This suggested that the high level was maintained only by virtue of the diminishing weight of the tissue.

Although surrounded by these extremely high concentrations, the seed (Fig. 4) showed very low levels, and the woody shell showed a steadily decreasing amount of ascorbic acid.

If the fruit is considered in conjunction with the rest of the plant, it is possible to follow the concentration gradient of ascorbic acid from the mesophyll of the leaves to the fruit stalk and possibly into the mesocarp. As the epicarp contains chlorophyll, it can supply further quantities of ascorbic acid by photosynthesis. These facts, however, do not explain the high levels in the endocarp which can be maintained by preformed ascorbic acid only if it is translocated against a steep concentration gradient, which is improbable (Wokes & Melville, 1948).

An alternative suggestion is that the ascorbic acid is synthesized in the endocarp. The intermediates in this proposed synthesis are first, precursors which are translocated from other parts of the plant, secondly, glucose (which is found in very high concentrations in the fruit), and thirdly, dehydroascorbic acid.

Zilva, Kidd & West (1938) showed that there was a change in the relative proportions of ascorbic and dehydroascorbic acids in Bramley's Seedling apples as the fruit developed, with the equilibrium shifting in favour of ascorbic acid. In the walnut, when the fruits are small, a concentration gradient of the

oxidized form exists both from the outer tissues of the fruit and from the stem, into the endocarp.

It will be shown in a future paper, that in the presence of ascorbic acid, the epicarp and mesocarp are able to synthesize hydrojuglone glucoside. Examination of extracts failed to reveal sufficient dehydroascorbic acid to account for the high concentration of the glucoside in these tissues. It is presumed, therefore, that the dehydroascorbic acid is rapidly removed and utilized by the endocarp.

SUMMARY

1. A spectrophotometric assay for ascorbic acid and dehydroascorbic acid has been applied to extracts prepared from various parts of the walnut (*Juglans regia*).

2. Definite seasonal variations have been found for the concentration and amount per unit of these substances.

3. In order to explain the high concentration of ascorbic acid in the endocarp, which appears to preclude its translocation as such from the other parts of the plant, it is suggested that dehydroascorbic acid may be utilized by this tissue.

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Non-protein Nitrogenous Constituents of Rye Grass: Ionophoretic Fractionation and Isolation of a 'Bound Amino-acid' Fraction

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About one-sixth part of the organically combined nitrogen of freshly picked green leaves is in the form of compounds of low molecular weight, in the main free amino-acids. Since these are probably the most reactive nitrogenous compounds when leaves are eaten by the ruminant animal, or made into silage and so forth, it is important to have better knowledge of their nature, quite apart from the importance of this knowledge for plant physiology.

During the latter part of the nineteenth century, studies of plant juices, especially those by Schulze, were very fruitful for biochemistry, and particularly the biochemistry of nitrogenous substances. Subsequently, however, such studies were rather neglected. Plant physiologists indeed made extensive series of measurements of the concentrations of individual compounds or groups of compounds, and their variation with changing conditions, but

there were few attempts at detailed characterization of the 'non-protein nitrogen' of particular plant tissues. Outstanding exceptions are Vickery's extended study of alfalfa constituents (Vickery, 1924*a, b*, 1925*a, b*; Vickery & Leavenworth, 1925; Vickery & Vinson, 1925) and a study of potato constituents by Neuberger & Sanger (1942; cf. Slack, 1948). In recent years, however, chromatographic methods have been used in a number of studies which have given a good general idea of the distribution of amino-acids in plant juices, confirming and extending the earlier observations, and in some cases providing rough quantitative data. Reference may be made to Allsopp (1948), Dent, Stepka & Steward (1947), Hulme & Arthington (1950), Joslyn & Stepka (1949), Steward, Thompson & Dent (1949), Westall (1950) and to a review by Steward & Thompson (1950).

In view of the difficulty of obtaining quantitative data on amino-acids from filter-paper chromatograms it has seemed useful to combine studies of this kind with quantitative fractionations by electrical-transport methods, and to base the amino-acid and peptide figures on the highly specific ninhydrin-carbon dioxide determination of Van Slyke and colleagues. In the present paper are described some data obtained in this way with rye grass. The results give a synopsis of the distribution of several categories of non-protein nitrogen in a typical forage material. There emerges a fairly detailed picture of the relative proportions of the main nitrogenous compounds when these figures are collated with the data from the filter-paper chromatograms, and with information about γ -aminobutyric acid (Synge, 1951). Since that paper was sent for publication, Beevers (1951) has demonstrated formation of γ -aminobutyric acid from L-glutamic acid by enzymic preparations from barley roots, and relatively large quantities have been found in mammalian brain by Awapara, Landua, Fuerst & Seale (1950) and by Roberts & Frankel (1950). These last authors produced evidence for its enzymic origin from glutamic acid, and their product was identified with authentic γ -aminobutyric acid by Udenfriend (1950), using an isotopic-labelling method.

