CHANGES IN PEPTIDES AND OTHER NON-PROTEIN NITROGENOUS CONSTITUENTS OF HERBAGE DURING CONSERVATION

by

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GENERAL INTRODUCTION

Crop conservation

Conservation of forage crops is practised in all countries where climatic conditions cause growth of herbage to stop for a period of the year, thus depriving grazing animals, especially cattle, of a sufficiency of their natural food. This explains the need for crop conservation in Britain, where grass may stop growing for up to five months during the winter. Our agricultural economy is extensively based on the sale of stock and animal produce. Thus it is important for winter food to be of good quality and for as much as possible to be derived from home-grown crops.

The aim of crop conservation is to retain as nearly as possible the nutritive value of the starting material at its optimum stage of growth. However, the ideal of unimpaired food value has not been achieved by any economic method of conservation. The limitations of our conservation techniques force us to supplement rations by root crops, which are unnatural foods, and concentrates, which are expensive. Thus we must use the methods available to the fullest advantage, and at all times must seek improvement by technical and fundamental research.

Grass, clover and lucerne are the crops most commonly used in conservation, and the present work deals with chemical changes in grass silage, and in wilted grass, which simulates hay, and is often recommended as a preliminary to ensilage.

After grass has been cut the cells will live for a considerable time provided moist aerobic conditions are maintained. Respiration of carbohydrate causes depletion of dry matter, and there is simultaneous proteolysis with production of peptides, amino acids, amides and ammonia. Protein breakdown

is a typical feature of the chemistry of excised leaves. Growth of bacteria and fungi on the grass can cause further destruction of cell nutrients. The activities of cell enzymes and micro-organisms can be arrested by desiccation or by acidification. The basic principle applied in the common methods of conservation is that either of these conditions should be created as soon as possible after reaping. The former condition is aimed at in haymaking and the latter in ensilage. In these two commonest techniques, the drying or acidifying are achieved naturally, but other processes simulate ideal hay or silage by rapid artificial adjustment to the required state.

Artificial drying of the crop is the most efficient method of conservation in that there is higher retention of nutrients than by any other process (1a). This technique is however expensive to apply, and is often uneconomical.

Although haymaking is the most widely practised form of conservation in Great Britain it is also the least satisfactory. The rate of drying in the field is governed by the humidity of the atmosphere. Even in ideal weather, losses of dry matter will be at least 10-15%, and in humid weather this figure is often doubled or trebled (1b). When rain falls on the swathes much of the soluble nutrients may be leached out. These losses represent the most digestible substances in the plant, so that nutrient losses are much more than the depletion of dry matter indicates. In the drying stage losses of crude protein need not be heavy, but since hay is usually made in the late flowering or seeding stage, mechanical losses of high-protein seeds are unavoidable when the dry material is transferred to the rick. If hay is stored before the plant cells have been killed by desiccation, continued respiration will cause overheating, and subsequent fermentation may spoil the product completely. Hay has the inherent disadvantage that the starting material has higher fibre content, higher starch equivalent, lower protein content and lower digestibility

than younger grass (2a). In fact, grass is not used for hay at its optimum period of growth which is in the preflowering stage. This is because hay is not intended to replace grass completely as a food, but largely to provide bulk in a ration supplemented with roots and concentrates.

In contrast, silage can be made from grass at this nutrient-high stage, and may be used as the sole winter feeding-stuff for milk production and fattening. Ensilage is also much less dependent on the weather for success, and is intermediate in efficiency between artificial drying and haymaking (1a).

Ensilage means the compaction of the crop into air-tight containers, the material being preserved by bacterially produced lactic acid which at best should lower the pH of the mass to 3.8 to 3.9. At this pH plant proteolytic enzymes are inactivated, respiration is reduced or inhibited, and undesirable bacterial fermentations are prevented. Aciduric fungi do not grow because of the anaerobic conditions.

After the grass has been packed into its container, or silo, respiration continues until the oxygen which has been unavoidably trapped during packing is exhausted. During this time energy released in the respiratory oxidation is dissipated as heat, causing the temperature to rise more or less depending on the amount of oxygen available. There then follows a short period of anaerobic respiration during which cell enzymes use chemically bound oxygen to oxidise carbohydrate to short chain fatty acids, other organic acids, alcohols and carbon dioxide. Heat is also evolved in this process. Anaerobic respiration ceases when the cells die due to either the elevated temperature or lack of certain metabolites. Micro-organisms which were present on the surface of the fresh grass then begin to multiply, using the dead cells as a medium. The course taken by the subsequent microbial fermentation will determine the final quality of the silage (1c).

In 1912 Esten and Mason (1d) concluded that bacterial action was responsible for acid formation in silage, and added that acidity prevents harmful bacterial fermentations. This latter fact has been continually stressed, and it is the basic principle of all silage making that acid conditions must be created as early as possible.

The bacteria which produce lactic acid in silage belong principally to the genus Lactobacillus, of which most species have been detected. Other lactic acid producers have been found such as Leuconostoc and Streptococcus (1e,3). Lactic acid bacteria are present on the surface of fresh herbage and in silage they multiply most actively in the first three days after packing (1f, 4), although the rate and amount of growth depend on properties of the herbage and temperature (3, 4). Lactic acid bacteria show little or no proteolytic activity, and lactic acid is itself a food for animals.

The organisms which it is most necessary to inhibit are true anaerobes, mostly species of Clostridium. These bacteria are also present on fresh grass. Like lactic acid organisms, they begin to multiply soon after the silo is filled and their rate of growth & maximum count are similarly dependent on conditions in the silage. Of the Clostridia found on herbage some species are strongly proteolytic and others ferment sugars and lactic acid to butyric acid. Butyric acid gives silage an unpleasant odour and milk may be tainted by feeding butyric acid-containing silage to cattle. The proteolytic organisms destroy proteins and degrade amino acids to ammonia and amines. Certain amines, especially histamine from histidine, cadaverine from lysine and putrescine from arginine via ornithine are toxic, and the last two have been reported in bad silage (1g, 2b). In addition, Cl. Welchii, which is sometimes found in bad silage, has pathogenic tendencies.

The destruction of silage by these bacteria can be prevented if the pH of the mass is rapidly lowered. Virtanen (1h) showed that vegetative Clostridia could not multiply below pH 4.2, although a pH of 3.5 is necessary to kill the spores (1i).

The whole success of silage making hinges on creating a medium which is optimum for rapid and sufficient growth of lactic acid bacteria, with Clostridia being kept in subjection. Temperature, original microbial population, fermentable carbohydrate, carbohydrate-protein ratios, moisture content and oxygen tension have all been listed as important in controlling the fermentation. It is now clear that no single condition is essential for success, and that although one variable, if unfavourable, may lead to bad silage it is often possible to adjust it or others to offset the initial disadvantage.

The lactic acid of silage is produced from fructosan, oligosaccharides and monosaccharides present in the herbage. It is axiomatic that unless the crop contains enough fermentable carbohydrate, the lactic acid formed will not reduce the pH to a safe value. When the crop is low in fermentable sugars a carbohydrate source such as molasses is usually added during packing. This procedure is necessary with young leafy grass and with lucerne and clover. These plants have high protein contents, and unless sugar is added, successful ensilage is difficult. Recent work has indicated that the ratio carbohydrate to protein is more important than the actual level of carbohydrate (5). This view is supported by results from Edinburgh, where lactic acid bacteria and proteolytic bacteria were found to multiply actively and reach their maximum count simultaneously (3). Since Lactobacilli require a higher carbohydrate-protein ratio than Clostridia it may be deduced that the lower this ratio the more vigorous will be the production of ammonia and bases, and the weaker the production of lactic acid. If lactic acid accumulates too slowly the bases

will maintain a high pH. The conditions will thus continue to favour the growth of Clostridia which must ultimately destroy the greater part of the protein and free amino acid of the silage. All other conditions being equal it may be possible to define a ratio of carbohydrate to protein, which must be exceeded if the required degree of acidity is to be achieved.

