances from yeast, one of which they consider to be arginylalanylarginylalanyl-5'-uridyate. Gilbert & Yemm (1958) have made similar observations on *Torulopsis*. Medvedev & Zabolotskii (1958) found 2-3 % of a similar group of amino acids bound in ribonucleic acid isolated from higher plants.

In further investigation of the possible occurrence of peptide-nucleotides it would be desirable to develop methods that subject the material being fractionated to less-extreme pH values than those used here, since the possibility of breakdown of such compounds in the course of the fractionation has by no means been excluded. The further possibility that these peptide-like substances form a complex in some way with perhaps carbohydrate or lipid moieties cannot be ruled out until they have been more highly purified.

Fractions *SVIIB₂* and *SVIID₁* differed from *SVIIA₄* in their higher proportion of aspartic acid and glutamic acid. Their properties likewise were consistent with those of oligopeptides.

Since the methods used were designed to recover, as quantitatively as possible, neutral oligopeptides, then it can be estimated that they account for less than 1 % of the nitrogen of the expressed juice. Synge & Wood (1958) obtained similar results with a different fractionation procedure. It was possible that some of these peptides were produced by autolysis of protein during the time the grass was standing in ether. However, similar 'peptide' fractions were isolated from extracts prepared by plunging grass into boiling water immediately after cutting. Also in experiments by Synge & Youngson

(1961), with [^{14}C]valine, a similar 'peptide' fraction was obtained when either the ether procedure or extraction with boiling water was used. The yield of bound amino acids in the fraction from the ether procedure was, however, higher than in that from the boiling-water extraction. No single substance was found to be present in a much higher concentration than the others. The material isolated seems to be composed of a mixture of closely related substances differing slightly in their amino acid composition.

Synge & Wood (1958) observed that only a small proportion of the bound amino acids could be genuine oligopeptides, the greater part being in acidic compounds devoid of ionizing basic groups. The experiments described here with a different fractionation procedure confirm their observation. Several of the more acidic bound amino acids which were associated with dark-brown pigment were adsorbed on the cation-exchange resin and displaced during the ammonia development. Their presence in the supposedly cationic fraction should serve as a warning in assessing the results obtained when ion-exchange resins are used to isolate compounds from biological materials. The adsorption of polyphenolic compounds from plants on a cation-exchange resin and their elution with ammonia was observed by Thompson, Morris & Gering (1959).

Other reports of peptide-like substances in plants

This topic has been reviewed (Bricas & Fromageot, 1953; Synge, 1955, 1959; Synge & Wood, 1958). Apart from instances dealt with by these authors and the 'peptide-nucleotide' substances mentioned above, the following better-defined reports may be added. γ -Glutamylvalylglutamic acid was isolated from *Juncus conglomeratus* by Virtanen & Ettala (1958). Montant (1956) mentioned a fraction from the latex of *Euphorbia characias*, which yielded on hydrolysis glutamic acid, leucine, valine and glycine. Rinderknecht, Thomas & Aslin (1958) (cf. Zacharius, Morris & Thompson, 1959) isolated γ -glutamyl-S-methylcysteine from extracts of beans. Two other peptides, γ -glutamyl-S-methylcysteine sulfoxide and γ -glutamyl-leucine, were tentatively identified. Peptides of arginine, citrulline, glycine, threonine and ornithine have been isolated from Irish moss (Young & Smith, 1958; cf. Morita, 1955). Ohira, Imai & Kido (1955), Ohira, Kido & Imai (1955) and Rudinger & Pravda (1958) have reported work bearing on the structure of eisenin and related seaweed peptides. Makisumi (1958) isolated arginylglutamine from *Cladophora* sp. Butler & Bathurst (1958) recognized rather large quantities of bound γ -aminobutyric acid in root nodules of *Trifolium* spp. A toxic peptide isolated from a blue-green

alga *Microcystis aeruginosa* was found to contain L-aspartic acid, L-glutamic acid, D-serine, L-valine, L-ornithine, L-alanine and L-leucine, probably in a cyclic structure (Bishop, Anet & Gorham, 1959). In further purification of viscotoxin Samuelsson (1958, 1959) has eliminated carbohydrate claimed to be present by earlier workers and obtained a substance diffusible through cellophan and yielding 11 different amino acids after hydrolysis (see also Porath, 1960). Kauffmann & Ködding (1957) found bound glycine, alanine, serine, aspartic acid and glutamic acid in lipid fractions from spinach leaves and Kauffmann (1957) found the same amino acids 'loosely bound' to grana-protein of various plants. Reindel & Bienenfeld (1956) found, in virus-infected potato leaves, a peptide composed of glycine, aspartic acid, glutamic acid, threonine, alanine, valine, leucine, a little methionine and some phosphorus. Powden (1958) has established that the acetylornithine found in plants is the N^{δ} -derivative, and has found it in a number of grasses. Guha & Das (1957) (cf. Das & Ghosh, 1957; Das & Guha, 1957, 1958) isolated from bran a crystalline substance, mainly peptide in character, containing bound nicotinic acid. Two peptide-like compounds have been observed in corn sap by Fejér & Kónya (1959), who give references to earlier similar observations. Bollard (1957) observed the presence of peptides in apple tracheal sap. Schon & Richter (1959) isolated an allergenically active peptide-like fraction from ragweed pollen. These last three groups were not well characterized chemically. Miettinen (1959), in an investigation of the incorporation of DL- ^{14}C -alanine by pea roots, isolated a peptide composed of equimolar amounts of alanine and glutamic acid with less glycine and serine and traces of threonine, valine and leucine, a mixture rather similar to that from several of the fractions discussed here.

SUMMARY

1. A complex mixture of neutral amphoteric peptide-like compounds of low molecular weight has been isolated from the expressed juice of ryegrass. The bound amino acid residues account for less than 1 % of the nitrogen of the expressed juice.

2. The techniques used in the isolation included ion-exchange chromatography, partition chromatography and preparative paper electrophoresis. Pigments were difficult to remove. Nitrogenous bases similar in some of their properties to purines and pyrimidines occurred in several of the fractions.

3. A group of substances with amino acid content mainly of leucine, isoleucine, valine, tyrosine and phenylalanine was isolated. They had similar chromatographic and electrophoretic properties to leucyl peptides of low molecular weight. The

presence of other groups of substances, containing a higher proportion of glutamic acid and aspartic acid and smaller amounts of most of the common amino acids, was observed. It was not possible, because of the small quantities available, to make a full characterization.

4. The occurrence of adenine as a free base in grass is confirmed.

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