Throughout the present work special attention has been paid to the recognition of possible peptide material ('bound' amino-acids). Such material has been reported in plant juices by a number of

authors, but few have undertaken any detailed characterization. What has been learnt on this subject in the present work is discussed below in relation to these previous studies. Such substances may have importance as intermediates in the biological synthesis and breakdown of protein.

MATERIAL

Rye grass (*Lolium perenne* L.), strain S23, was obtained through the kindness of Mr R. Bain from the grass plots of the North of Scotland College of Agriculture at Craibstone. The freshly picked material was treated with ether according to Chibnall (1939). It was hoped that this procedure, in which the cell walls are thought to function as dialysing membranes, would give a fairly representative mixture of the low-molecular components of the cell sap without allowing much opportunity for enzymic proteolysis and other changes that might occur on mincing or milling the tissues (cf. Tracey, 1948; Holden, Pirie & Tracey, 1950). At the same time possible alterations due to heating or drying (see Discussion) would be avoided.

The grass was placed in ether (anaesthetic, B.P.) for 20 min. The juice was then squeezed out in a hand press and filtered through paper pulp. Very different quantities of clear brown juice were obtained in this way from the grass in its various states, but the concentrations of components found in the juice did not vary strikingly. Nine samples of juice obtained on different dates from May to November had total N (Kjeldahl) in the range 0.51–1.33 mg./ml. The juice was placed in a cellophan sac and was dialysed against 4–5 vol. of CHCl_3 water for 96 hr. at 0–2° with occasional stirring. 72–94% of the N was diffusible under these conditions, assuming even distribution of diffusible material inside and outside the sac. All analyses given below are referred to the total N (Kjeldahl) of such diffusates. These were stored in the dark, saturated with CHCl_3 , at 0–2°. No observations made during the necessarily protracted analyses suggested any changes in the nitrogenous compounds during storage. The pH of these diffusates was approx. 6. Three preparations specified in Table 1 were analysed in full detail.

METHODS

Ionophoretic fractionations

The first stage in the fractionation aimed at the separation of the non-protein nitrogenous substances into groups according to the net charge on the molecule at about pH 6, avoiding altogether the use of such empirical precipitants as lead acetate or phosphotungstic acid. Electrical-migration procedures seemed preferable to the various ion-exchange procedures that are available, since some of the compounds being studied were of unknown chemical nature and might be

Table 1. *Specimens of juice obtained from rye grass by the ether procedure*

Specimen	Hour of picking	Date of picking	N content of filtered juice before dialysis (mg. N/ml.)	Remarks
I	09.00	19. vi. 50	0.77	At flowering stage
II	14.00	1. ix. 50	0.73	Very dry; low yield of juice
III	—	30. xi. 49	1.08	Green and juicy; had been exposed to frosts

obtained in the wrong fraction or be irreversibly adsorbed during ion-exchange procedures.

The diaphragm-cell procedure of Theorell & Åkeson (1942) showed promise, particularly on account of their successful handling of glutamic and aspartic acids. By adding a special compartment for the anode, as in the electrolytic desalting apparatus of Consden, Gordon & Martin (1947), it was possible to collect these and other weak acids as a separate fraction without danger of them suffering anodic changes as in the original apparatus. The necessity for preliminary removal of chloride was thus also avoided. Fractionations of this kind may be of wide usefulness for biochemical separations, especially when weak acids are to be separated from bases, neutral substances and strong acids in a single operation. Work (1951; private communication) has evolved a similar arrangement for study of bacterial extracts and hydrolysates, and Sanger & Tuppy (1951) have made use of such a procedure in fractionating partial hydrolysates of insulin.

constructed from Perspex sheet. Each compartment had approx. 50 ml. capacity. The details of construction and assembly are shown in Fig. 2. The membranes were held between rubber gaskets. The two terminal membranes were of cellophan, and the middle membrane of commercial sheepskin parchment which had been kept overnight at room temperature in a 1:5 (v/v) aqueous dilution of B.P. formalin and then washed thoroughly with distilled water. Since the higher current densities led to weakening and perforation of the parchment membrane, perhaps on account of local heating, a greater hole area was used for it than for the cellophan membranes. The platinum-wire electrodes were placed close to the terminal membranes as recommended by Theorell & Åkeson. The contents of each compartment were stirred by a slow stream of air or N_2 . Cooling coils could be inserted as required into any compartment and temperatures were maintained at all times below 30° .