Since we are dealing with a bacterial process the temperature of the mass will determine in part the types of micro-organisms which multiply most actively. Several workers have advocated silage-making at either high temperatures (warm fermentation) or low temperatures (cold fermentation). Fry (1j) was the first exponent of warm fermentation and achieved temperatures of 50°C by loose filling of the silo. Such high temperatures prevent growth Clostridia, but at the expense of carbohydrates and with loss of protein digestibility. In cold fermentation the herbage is very tightly packed and is often crushed or chopped beforehand. The free oxygen is reduced to a minimum and the temperature does not exceed 30°C. Both these processes are designed to avoid the temperature range in which butyric organisms are most active, but this is also the optimum range for lactic acid bacteria, and good silage can be made in the 30°-50° range. In a large number of experiments at Jeallott's Hill (1k) good silage was made at temperatures between 27° and 38°. This has been called low temperature fermentation. Silage made in this range is easier to prepare than cold silage and is less expensive on nutrients than warm silage. Control of respiratory heating is most important for high-protein herbage, which should be packed very tightly, in order to keep the temperature at a minimum. If molasses is added the silage temperature can safely be raised by looser packing.

Gibson et al. (5) have made a close study of the bacteria found on fresh grass at different seasons and stages of growth. They have also attempted

to relate the original bacterial seeding to the development of each group of organisms during ensilage. The dominant organisms of fresh grass are strict aerobes, with Lactobacilli and Clostridia present in small, variable amounts. It was found that the greater the original Lactobacilli count, the more rapidly did the pH fall. It must be concluded that the original concentration of Lactobacilli determines in part the rate of fall of pH, probably by limiting the maximum count. One obvious way to assist rapid acidification is to inoculate the silage with a suitable lactic acid bacterium. This procedure could not be expected to succeed unless the herbage contained enough fermentable sugars. The inoculum is usually added in a carbohydrate medium.

Wilting of grass before ensiling has been recommended. The moisture content of fresh grass is usually about 80%, and this should be reduced to 65-70% before packing. Prewilting reduces the flow of effluent from the silo and in practice a high dry matter content of silage is desirable. If very wet herbage is packed directly anaerobic conditions are rapidly created, and this seems to favour growth of Clostridia over Lactobacilli in the early stages.

Several workers have called for closer collaboration between biochemists and bacteriologists to discover more about the exact nutrient supply to Lactobacilli in ensilage (3, 6). In the important early stages the bacteria grow on the surface of the herbage and use nutrients exuded from the cells. The composition of the cell fluids is known to vary with season and age of grass and most information is available on seasonal fluctuations of free sugars (7). Seasonal changes of amino acids have not been studied in this context in spite of the fact that all Lactobacilli show requirements for specific amino acids. From the lack of variety of Lactobacilli species found in any particular crop (3), one would expect that deficiency of certain amino acids could affect growth of Lactobacilli. However, the results of Kemble (8) show that there is no

lack of neutral amino acids except tryptophan one day after packing. Virtanen found very little tryptophan in ordinary silage, but only half of the tryptophan released in proteolysis was destroyed in A.I.V. silage (see below).(9)

Much work has been done on the preservation of herbage by addition of acid or other bacteriostatic agents while the silo is being packed. The most outstanding contribution in this field is that of A.I. Virtanen and others in Finland, who have developed a method in which a mixture of sulphuric acid and hydrochloric acid is sprayed on to the crop during filling. When the pH of the mass is adjusted in this way to 3.5-4.0 there is much less loss of true protein than ordinary silage of the same pH because of the relatively rapid inactivation of cell proteolytic enzymes.

Many other additives have been tried which are purely bacteriostatic in their function. These include sodium metabisulphite which is widely used in the United States. Treatment with formic acid, phosphoric acid, sulphur dioxide, urea, carbon dioxide and inorganic salts have also been tested, but none of these substances are more efficient preservatives than A.I.V. acid or lactic acid.

Proteolysis and amino acid distribution in conservation

The true protein of fresh grass usually amounts to 80-90% of the total nitrogen and from 2-4% of the dry matter, depending on the season. According to Watson (11) the loss of true protein in ordinary silage is 30-40%. In silage the hydrolysis of protein to amino acids is thought to be primarily caused by plant enzymes, although in bad silage bacterial proteolysis takes place. Amino acids are deaminated by both plant and bacterial enzymes. Of the various stages in protein degradation it is most important to limit bacterial

deamination. This is because of the neutralising effect of ammonia and also because ammonia as a source of nitrogen is inferior to amino acids, being less efficiently utilised by the animal.

Proteolysis and deamination are prevented by acidification and the more rapidly the pH falls at a particular temperature, the less will be the protein and amino acid breakdown. In practice it is easier to limit bacterial processes than plant enzyme action, and some degree of breakdown by plant proteases is almost inevitable. Rapid acidification of the crop to 3.5-4.00 as in the A.I.V. process can reduce protein losses to 10-20% (11). Any breakdown which does occur in A.I.V. silage occurs during the percolation of the acid through the mass. Using small scale silos made from grass sap Macpherson was able to eliminate proteolysis completely by adjusting the pH to 3.8 with hydrochloric acid (10). In this case of course the acidification was instantaneous. Macpherson also found that with sap silage prepared without any additives the normal fermentation of sugar to lactic acid took place, and proteolysis ceased when the pH reached 4.3. The role of acid in inactivating leaf proteases is emphasised by later experiments in which metabisulphite was added to prevent growth of bacteria (11). In metabisulphite silage, which has a pH higher than ordinary silage, 90% of the protein was degraded whereas in ordinary silage made from the same grass, but inoculated with Lactobacilli a vigorous lactic fermentation reduced the pH to 4.0 in three days, and then proteolysis was retarded, the final protein breakdown being 75%. This figure is higher than usual for ordinary silage, whereas the 90% loss in metabisulphite silage is common.

Watson states that in ordinary silage ammonia usually represents 5-10% of the total nitrogen (11). The relative importance of plant and bacterial deamination is shown by the metabisulphite experiment referred to above, in

which, although 90% of the original protein had disappeared, ammonia accounted for only 2.5% of the total nitrogen. Watson's collected figures for A.I.V. silage show that only 2-4% of the total nitrogen is ammonia nitrogen, and in molassed silage, where lactic acid accumulation is rapid, the figure is similar. This low ammonia content is sometimes found when the silage is inoculated with lactic acid bacteria (8).

In assessing silage quality by chemical means, pH, volatile base nitrogen and volatile acidity are considered the most important measurements. Watson has established an inverse relationship between pH and ammonia content (11). Virtanen lays most stress on the ammonia content for estimating quality (1m), having found as much ammonia in silage at pH 5.6 as 4.5. Other workers consider that pH, ammonia and volatile acids should all be measured for accurate rating of silage (12).

Kemble and Macpherson (13) have demonstrated that the neutral amino acid composition of protein in silage is similar to that in fresh grass. However, Kemble (8) found that the free neutral amino acid composition is very different from that of protein in silage. Since non-protein nitrogen in silage can comprise 75% of the total nitrogen it follows that the overall amino acid composition may be considerably changed compared with the fresh crop. From Kemble's results, the overall neutral amino acid losses vary between 4% (Leucine) and 27% (Glycine). However, these losses must be considered as apparent until chemically bound amino acids in the non-protein fraction have been measured. Kemble and Macpherson have measured the free neutral amino acid composition of wilting grass, and the results probably indicate the nitrogen distribution in hay (14). As with silage there were considerable changes in the overall amino acid composition with apparent depletion of many amino acids, although the reservation concerning bound amino acids must again be made.

For all animals certain amino acids, which vary from species to species, must be supplied in the diet, for they cannot be synthesised by the animal. It has been amply demonstrated that the feeding value of any protein source is determined by the concentration of these essential amino acids.

Nearly all the silage made in Britain is fed to ruminants. In these animals most of the protein digested in the true stomach is contributed by the bodies of micro-organisms which have developed in the rumen, and which contain the full range of amino acids required by the animal. Thus although a range of essential amino acids exists for ruminants (15), the need to supply them in the diet appears to be eliminated. In assessing the protein value of a certain food it may be more important to consider the essential amino acids of the rumen micro-organisms. Sheep and cattle can thus live on a diet which is unbalanced as regards amino acid composition, or even one in which much of the nitrogen is non-protein and non-amino acid in nature. This is well shown by the increasing use of urea as a protein sharer in ruminant feeding. Sheep have been reared on diets containing 30% of the nitrogen in urea or ammonium salts (16).

It has been pointed out that the independence of ruminants for the amino acid balance of their diet has only been clearly demonstrated by urea feeding trials (17). Very few detailed comparisons have been made of the relative values of different protein sources. It seems wise to extend our knowledge of the precise nitrogen distribution in silage or hay, and from time to time relate the findings to other biochemical, physiological and micro-biological studies of rumen function.

One object of the present research was to continue the work of Kemble and Macpherson by measuring changes in distribution of the basic and dicarboxylic amino acids in silage and wilted grass. It was also intended to estimate the amounts of these amino acids existing in chemically bound form in protein-free

fractions. The latter measurements are considered important if the total content of any particular amino acid in silage or hay is required.

Contributions on the Nitrogen metabolism of excised leaves

One of the greatest problems in plant physiology has always been to establish the mechanisms by which protein is synthesised and degraded in the living plant. In the normal plant at least a part of the protein is subject to continual turnover, with breakdown being balanced by synthesis. The hypothesis of Alcock (18) suggested that synthesis is a reversal of hydrolysis, but few workers subscribe to this view, mainly on the grounds of non-specificity of hydrolytic enzymes, and many alternatives have been proposed. When a thorough search has been made, all the constituent amino acids of protein have usually been found in deproteinised cell fluids. This contributes to the view of some plant physiologists that all these amino acids must be present simultaneously for protein synthesis to proceed, and that amino acids are necessary precursors of protein. Circumstantial evidence in favour of this idea is the existence of essential amino acids for most organisms and it seems logical that these at least are used directly in protein synthesis. In the extreme theories of Haurowitz (19) and Chantrenne (20) the amino acids are considered to come together on a template, and then be fused in a single multimolecular reaction. Such hypotheses diverge from modern biochemical reasoning, for all processes which have been studied in detail take place through a series of simple chemical reactions.

Yemm and Folkes support the theory that amino acids are precursors in protein synthesis and have reviewed most of the recent evidence in the light of this concept (21).

Interest has been focussed on attempts to identify intermediates in the synthesis of protein from amino acids. In many studies the presence of a

peptide or bound amino acid fraction has been recognised, usually by indirect means in which amino acids are determined before and after hydrolysis of a protein-free extract. Synge (22) has reported that 5-10% of the nitrogen of diffusates from perennial rye-grass juice is present in chemically bound amino acids. Most of these bound amino acid compounds are strongly anionic, containing organic acids, and only a small proportion are true peptides (23). Bathurst (24) has made a quantitative survey of bound amino acids in various grasses and found all the protein amino acids in his "peptide" fraction. It is not certain yet whether peptides are important in protein synthesis. Work discussed by Yemm and Folkes has shown that C^{14} amino acids enter the peptide fraction more rapidly than the protein fraction, but turnover of these amino acids from peptide to protein was not proved conclusively, and the results may arise from a parallel labelling of separate metabolic routes (21a).

An opposing theory of protein synthesis is proposed by Steward and Pollard (25). Carbon skeletons derived from sugar, and nitrogen supplied by donors such as glutamine are considered to condense at a site in the cell which supplies the necessary template and energy, but which precludes direct participation of free amino acids. The free amino acids of plant fluids are therefore not precursors of protein, but are catabolic products.

The mode of action of many plant proteolytic enzymes is well known, and the main difficulties in the whole problem of protein metabolism are the synthesis itself and the mechanisms by which amino acids released in the catabolic stage are metabolised and their nitrogen made available again for protein synthesis.

Bonner (26), and Chibnall (27) have reviewed work on the nitrogen metabolism of excised leaves. We have seen that proteolysis is a typical feature of the chemistry of excised leaves. If the leaves are left in the light with their petioles in water, respiration and photosynthesis continue, and there are

isolated reports of protein synthesis when leaves from plants maintained in a nitrogen-deficient regime are floated in a medium rich in nitrate (26a). For normal leaves however, proteolysis appears to be a direct result of excision. Chibnall has suggested that protein synthesis could be subject to hormonal control, and that if this is so, the hormone must be supplied from the stem or roots (27). Wood and Cruickshank (28) have measured glutamic acid, tyrosine, arginine and tryptophane in excised leaves of Kikuyu grass and Avena sterilis, and found that all four appeared to be rapidly metabolised after release from protein. This led to the suggestion that amino acids which are unstable in the free form in the leaf are the factors which control protein synthesis since they may need to be supplied from other parts of the plant.

The study of nitrogen metabolism in wilted grass and silage provides an opportunity to extend our knowledge of the metabolism of amino acids formed in protein breakdown. By excision of leaves we prevent protein synthesis and unmask the hydrolysis which balances synthesis in the whole plant. By studying changes in the nitrogen distribution of excised leaves we might therefore gain valuable data on the initial pathways in recycling the nitrogen back into the anabolic scheme.

Since the present work is part of a programme of crop conservation research we were most interested in studying nitrogen metabolism under three conditions. These are anaerobiosis in darkness (silage), aerobiosis with loss of moisture ("dry wilting") and aerobiosis without loss of moisture ("moist wilting"), all conditions met with in conservation practice.

The results of Kemble and Macpherson (8, 14) show that each condition provides a distinctive nitrogen distribution. In all cases, most amino acids appeared to be depleted after proteolysis. In silage, deamination is important, and ammonia accumulates. All the losses of amino acids cannot be accounted for by deamination because the amount of ammonia they represent

exceeds the total ammonia found in the silage, and in the above work no account was taken of changes in basic and dicarboxylic amino acids. It follows that at least some of the amino acids undergo conversion to different nitrogenous compounds after release from the protein. It is possible that some amino acids are not destroyed, but exist as peptides or are bound to non-amino acid residues. Kemble considers that in silage very little of the non-protein nitrogen is present in bound amino acids, since he found only 1-2% precipitable in 75% ethanol, and no apparent increase in amino acid nitrogen after hydrolysis of the alcohol soluble fraction.

In moist wilting very large amounts of amide, especially asparagine, are found, and may contribute up to 40% of the non-protein nitrogen.

In dry wilting little free ammonia is formed and the amide synthesised is much less than in moist wilting. All the free amino acids again appear to be depleted, with the exception of proline which accumulates in very large amounts. Kemble found that after 8 days the free proline was 50% more than that present in the original protein, even although only one-fifth of that had been degraded. No information is available on bound amino acids in wilted grass.

An interesting feature of recent studies of amino acid metabolism has been the recognition of families of closely related amino acids (21). These are:

a) glutamic family - glutamic acid, proline, arginine

b) aspartic family - aspartic acid, methionine, threonine, isoleucine

c) serine family - serine, glycine, cysteine

d) pyruvic family - alanine, valine, leucine

e) aromatic family - tyrosine, phenylalanine, tryptophan.