In operation, the cathode compartment was filled with 1% (w/v) aqueous NH_3 ; the solution for analysis was

Fig. 1. Scheme of fractionation of grass-juice diffusates.

During preliminary study it was found that an important fraction of the 'bound' amino-acids stayed with the neutral amino-acids during fractionation in the four-compartment cell, but did not move with these into the cathode compartment when the mixture was acidified and subjected to ionophoresis as for the isolation of ω -amino-acids by Synge (1951), and of diketopiperazines by Gavrilov and colleagues (Gavrilov & Balabucha-Popzova, 1934; Balabucha-Popzova, Gavrilov & Rikalewa, 1935; Gavrilov, Paradashvili, Balabouha-Popzova & Ljapounzowa, 1938; Balabouha-Popzova, Gavrilov, Paradachwili & Jakounine, 1938; Antonovich & Gavrilov, 1941). This observation formed the basis for obtaining these bound amino-acids free from other known N compounds by a second fractionation, this time in a three-compartment cell. The scheme of fractionation adopted is set out in Fig. 1.

Fractionation in four-compartment cell. The cell was of similar design to that of Theorell & Åkeson (1942), except that the compartments were open at the top. They were con-

adjusted to pH 6 and inserted in the next (specimen) compartment. The next (acetic acid) compartment was filled with 1% (v/v) aqueous acetic acid, to maintain its conductivity, and the anode compartment was filled with 0.1N- H_2SO_4 . 200 V. were then applied to the electrodes, though if much electrolyte was present such a lower voltage was applied in the early stages of the experiment as would keep the current below 200 ma. Where strong bases were present in the solution being analysed the current first rose as these entered the cathode compartment and increased its conductivity. The current then gradually fell as the specimen compartment was denuded of ions. The pH of the specimen compartment was checked repeatedly and maintained at pH 6 by additions of dilute NH_3 or acetic acid. Acetic acid was most often required in the early stages of a run and NH_3 later, but on many occasions the pH remained unchanged throughout. After 7 hr. the current had usually fallen to a constant value less than 5 ma., at which stage the fractionation was judged to be ended. Electroendosmosis took place into the cathode and

acetic acid compartments, but was not usually sufficient to cause overflowing or necessitate the withdrawal of solution. The contents of the cathode compartment could be removed at any stage and replaced with fresh aqueous NH_3 if it was desired to shorten the exposure of compounds which had migrated thither to the strongly alkaline conditions prevailing when salts had been present in the specimen. NH_3 was removed from the withdrawn solution by a short evaporation *in vacuo*, and then inorganic cations by subsequent treatment in the desalting apparatus of Consden *et al.* (1947). At the end of the run the contents of each compartment, with washings, were collected separately.

(aggregate recovery 101.0%). No Kjeldahl-reacting N was ever found in the anode compartment during any of the work described here. Presumably any nitrate present in the specimen migrated thither.

It was thus possible, by subjecting the contents of the cathode and acetic acid compartments to repeated fractionation, to obtain a very sharp quantitative separation of the basic, neutral and acidic components of the original mixture. For the purposes of the present exploratory work, however, a single fractionation only was used. The figures reported are without correction; the effect of correction would be to reduce still further the already low apparent quantities of

Fig. 2. Diaphragm cell assembled with four compartments: (a) sectional view from side. (b) end elevation of a compartment. A, cathode compartment; B, specimen compartment; C, acetic acid compartment; D, anode compartment; E, inlets for stirring gas.

On fractionating a mixture of alanine, ornithine and aspartic acid in this way the specimen compartment was found entirely free of ornithine and aspartic acid as judged by paper chromatography; ornithine with some contaminating alanine was found in the cathode compartment and aspartic acid, with a considerably smaller contamination by alanine, was found in the acetic acid compartment.