These relationships are most strongly based on work with bacteria and yeasts, in which secondary synthesis of the lower members from the heads of each family has been shown by isotope tracer work.

In determining interconversions of the amino acids which accumulate or disappear in grass conservation it was felt that important information might come from comparing the changes of amino acids belonging to these metabolically related groups. For example, in dry wilting, proline synthesis may be accompanied by loss of arginine and glutamic acid, as routes from both of these amino acids to proline are known.

The analyses of conservation products for dicarboxylic and basic amino acids have therefore been supplemented by measurements of chemically related substances which can be produced from these amino acids. In addition the acidic and basic amino acids were re-estimated after acid hydrolysis of the extracts in order to measure the amounts present in peptides or otherwise chemically bound.

DEFINITIONS AND GENERAL METHODS

The following terms and methods are referred to constantly throughout this thesis.

Dry matter of plant material (d.m.) The sample is well mixed and about 100 gm are transferred to a large crystallising dish and weighed accurately. The dish is then placed in a convection oven set at 105° and dried to constant weight. This usually takes 3-4 hr. With silage, losses of volatile acids and ammonia in drying may be considerable, and corrections should be made. However, as the analytical results have always been referred to the total nitrogen content of the sample, the dry matter did not have to be determined with great accuracy.

Nitrogen determination. Organic nitrogen in protein-free extracts has been determined throughout by the modified Micro-Kjeldahl technique described by Chibnall, Rees and Williams (29). The sample for Nitrogen analysis is digested in 2 ml. conc. H_2SO_4 (N-free grade) containing 0.2 gm of catalyst taken from a mixture of 80 g. anhydrous K_2SO_4, 20 g. $CuSO_4.5H_2O$ and 1 g. sodium selenate (N-free grade). Certain amino acids, notably lysine and histidine are particularly resistant to destruction in boiling sulphuric acid and the digest should be boiled for at least 8 hr. after clearing to ensure quantitative results. After cooling the solution is transferred to a Pregl micro-distillation apparatus, 10 ml 10N-KOH are run in, and the ammonia is steam distilled into $\frac{N}{35}$ or $\frac{N}{70}$ hydrochloric acid. For the back titration with NaOH, Tashiro's indicator is used (30).

Total Nitrogen (T.N.) About 25 g. of the undried plant material are transferred to a 250 ml. flask and 125 ml. conc. H_2SO_4 (N-free) added. The digest is heated under reflux until homogeneous, cooled, and made to the required volume with conc. H_2SO_4. 2 ml. aliquots are taken for nitrogen determination by the micro-Kjeldahl technique.

pH 10 g. of the plant material are chopped and mixed with 25 ml. distilled water. After 30 minutes the pH is determined using a Cambridge pH meter.

Preparation of protein-free extracts. Extraction is always carried out on undried material as soon as possible after sampling. Very often, particularly where a large number of samples is involved, drying would be a convenient method of preparation for storage, and sampling errors could be reduced by milling. However, while there is little or no loss of nitrogen on oven-drying, except where the ammonia content is high as in some silages, changes of nitrogenous and other substances may be considerable, and some sort of forced-draught drying is preferable. When elevated temperatures are used without rapid dissipation of moisture, much protein may be degraded owing to an increased rate of enzyme action (31).

Boiling water is poured on to the sample which is then macerated or kneaded for a few minutes, and the liquid is poured off and cooled rapidly. This treatment is repeated twice, and the combined extracts are concentrated in vacuo to the required volume. The extracts are then filtered to remove cell debris, shaken with toluene, and stored at $0^{\circ}C$.

Macpherson has shown that in extracts prepared in this way 92-98% of the nitrogen is rapidly dialysable through cellophane (10).

Soluble Nitrogen (S.N.) This is the total nitrogen extracted from the sample by boiling water. It is determined on aliquots of the extracts by the micro-Kjeldahl method.

Volatile bases (V.B.) An aliquot of the S.N. extract is run into the Pregl distillation apparatus, and the pH is adjusted to 10.5 with borax buffer. The volatile bases are then distilled into standard hydrochloric acid, and have been quoted throughout in terms of ammonia-N.

Laboratory ensilage experiments. The present studies have necessitated measuring day-to-day changes of certain amino acids during the ensilage process. There are many sampling difficulties inherent in studying the changes in a large-scale silo, and it is much better to prepare a series of small silos from the same grass and to extract them one by one at suitable intervals. This method has been used by Kemble (8) and he and other workers have noted that chemical changes in laboratory scale silage are similar to those in full-scale silage (2c).

The sample for ensiling is carefully mixed and tared half-pint milk bottles are packed tightly with about 200 g. of the grass. The bottles are reweighed, closed with a mercury seal, and placed in an incubator at 35°C. After opening the bottles most of the silage is extracted. It has been assumed that no nitrogen is lost in the gaseous effluent, in view of the low pH and the fact that almost all of the volatile nitrogen is ammonia. The total nitrogen of the silage has therefore been calculated from the known T.N. content of the fresh grass.

A few metabisulphite silages have been prepared on the laboratory scale. During packing the grass is mixed intimately with dry sodium metabisulphite (0.2 g. per 50 g. grass).

With the cooperation of the Bacteriology Department of the Edinburgh and East of Scotland College of Agriculture, amino acid distribution in "silage" made from microbe-free grass has been studied. Grass grown in aseptic conditions is packed into test tubes containing 5-10 gm., closed with mercury

seals, and incubated at various temperatures. By elimination of micro-organisms, knowledge has been gained about the changes in nitrogen distribution caused by plant enzymes under anaerobic conditions.

Laboratory wilting experiments. These have been designed to study nitrogen metabolism of excised leaves in aerobic conditions. The contribution of bacteria to changes in the overall nitrogen distribution is probably very small.

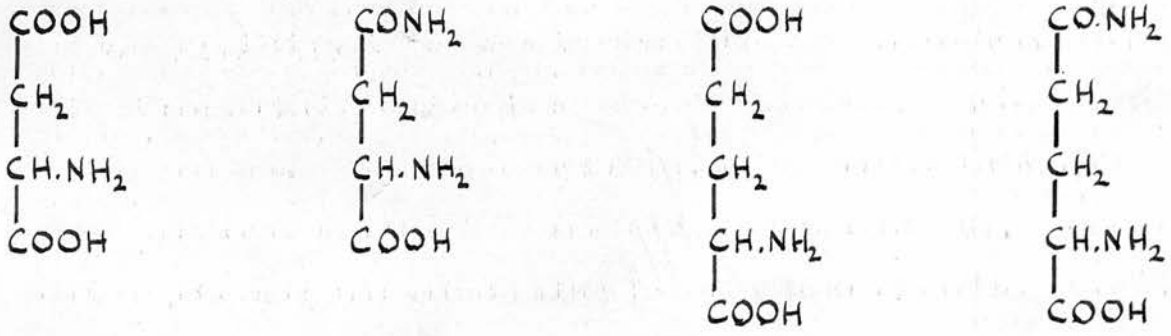
a) Moist Wilting. By this is meant wilting of grass with no loss of moisture. Weighed samples of grass are placed in crystallising dishes covered with clock glasses. These are kept at room temperature, and extracted separately at suitable intervals.

b) Dry Wilting. The grass is spread thinly on grease-proof paper and left (if possible in a dust-free atmosphere) at room temperature. The rate of drying is found by measuring the dry matter of a part of each sample just before extraction. In both cases the grass is mixed from time to time.

SECTION 1

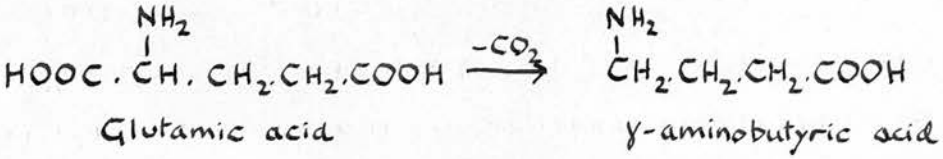
THE DICARBOXYLIC AMINO ACIDS IN CONSERVATION

The dicarboxylic amino acids, glutamic acid and aspartic acid, together account for almost one seventh of the nitrogen of grass protein. They often contribute a much greater proportion of the non-protein nitrogen of leaves and several workers have found that they are the most abundant free amino acids in higher plants (22, 24). Glutamic acid, aspartic acid, and their amides, glutamine and asparagine have a central role in the assimilation of nitrogen, and in the synthesis of amino acids and proteins in plants.

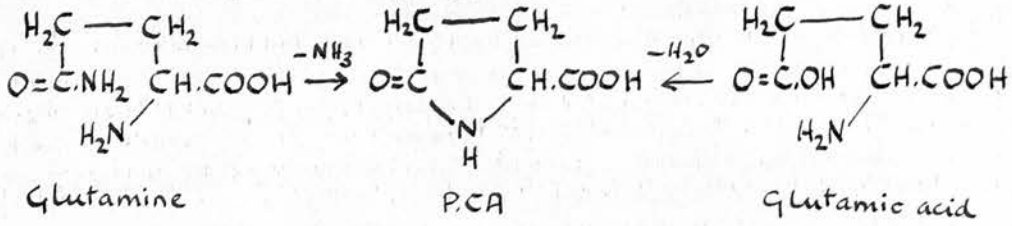


In common with other amino acids, glutamic and aspartic acid are released from protein in excised leaves. Preliminary work on silage and wilted grass indicated that they do not accumulate as the free amino acids in the amounts expected from the degree of proteolysis. However, certain chemically related compounds do occur in conservation products, often in considerable amounts, and it was desirable to determine these quantitatively in order to elucidate the metabolism of glutamic and aspartic acid formed in protein breakdown. A great deal of research has already been done on the formation of asparagine and glutamine in cut leaves, and mention should be made of the work of Chibnall, Mothes and many others, which has been reviewed by Chibnall (27) and Bonner (26). From the amounts of asparagine and glutamine found in excised leaves,

all these workers have shown that the amides cannot arise entirely from glutamic and aspartic acid released from the protein. Kemble and Macpherson have come to the same conclusion from measurements of amide in wilted grass, and add that the nitrogen of the amide group must come from the deamination of other acids. Attempts have been made to find precursors of asparagine and glutamine, and Chibnall has shown a correlation between the disappearance of infiltrated α-ketoglutarate and synthesis of glutamine. No such evidence exists as yet for the pathways of asparagine synthesis. γ-amino-n-butyric acid has been reported in silage by Kemble, and in slurries of kale kept under anaerobic conditions, by Barnett (2d). This compound may arise by decarboxylation of glutamic acid.



Another compound which may arise either from glutamine or glutamic acid is pyrrolidonecarboxylic acid (PCA).



PCA is formed by heating both glutamic acid and glutamine in neutral solution, and much more readily from the latter. It has not yet been established whether PCA occurs naturally in plants, or whether it is formed as an artefact during extraction.

The compounds measured in the following experiments were glutamic acid and aspartic acid, glutamine and asparagine, γ-amino-n-butyric acid, and pyrrolidonecarboxylic acid. In addition many of the silage and wilted grass extracts were hydrolysed in order to measure bound glutamic and aspartic acid.

For the estimation of glutamic and aspartic acid a single stage ion-exchange method based on that of Hirs, Moore and Stein (32) has been used. Pyrrolidonecarboxylic acid, for which no simple analytical method appears to exist, was determined as glutamic acid after removal from the same column and acid hydrolysis. A new ion-exchange method for γ-amino-butyric acid has also been developed.

The method of Vickery, Pucher, Clark, Chibnall and Westall was used to estimate amides, and the results could often be checked by determining the aspartic acid and PCA produced in hydrolysis of asparagine and glutamine.

These methods have been applied to protein hydrolysates, to fresh grass and to grass kept under various conditions met with in conservation practice. The results obtained have been used to draw some conclusions about the metabolism of these substances in conservation.

EXPERIMENTAL

Determination of Glutamine and Asparagine. These are determined by the methods of Vickery et al. (33) both being measured by the amount of ammonia released on hydrolysis.

For glutamine, the sample is adjusted to pH 6.5 and heated for 2 hr. at 100°C. Silage extracts, which may have a pH as low as 4.0, are brought to pH 6.5 with 0.5 M phosphate buffer. The pH of fresh grass and wilted grass is usually close to 6.5 and is not normally adjusted.

For asparagine + glutamine the sample is mixed with an equal volume of $2N\text{-}H_2SO_4$ and heated for 3 hr. at 100°C.

After hydrolysis the samples are brought to pH 10.5 with sodium hydroxide-borax buffer, and the ammonia is steam distilled into standard hydrochloric acid. N-acid hydrolysis gives the total amide content, and asparagine is determined by subtracting the glutamine value.

In the analyses for amides the N-values refer to the sum of amide-group N and α-carboxyl-N, being obtained by doubling the value for amide-group N found by hydrolysis.

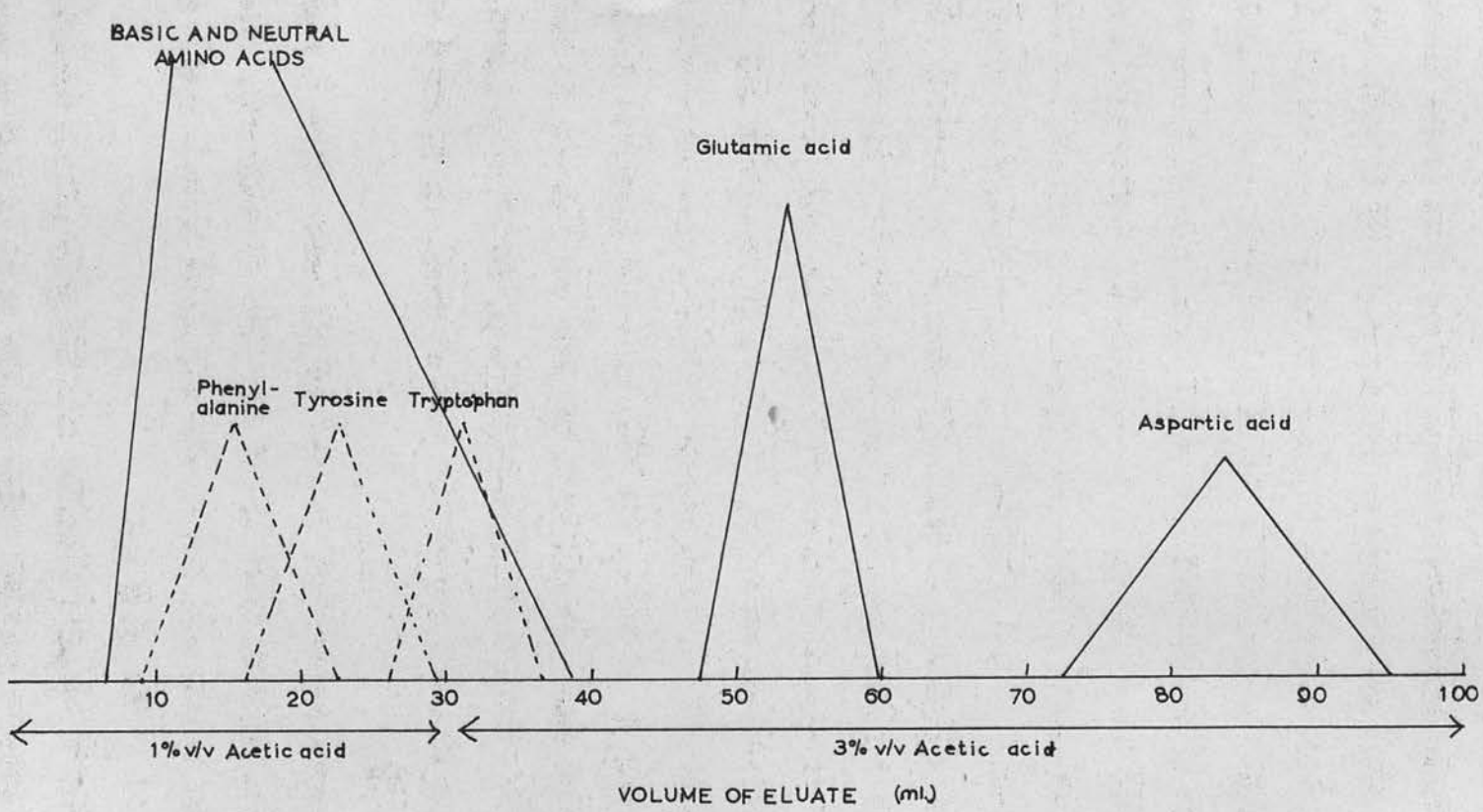
Determination of Glutamic acid and Aspartic acid. Wiltshire (34) has described a separation by the two column method of Consden, Gordon and Martin (35) followed by Van-Slyke amino-N determination. Hirs et al. (32) have used a Dowex-1 acetate column but report poor recoveries, and Campbell (36) has investigated the separation of dicarboxylic from other amino acids using acetic acid and resins of varying cross-linking. The following method, which has been devised by H.T. Macpherson employs a single short column of De-acidite FF in the acetate form eluted with dilute acetic acid at a comparatively rapid flow rate, the determination of the acids being completed in about 4 hr. The method has proved thoroughly reliable in a very large number of analyses.