To obtain a quantitative picture of the amount of neutral material carried into the adjoining compartments a solution was made up to simulate crudely the non-nitrogenous dialysable constituents of a plant juice, with a single amino-acid, alanine, present: DL-alanine, 0.13; trisodium citrate dihydrate, 0.6; NaCl, 0.3; sucrose, 0.2% (w/v). At the end of the run, 3.8% of the alanine N was recovered in the cathode compartment (after removal of NH_3 by evaporation); 96.5% was found in the specimen compartment, 0.7% in the acetic acid compartment and nil in the anode compartment

basic amino-acids, but would otherwise not greatly affect the figures given.

As might be expected, histidine and the ω -amino-acids β -alanine and γ -aminobutyric acid were not always sharply separated into the cathode and specimen compartments respectively (cf. Sanger & Tuppy, 1951). However the filter-paper chromatograms showed very little histidine to be present and γ -aminobutyric acid and β -alanine made only small contributions to the ninhydrin- CO_2 figures (see below).

Fractionation in three-compartment cell. A three-compartment cell constructed as above was fitted with two cellophane membranes. The cathode compartment was filled with 1% (w/v) aqueous NH_3 , the anode compartment with 0.1N- H_2SO_4 . In the middle compartment was placed a solution of fraction 4-N, to which had been added 1% (w/v) of acetic acid. On applying 200 V. the resulting current remained substantially constant (approx. 50 ma.), and at

the end of 7 hr. all free monoamino-acids had entered the cathode compartment (cf. Synge, 1951). The greater part of the bound amino-acids remained in the centre compartment together with much carbohydrate material.

Application of the ninhydrin-carbon dioxide determination to the study of plant extracts

Despite its high specificity for α -amino-acids little use has been made so far of the ninhydrin-carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for studies of plant material. Neuburger & Sanger (1942) used it for the determination of glutamine and other amino-acids in potato. The present work has shown that difficulties, so far not adequately explained, are encountered, but that nevertheless its usefulness is great.

As a routine, the determinations were done with 50 mg. triketohydrindene hydrate and citrate buffer (pH 4.7) at a vol. of 3 ml., as described by Van Slyke *et al.*, except that sealed evacuated hard-glass test tubes ($6 \times \frac{5}{8}$ in.) were used as the reaction vessels. These were drawn with a shoulder (as shown in Fig. 3) so that they could be broken at a file scratch after connecting to the reaction chamber through a piece of pressure tubing (previously boiled out in dilute lactic acid).

Fig. 3. Reaction tube connected to side arm of Van Slyke-Neill manometric apparatus for ninhydrin- CO_2 determination (before breaking off tip from tube).

The reaction chamber, side arm and tubing were evacuated before breaking the reaction vessel. The reaction was done at pH 4.7 rather than 2.5 to avoid, as far as possible, hydrolysis of peptides or CO_2 formation from uronides during heating under the more acid conditions. However, there was the disadvantage that at pH 4.7 γ -aminobutyric acid gave 0.13 mol. CO_2 , whereas at pH 2.5 it gave no CO_2 ; Van Slyke *et al.* (1941) showed that β -alanine behaves similarly. These facts, as well as the yield of 2 mol. CO_2 from aspartic acid, should be borne in mind when interpreting the figures obtained.

Determinations were carried out on the original juices, and the fractions derived from them (a) without hydrolysis and (b) after hydrolysis for 24 hr. in 6N-HCl at 105° in sealed evacuated tubes. The HCl hydrolysates were evaporated to dryness *in vacuo* before analysis, but no further attempts were made to adjust the pH, the amount of citrate buffer used being regarded as adequate. All determinations were in duplicate, about 0.1 mg. carboxyl N (Van Slyke *et al.* 1941, Table 1) being taken for each.

Determinations on unhydrolysed materials. When the plant materials were subjected to blank determinations without adding ninhydrin to the reaction mixtures considerable CO_2 was evolved. This was not due to dissolved CO_2 , since prolonging the evaporation *in vacuo* before sealing the tubes did

not diminish the amount evolved. Accordingly, all determinations on unhydrolysed material were done by measuring the difference of CO_2 evolution in the presence and absence of ninhydrin, samples being treated similarly in every other way. During the fractionations it was found that the material responsible for the blank CO_2 migrated into the cathode compartment; blank determinations on neutral and acidic fractions rarely differed much from the reagent blank. It was sometimes observed during the three-compartment stage of the fractionation that, while the material taken gave no blank, that recovered from the cathode compartment gave a considerable blank. The effects are tentatively attributed to carbamino compounds, either originally present or formed by interaction with atmospheric CO_2 under the conditions prevailing in the cathode compartment. In support of this view it was repeatedly observed, while analysing cathode-compartment contents from the four-compartment fractionations, that the blank CO_2 actually exceeded that evolved in the presence of ninhydrin, recalling the effect of ninhydrin in inhibiting the evolution of CO_2 from urea (Van Slyke *et al.* 1941). In any case this effect completely prevented calculation of carboxyl N in the unhydrolysed fractions 4-B, and introduced some error into the corresponding figures for fractions 3-C and for the unfractionated juices. Fractions from other compartments probably gave more nearly correct results, the small amounts of CO_2 evolved from uronides, ascorbic acid, etc., being allowed for by the blanks.