De-acidite FF, 3.5% cross-linked, 16-50 mesh is broken down for a few minutes in a ball mill and the fraction of mesh size 90-120 is selected by sieving. Crushing does not appear to affect the functioning of the resin. The resin is washed with N-NaOH and then water washed three times, with settling and decanting after each treatment. After washing once with 20% ($^{V}/_{V}$) acetic acid and twice with water it is finally stored under 1% ($^{V}/_{V}$) acetic acid. Enough to fill several columns is usually prepared. Regeneration is carried out in the same way, and a fresh column is poured for each analysis. The useful life of the resin appears to be considerable. A 15 cm. depth is poured from the 1% suspension into a 1.2 cm. diameter chromatography tube with a very small cotton-wool plug at the bottom. The column is tapped to ensure close settling, the sides are washed with 1% acetic acid, and a small glass-wool plug is firmly tamped on top. Finally, 4-5 bed volumes of 0.1% acetic acid are run through and the column is ready for use; it should be used immediately.

The sample of extract, 2-3 ml. containing up to 2 mg. of nitrogen is put on with a bent tip pipette and allowed to drain in slowly, being washed in with 1-2 ml. of 0.1% acetic acid. Separation of the dicarboxylic acids from other ninhydrin-reacting substances and from each other can be varied by changes in the strength of the eluting acid and by promotion or postponement of such changes. The following procedure is satisfactory. When the sample has been washed on, 1% acetic acid is applied to the column at 40-45 ml./hr. When a total of 30 ml., including sample and washing effluent, has been collected, the solvent is changed to 3% ($^{V}/_{V}$) acetic acid flowing at a similar rate. A fraction collector may be used, but in most of this work fractions were collected via a syphon delivering 3.2 ml. ($\pm$ 0.1 ml.) into 6 in. x 5/8 in. tubes graduated at 10 ml. Usually 25 fractions are collected after the change to 3% acid and the position of the peaks is very consistent, glutamic acid coming off in a very sharp band at the seventh and eighth fractions, followed

FIG 1

ORDER OF ELUTION OF AMINO ACIDS FROM DE-ACIDITE FF (ACETATE)



closely by the somewhat wider aspartic acid band. The amino acids are then measured by ninhydrin colorimetry, 2 ml. of the Moore and Stein (37) strongly buffered ninhydrin-hydrindantin reagent being added to the contents of each tube. The tubes are immersed in boiling water for 20 min., cooled to room temperature, made to volume with 50% ($^{V}/_{V}$) ethanol and shaken vigorously for a few seconds; the intensities are then read after about 20 min. A wavelength of 570 mμ may be used, but in this case a small Unicam colorimeter with a green Ilford filter 404 was more convenient. Comparison is made with an appropriate series of glutamic and aspartic acid standards freshly made in 3% ($^{V}/_{V}$) acetic acid and developed at the same time, which eliminates the need for stringent precautions in the preparation of the reagent. As a refinement, the standards are read against a blank of acetic acid and reagent, and the column fraction against a column blank, plus reagent, taken after the aspartic acid has emerged; generally the blanks are almost identical. As long as a fresh column is used the high blanks obtained by the use of HCl (Wiltshire - 34) are not found. Usually, however, the blank preceding the glutamic acid peak is greater than that following the peak and this may be due to the fact that even with the strongly buffered reagent, the pH in the presence of 1% acetic acid is slightly higher than with 3% acetic acid, and a slightly deeper colour results.

In a typical separation, the last of the amino acids to appear before glutamic is tryptophan, which is not separated from glutamic acid on the column of Hirs et al. (32) but is well separated here, preceded by tyrosine and phenylalanine (See Fig. 1).

Recoveries were: for glutamic acid 100 ± 1% and for aspartic acid 99 ± 1%. In the present instance there appears to be no need to correct for "destruction" of glutamic acid, as is often done when buffered columns are employed. Glutamic acid left on the column for 24 hr. before elution has been

recovered quantitatively. The recovery of aspartic acid is nearly always just less than 100% and very faint traces are sometimes found on elution of the column with formic acid (see below). The purity of the glutamic and aspartic acid bands was checked by paper chromatography and no other ninhydrin-reacting substances were found, either before or after hydrolysis.

Determination of pyrrolidonecarboxylic acid. By an extension of the above method, pyrrolidonecarboxylic acid can also be determined as glutamic acid. After the fractions above have been collected, the eluent is changed to 5% ($^{V}/_{V}$) formic acid and 50 ml. run through. The eluate is taken to dryness on the waterbath and a few millilitres of 6N-HCl are added; the vessel is covered with a watch glass and heating is continued for 1.5 hr., the glass then being removed and the solution evaporated to dryness. This treatment has been shown to be sufficient for complete conversion into glutamic acid. The residue is taken up in a little cold water and the glutamic acid determined on a fresh column as described above. Ninhydrin colorimetry cannot be applied directly to the residue because the HCl treatment gives rise to amine from column residues appearing in the formic acid eluate; this basic material comes off early on the second column and a clean glutamic acid fraction is obtained.

Recovery of pyrrolidonecarboxylic acid by the method described has been tested on an authentic sample containing 7% free glutamic acid. The free glutamic acid was determined directly in the sample after column chromatography, the residual pyrrolidonecarboxylic acid being recovered quantitatively as glutamic acid from the formic acid eluate. The method for pyrrolidonecarboxylic acid is valid as long as (a) all the glutamic acid is eluted by 3% ($^{V}/_{V}$) acetic acid and (b) no other glutamic acid - or glutamine-containing material - is present in the formic acid eluate. The first point has been checked by heavily loading a column with glutamic acid, and the second by running paper chromato-

grams of the formic acid fraction. The presence of pyrrolidonecarboxylic acid was demonstrated by the appearance of an acid spot of the correct R_F, hydrolysis of which yielded only glutamic acid. The remainder of the chromatograms showed no trace of glutamic acid either free or combined, though very faint spots of other amino acids sometimes appeared after hydrolysis. However, since pyrrolidonecarboxylic acid is determined as glutamic acid on a fresh column, these do not affect the determination. In the paper chromatography of the pyrrolidonecarboxylic acid fraction the formic acid eluate was dried *in vacuo* in the cold to prevent hydrolysis.