Determinations on HCl hydrolysates. When ninhydrin was omitted these never gave CO_2 differing significantly from the reagent blank, and blank determinations were accordingly not done in every case. Here the main source of error is that due to destruction of amino-acids during boiling with acid in the presence of carbohydrates. This has been discussed frequently in connexion with the hydrolysis of proteins. Vickery, Pucher, Wakeman & Leavenworth (1937) quote figures for amino N after hydrolysing edestin with 6N-HCl in the presence of various carbohydrates. These show rather small losses. Tristram (1939) observed somewhat greater losses of basic amino-acids under similar conditions. Since the proportions of the more destructible amino-acids to total amino-acid residues are lower in the non-protein fractions than they are in common proteins, the errors here may be smaller still. At any rate the carboxyl-N figures for the acid hydrolysates of the various fractions on adding together agree rather well with the corresponding figures for unfractionated material, and it is felt that greater confidence can therefore be placed in these than in the carboxyl-N figures for unhydrolysed materials (see Table 2).

Determination of ammonia and amide N

Solutions were treated with at least 2 vol. of 10N-HCl and kept at 37° for 10 days (cf. Gordon, Martin & Synge, 1941; Rees, 1946), after which the mixtures were evaporated at low temperature *in vacuo* and analysed for NH_3 according to Conway & O'Malley (1942), liberating the NH_3 with K_2CO_3 . Free NH_3 was determined in the same way on the untreated solution, and the difference was recorded as amide N.

Determination of total N

This was done by a micro-Kjeldahl method. In view of the use of NH_3 in the cathode-compartment solutions, specimens from these were evaporated *in vacuo* at room temperature in the presence of excess NaOH before incineration.

*Two-dimensional filter-paper chromatography
of amino-acids*

Chromatography was done by the ascending method (Williams & Kirby, 1948) successively with ammoniacal phenol-water and commercial collidine-water (Yorkshire Tar Distillers Ltd., Cleckheaton) using 35 cm. squares of Munktell OB paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden). Samples usually contained 0.1 mg. N and were treated with H_2O_2 and ammonium vanadate on the paper (cf. Dent, 1948) to render visible methionine as the corresponding sulphone and cystine-cysteine as cysteic acid. The papers were dried below 40° , and the amino-acids were made visible by treatment with ninhydrin as usual, also below 40° ; this was found to give the greatest differences between the colours given by the various amino-acids (cf. Dent, 1948).

Fractions containing salt or alkali were usually desalted according to Consden *et al.* (1947) before chromatography. Neutral fractions all gave excellent chromatograms without special treatment. The acidic fractions, owing to the presence of plant acids, often gave spots that were streaky in the phenol direction. Leucine and isoleucine were not resolved, and are not differentiated in what follows. In nearly all chromatograms studied there were sufficient of the well known amino-acids for ready identification simply from their relative positions and characteristic shades of colour in the early stages of reaction with ninhydrin. In doubtful cases mixed chromatograms with authentic amino-acids were run.

RESULTS

The quantitative data obtained in analyses of the diffusates from grass-juice preparations I, II and III (Table 1) are set out in Table 2. During fractionation in the four-compartment cell most of the orange-brown colouring matter of the diffusates tended to deposit itself on the parchment membrane; however, with some preparations, a bright yellow-green pigment entered the cathode compartment. The contents of the specimen and acetic acid compartments were usually pale yellow at the end of a run.

All the fractions were examined for ninhydrin-reacting substances by filter-paper chromatography before and after hydrolysis with 6N-hydrochloric acid for 24 hr. at its boiling point. Since the individual fractions gave better chromatograms than the unfractionated material, and since nothing was seen with the latter that was not seen with the former, only the observations on the fractions are described.

Fractions 4-B

Before hydrolysis. Arginine, lysine and γ -aminobutyric acid were always present, together with sometimes faint traces of histidine and of the two abundant neutral amino-acids alanine and glutamine.

After hydrolysis. The same, with additional very faint spots of nearly all the neutral and dicarboxylic amino-acids, among which leucine-isoleucine and valine were especially prominent.

Fractions 4-A

Before hydrolysis. Only glutamic and aspartic acids, with occasional faint traces of alanine and glutamine.

After hydrolysis. The same dicarboxylic acids; glutamine disappeared, alanine appeared or if previously present increased in intensity, and well defined, though not strong, spots due to phenylalanine, leucine-isoleucine, valine, β -alanine and glycine appeared. On one occasion a weak proline spot appeared.

Table 2. *Distribution of nitrogen in rye-grass juice diffusates and ionophoretic fractions therefrom*

(For methods of fractionation and determinations see text.)

	N as % of total N of diffusate from specimens (Table 1)		
	I	II	III
Unfractionated diffusate:			
Free ammonia	Nil	Nil	—
Amide N	28	22.4	—
Carboxyl N before hydrolysis	52.4	54	42
Carboxyl N after hydrolysis	54.5	58	48
γ -Aminobutyric acid (Synge, 1951)	—	—	6.2
Fraction 4-B (bases):			
Total N	25	38	25
Carboxyl N before hydrolysis	*	*	(1.9)*
Carboxyl N after hydrolysis	2.6	4.9	2.5
Fraction 4-A (weak acids):			
Total N	16.5	13	26
Amide N	Nil	2	—
Carboxyl N before hydrolysis	16.1	10.7	20.2
Carboxyl N after hydrolysis	15.9	11.3	21.6
Fraction 4-N (neutral substances):			
Total N	55	47	46
Amide N	11.8	10	—
Carboxyl N before hydrolysis	24.4	19.3	16.7
Carboxyl N after hydrolysis	39.6	32.4	24.6
Fraction 3-C (zwitterions):			
Total N	49.0	37.2	37.6
Carboxyl N before hydrolysis	19.6	23.6	21.8
Carboxyl N after hydrolysis	19.1	28.0	22.5
Fraction 3-M (uncharged substances):			
Total N	5.5	5.2	9.9
Amide N	1.0	1.3	—
Carboxyl N before hydrolysis	0.6	0.2	0.06
Carboxyl N after hydrolysis	9.0	3.7	5.2

* Not calculable (see text).

Fractions 4-N

Before hydrolysis. The following amino-acids were always seen: (strong) alanine, glutamine, γ -aminobutyric acid and serine; (medium) leucine-isoleucine, valine, proline; (weak) phenylalanine, tyrosine, β -alanine, glycine, threonine. Asparagine was variable, ranging from absent to medium; cysteic acid ranged

from absent to faint, and no indication of methionine was obtained.

After hydrolysis. Glutamic and aspartic acids appeared, glutamine and asparagine (if present) disappeared. No other new spots were seen, but the spots corresponding to leucine-isoleucine, valine and glycine nearly always showed obvious increases in intensity.

Fractions 3-C

Before hydrolysis. Same as 4-N.

After hydrolysis. Glutamine disappeared and glutamic acid appeared; if asparagine had been present it disappeared and aspartic acid appeared. Otherwise unchanged by hydrolysis.

Fractions 3-M

Before hydrolysis. Usually no material colouring with ninhydrin (nor was any colour seen during ninhydrin-carbon dioxide determinations on these fractions). Occasionally traces of aspartic acid were seen, which could be attributed to slight hydrolysis under the acid conditions of fractionation in the three-compartment cell (cf. Partridge & Davis, 1950).

After hydrolysis. Glutamic and aspartic acids, leucine-isoleucine, valine, alanine, proline and glycine appeared as spots of fairly equal intensities, although it was obvious that the relative proportions varied somewhat from preparation to preparation.

DISCUSSION

Free amino-acids

The free amino-acids found in the rye-grass diffusates are in general similar in nature and relative proportions to those noted by other workers with other plant juices (for references, see Introduction). Glutamine, alanine, serine and glutamic, aspartic and γ -aminobutyric acids predominate. In this connexion it is of interest that Vickery (1925*b*) isolated alanine and the then little-known serine from alfalfa juice. These two amino-acids also seem to be the first into which the carbon of newly assimilated carbon dioxide is incorporated by the plant (Calvin, Bassham & Benson, 1950; Vernon & Aronoff, 1950). Other common amino-acids are present in lower concentrations. Methionine and hydroxyproline were not seen. It has long been known that the proportions of glutamine and asparagine vary very much from species to species. It has more recently become apparent that the other free amino-acids of plants also differ more from species to species in their quantitative distribution than does the amino-acid composition of the tissue proteins (cf. Allsopp, 1948). Nevertheless, the data that have accumulated suggest a general similarity between the various species studied, and it is perhaps permissible to speak of a typical pattern of concentrations of free amino-acids in plant juices.