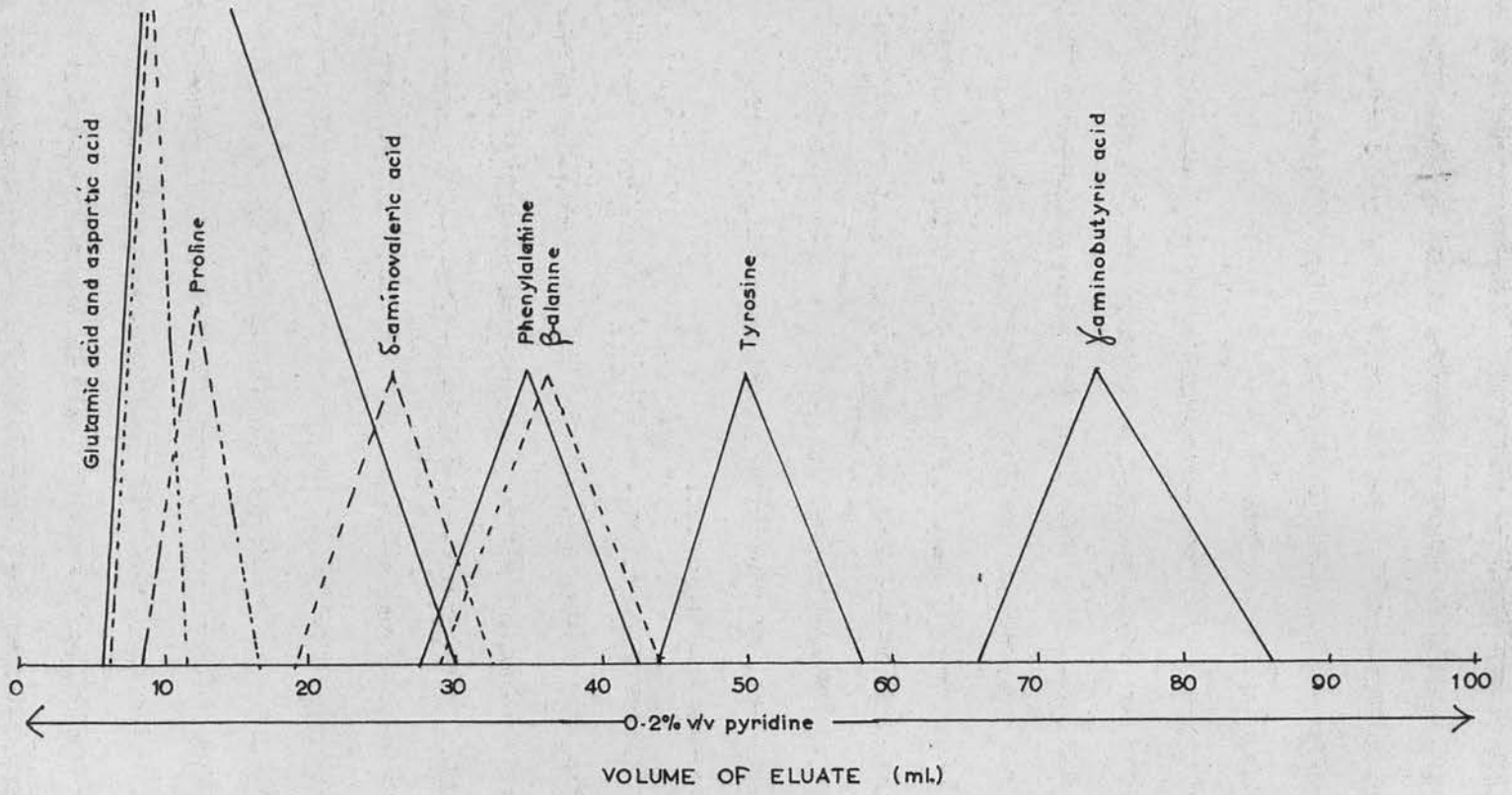
Determination of γ-amino-butyric acid. Waksman and Bigwood (38) have described a separation of this compound on a long buffered column of Dowex-50, but the procedure now described is quicker, and uses a volatile solvent. The sample is applied to a short column of cation exchanger in the pyridine form and the elution is performed with weak aqueous pyridine. The separation and determination occupy about 5 hr.

The resin used is Zeo-Karb 225 4.5% cross-linked, 200-400 mesh. Preliminary treatment followed closely that of Moore and Stein (39) for Dowex-50, except that selection of the particles by sieving was omitted. 1 lb. of the material as supplied was suspended in 2 l. of 4N-HCl and allowed to settle for 15 min., the supernatant being discarded; after three such treatments the resin was washed with water and suspended in 2N-NaOH. The suspension was heated on a boiling-water bath with occasional stirring and replacement of the NaOH until the supernatant was clear and colourless (four or five changes). The resin was again washed with water, suspended once in 2N-HCl, washed till neutral and finally shaken up and stored in 5% ($^V/_V$) pyridine.

Some of the stock resin prepared as above is suspended in 0.2% ($^V/_V$) pyridine and a column 15 cm. x 1.2 cm. poured. Two bed volumes of 0.2%

FIG 2

ORDER OF ELUTION OF AMINO ACIDS FROM ZEO-KARB 225 (PYRIDINE FORM)



pyridine are then passed through and the column is ready for use. With columns prepared from a new batch of resin the flow-rate under a 40 cm. head is about 20 ml/hr., increasing to about 50 ml./hr. after several regenerations; however, this has no apparent effect on the position of the peaks. Regeneration is carried out by heating twice with NaOH on the bath, then the subsequent treatment is that described above.

A small sample of extract containing up to 2 mg. of nitrogen is applied to the top of the resin and washed in with a little 0.2% ($^{V}/_{V}$) pyridine. The column is then filled up with 0.2% pyridine, a reservoir fitted and 2 ml. fractions are collected. The γ-amino-butyric acid band generally appears in fractions 33-43 with a maximum at 37. The ninhydrin colour is developed and measured against standards as described for the dicarboxylic amino acids. The column and reagent blanks may differ slightly unless the pyridine used in the standards has been run through Zeo-Karb 225, H^{+} form, to remove traces of a ninhydrin-reacting impurity.

A typical elution pattern is shown in figure 2. By increasing the length of the column the dicarboxylic acids can be completely separated from the monocarboxylicmonoamino acids, and clean fractions of phenylalanine and tyrosine are obtained. The method could probably be made quantitative for the aromatic amino acids on a 20 cm. column. On a 100 cm. column, with 0.2% pyridine as eluent, proline emerged as a discrete band ahead of all the neutral amino acids. In the usual method, tryptophan is eluted much later with 1% ($^{V}/_{V}$) pyridine and histidine with 20% ($^{V}/_{V}$) pyridine.(cf 55) Of the less common metabolites, β-alamine overlaps with phenylalanine and δ-aminovaleric acid appears earlier; glucosamine and ethanolamine were not found in the 0.2% pyridine eluate.