'Bound' amino-acids

Many workers with plant juices have referred to 'peptide nitrogen' and have made estimates of the amount present. Thus Vickery *et al.* (1937) found 0.05–0.26 g. 'peptide N'/kg. of fresh tobacco leaves. Such figures were obtained by measuring the increase in amino nitrogen on hydrolysing material which had been freed from protein by the use of heat or precipitants, and leave little doubt as to the existence of fair amounts of such non-precipitable non-heat-coagulable material. The ninhydrin-carbon dioxide data in the present work suggest that only a proportion of this is of such low molecular weight as to be able to pass through cellophan. Furthermore, in agreement with other workers referred to in the Introduction, in the present work no materials colouring with ninhydrin were seen on the chromatograms which disappeared to give amino-acids after acid hydrolysis, excepting of course the amides glutamine and asparagine. Simple open-chain peptides of low molecular weight are therefore not present in quantity.

Such peptides seem to occur in marine algae. Haas and colleagues have isolated from a number of seaweeds diffusible products having amino nitrogen and yielding on hydrolysis various amino-acids (Haas & Hill, 1931, 1933*a, b*; Haas, Hill & Russell-Wells, 1938; Haas, 1950). One such preparation, from *Pelvetia fastigiata*, has recently been studied in detail by Dekker, Stone & Fruton (1949), who tentatively formulated their product as L-pyrrolidonoyl-L-glutamyl-L-glutamine, and thought that it might have arisen by lactam formation from L-glutamyl-L-glutamyl-L-glutamine during the isolation, which involved heat treatment. A peptide-like substance 'eisenine' was isolated by Ohira (1939, 1940*a, b*) from *Eisenia bicyclis*, using a similar procedure to that of Haas, and yielded on hydrolysis L-alanine, L-glutamic acid and ammonia. Ohira formulated eisenine as pyrrolidonoylglutamyl-L-alanine. This conclusion has been criticized by Tazawa (1949). If peptides of this kind were present in rye-grass, they would be found in fraction 4-A if in the pyrrolidonoyl form or in fraction 3-C if in the glutamyl form. However, the increase of carboxyl nitrogen on hydrolysing these fractions is slight.

Some positive conclusions can, however, be drawn as to the types of compound embodying bound amino-acids that occur in the rye-grass diffusates and these are set out below.

Basic compounds (fraction 4-B). Small amounts of a wide variety of neutral and dicarboxylic bound amino-acids occur in this fraction. These are therefore presumed to be linked to basic substances. Whether compounds are also present that yield basic but not other amino-acids on hydrolysis it is impossible to decide on the present evidence.

Vickery (1924*b*), using precipitation methods, obtained evidence for bound arginine and bound lysine in alfalfa juice, but, as already stated, there is no evidence that his materials were diffusible.

Acidic compounds (fraction 4-A). Judging from the ninhydrin- CO_2 figures for this fraction, only small quantities of bound amino-acids were present. As judged from the chromatograms, these consistently included phenylalanine, leucine-isoleucine, valine, alanine, β -alanine and glycine. Glutathione would be found in this fraction, and cannot therefore have been present in quantity. In addition to peptides, acyl derivatives should be considered as possibly responsible for the amino-acids liberated. Pantothenic acid is an obvious possible precursor of the β -alanine, and simpler acyl derivatives of amino-acids may occur (cf. Manske's (1937) isolation of δ -*N*-acetylornithine from *Corydalis ochotensis*).

Neutral compounds. The greater part of the bound amino-acids in the rye-grass diffusates stayed with the neutral amino-acids during fractionation in the four-compartment cell, and was recovered in fraction 4-*N*. It was unexpected to find that this material behaved as if devoid of ionizable groupings, a large part of it being recovered in fraction 3-*M* at the second stage of the fractionation. Some unaccounted losses of bound amino-acids occurred during this stage, if the carboxyl nitrogen figures are used as a basis of calculation, but the procedure used made it easy to isolate what is at least a substantial part of the bound amino-acids free from other known compounds of nitrogen, and the simplicity of the procedure should facilitate future studies.

This fraction accounts for 5–10% of the nitrogen of the diffusate. Only a few different amino-acids are set free on acid hydrolysis. Their relative proportions differ somewhat from specimen to specimen. They are leucine-isoleucine, valine, proline, alanine, glycine and glutamic and aspartic acids. From the presence of amide nitrogen it may be inferred that these last occur as glutamine and asparagine residues. The compounds in question are, judging by their ionophoretic behaviour, devoid of ionizing groups, and this is confirmed, as concerns amino groups, by the absence of colour reaction with ninhydrin by the unhydrolysed material.

The possibility of these amino-acid residues being present as cyclic peptides (Synge, 1949; Bailey, 1951) is one that immediately suggests itself. However, since much carbohydrate (probably mostly glucose, fructose, sucrose and lower fructosans) is present in the same fraction, the possibility of the amino-acid residues being bound to carbohydrate is not excluded. Such complexes might conceivably arise under natural conditions by a non-enzymic reaction between free amino-acids and carbohydrates. However, to judge from the observations of Gottschalk & Partridge (1950), the amino-acids in

question should not be very reactive with reducing sugars and the resulting compounds would possess ionizing groups. Nor would the conditions used during the isolation (avoidance of heat, high concentrations or alkaline pH) favour the formation of such compounds as artifacts (cf. Lea, 1950). Some of the diminution in amino nitrogen observed by Vickery *et al.* (1937, pp. 780–5) during the drying of tobacco leaves which had been exposed to light might well, however, be due to such a cause. These workers found that this amino nitrogen was liberated again on acid hydrolysis, and postulated interaction with some or other substances involved in the metabolism of carbohydrate.

The different categories of non-protein nitrogen

From the data presented here, taken with those on γ -aminobutyric acid (Synge, 1951), it is possible to draw up a very rough quantitative expression of the distribution of the non-protein nitrogen of rye-grass (Table 3). There is a considerable non-amino-acid basic fraction, presumably containing such bases as choline and purines (cf. Vickery's work on alfalfa already cited and Neuberger & Sanger, 1942).

Table 3. *Approximate quantitative distribution of non-protein nitrogen of rye-grass diffusates*

	N as % of total N of diffusates
Non-amino-acid bases	15–25
Basic amino-acids, peptides, etc.	6–15
γ -Aminobutyric acid	5–10
Glutamine	20–25
Other free neutral amino-acids	10–15
'Bound' amino-acids in neutral compounds	4–10
Free glutamic and aspartic acids	10–20
'Bound' amino-acids in acidic compounds	1–3

Presumably any betaines occur in fractions 4-*N* and 3-*C*. Apart from these it is evident, from comparing the chromatographic data with the carboxyl-nitrogen figures, that most of the nitrogen is accounted for as well known amino-acids. The predominance of 'non-essential' amino-acids in this mixture readily explains the poor nutritional results when plant non-protein nitrogen is substituted for protein in non-ruminant animals (e.g. Henriques & Hansen, 1907; Slack, 1948). In fact, subsequent work has done little to modify views on this problem since Schulze (1908) appraised it in the light of his long analytical experience.

SUMMARY

1. A diaphragm-cell arrangement has been adapted for the quantitative ionophoretic fractionation of plant juices by adding an extra compartment to the apparatus of Theorell & Åkeson (1942). While it has been used here for separating nitrogen compounds according to the net charges on their molecules it should also prove useful for separations

of carbohydrates, mineral constituents, phosphoric esters, plant acids, etc.

2. The ninhydrin-carbon dioxide determination has been used for measuring free and 'bound' α -amino-acids in plant juices, and in this certain difficulties have been encountered.

3. The above techniques, together with qualitative two-dimensional filter-paper chromatography of amino-acids, have been used in studying those organic nitrogenous constituents of the juice of rye grass (*Lolium perenne*) which can diffuse through cellophan.

4. The bulk of this non-protein nitrogen was made up by basic substances and by well known amino-acids in the free state.

5. Small quantities of bound amino-acids were

found in the form of basic and acidic compounds, but the greatest part of the bound amino-acids, representing 4–10 % of the diffusible non-protein nitrogen, was isolated ionophoretically in a fraction rich in carbohydrates but devoid of free amino-acids.

6. The material in this fraction seemed to be devoid of free amino or carboxyl groups, and on acid hydrolysis yielded ammonia, leucine and/or isoleucine, valine, proline, alanine, glycine, aspartic and glutamic acids.

7. The present observations are discussed in relation to previous work on the free and bound amino-acids of plant juices.

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