In identification of γ-amino-butyric acid from extracts of plant material paper chromatograms of the γ-amino-butyric acid fraction were developed in n-Propanol/Water, 80/20, Phenol/Water, 73/27, and n-Butanol/Acetic Acid/Water

4/1/5. No other ninhydrin reacting substance was detected in any of these solvents, and the fraction did not complex with $CuCO_3$. (Crumpler and Dent - 40).

Recoveries of γ-amino-butyric acid were determined on two amino acid mixtures and also on fresh grass extracts of known γ-amino-butyric acid content, to which γ-amino-butyric acid had been added.

Recovery of γ-amino-butyric acid from synthetic mixtures. Table 1

Each mixture contained glutamic acid, aspartic acid, glycine, alanine, valine, leucine, serine, threonine, phenylalanine, tyrosine, proline, methionine, cysteine, tryptophan, lysine, histidine, arginine and γ-amino-butyric acid.

Mixture	γ-amino-butyric acid-N		Recovery
	Taken mg.	Found mg.	%
1	0.0154	0.0156	101.2
2	0.0334	0.0337	100.8
2	0.0334	0.0330	98.9
2	0.0334	0.0326	97.7
2	0.0334	0.0347	103.9

Average recovery = 100.5 ± 1.8%

Recovery of γ-amino-butyric acid added to fresh grass extracts Table 2

γ-amino-N in extract	γ-amino-N added	Total γ-amino-N taken	γ-amino-N found	Recovery %
mg.	mg.	mg.	mg.	
0.0106	0,0103	0.0209	0.0208	99.5
0.0079	0.0239	0.0318	0.0312	98.0
0.0158	0.0176	0.0334	0.0337	100.9

Average recovery = 99.5 ± 1.0%

Ninhydrin reagent. This is the same as that described by Moore and Stein (37) except that ethyl Cellosolve (2-ethoxyethanol) has been used instead of methyl Cellosolve (2-methoxyethanol). 2 gm. ninhydrin and 0.3 gm. hydrindantin are dissolved in 75 ml. Cellosolve and mixed with 25 ml. 4N acetate buffer pH 5.5. The buffer is made up as follows:- 2760 gm. sodium acetate, 3 H_2O are dissolved in 2 l. water and cooled to room temperature. 500 ml. glacial acetic acid are then added, and the volume is adjusted to 5 l.

Cellosolve blanks. Some batches of ethyl Cellosolve show a considerable ninhydrin blank. This can be removed readily by running it through a column of Zeo-Karb 215, H^+ form, previously washed through with ethyl Cellosolve to remove traces of yellow colour from the resin eluate.

CONSERVATION EXPERIMENT A

The grass used was a lawn-grass mixture containing no rye-grass. It was cut on 8th. October 1956 at a height of 4-5 in. It had already been cut several times during the summer.

Dry matter — 19.3%

Total nitrogen — 606.0 mg. N per 100 g. fresh material, hence 3.14% of dry matter.

Soluble nitrogen — Weight of grass extracted = 50.0 g. TN = 303.0 mg.
Volume of extract = 100 ml.
2 ml. extract ≡ 2.97 ml. N/70 HCl, hence SN = 9.8% TN.

Volatile base — 5 ml. extract ≡ 0.22 ml. N/70 HCl, hence VB = 0.29% TN.

Total amide — 5 ml. extract ≡ 0.52 ml. N/70 HCl, hence TAN = 0.80% TN.

Glutamine — 5 ml. extract ≡ 0.37 ml. N/70 HCl, hence Glutamine-N = 0.40% TN.

∴ Asparagine-N = 0.40% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0606 mg. 1.00% TN.

Aspartic acid-N = 0.0182 mg. 0.30% TN.

PCA-N = 0.0091 mg. 0.15% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0121 mg. 0.20% TN.

Bound amino acids — 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. The HCl was removed in vacuo, and the volume made to 25 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0158 mg. 1.30% TN.

Aspartic acid-N = 0.0058 mg. 0.48% TN.

AMINO ACIDS IN SILAGE

Two silos were prepared in half-pint milk bottles and stored in an incubator at 35°C. One bottle was opened and extracted after one week, the other after five weeks.

ONE WEEK SILAGE — Weight of grass ensiled = 138.6 g. TN = 840.1 mg.

Weight before extraction = 137.8 g.

pH 4.45

Soluble nitrogen — Weight of silage extracted = 134.5 g. TN = 819.4 mg.

Volume of extract = 250 ml.

1 ml. extract ≡ 11.18 ml. N/70 HCl, hence SN = 68.2% TN.

Volatile base — 2 ml. extract ≡ 2.51 ml. N/70 HCl, hence VB = 7.66% TN.

Total amide — 2 ml. extract ≡ 2.76 ml. N/70 HCl, hence TAN = 1.53% TN.

Glutamine — 10 ml. extract ≡ 12.88 ml. N/70 HCl, hence Glutamine-N = 0.40% TN.

∴ Asparagine-N = 1.13% TN.

De-Acidite column — 1 ml. extract

Glutamic acid-N = 0.0610 mg. 1.86% TN.

Aspartic acid-N = 0.0852 mg. 2.60% TN.

PCA-N = 0.0111 mg. 0.34% TN.

Zeo-Karb column — 2 ml. of 1 in 10 dilution of extract

γ-aminobutyric acid-N = 0.0236 mg. 3.60% TN.

Bound amino acids — 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. The HCl was removed in vacuo, and the volume made to 20 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0410 mg. 2.50% TN.

Aspartic acid-N = 0.0540 mg. 3.30% TN.

FIVE WEEK SILAGE Weight of grass ensiled = 141.0 g. TN = 854.5 mg.

Weight before extraction = 139.4 g.

pH 4.30

Soluble nitrogen Weight of silage extracted = 132.8 g. TN = 813.9 mg.

Volume of extract = 250 ml.

1 ml. extract ≡ 11.31 ml. N/70 HCl, hence SN = 69.5% TN.

Volatile base 5 ml. extract ≡ 7.06 ml. N/70 HCl, hence VB = 8.69% TN.

Total amide 5 ml. extract ≡ 7.55 ml. N/70 HCl, hence TAN = 1.20% TN.

Glutamine 5 ml. extract ≡ 7.54 ml. N/70 HCl, hence glutamine is absent.

∴ Asparagine-N = 1.20% TN.

De-Acidite column 1 ml. extract

Glutamic acid-N = 0.0599 mg. 1.84% TN.

Aspartic acid-N = 0.0912 mg. 2.80% TN.

PCA-N = 0.0231 mg. 0.71% TN.

Zeo-Karb column 2 ml. of a 1 in 10 dilution of the extract.

γ-aminobutyric acid-N = 0.0220 mg. 5.38% TN.

Bound amino acids 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hrs. The HCl was removed in vacuo, and the volume made to 20 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0475 mg. 2.92% TN.

Aspartic acid-N = 0.0635 mg. 3.90% TN.

AMINO ACIDS IN WILTING GRASS

DRY WILTING — Weight of grass wilted = 50.0 g. TN = 303 mg.

Weight after 72 hrs. = 16.5 g.

Soluble nitrogen — The whole sample was extracted, and the volume made to 100 ml.

1 ml. extract ≡ 4.84 ml. N/70 HCl, hence SN = 31.9% TN.

Volatile base — 5 ml. extract ≡ 0.46 ml. N/70 HCl, hence VB = 0.61% TN.

Total amide — 5 ml. extract ≡ 3.61 ml. N/70 HCl, hence TAN = 8.30% TN.

Glutamine — 5 ml. extract ≡ 1.68 ml. N/70 HCl, hence Glutamine-N = 3.20% TN.

∴ Asparagine-N = 5.10% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0230 mg. 0.38% TN.

Aspartic acid-N = 0.0170 mg. 0.28% TN.

PCA-N = 0.0230 mg. 0.38% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0273 mg. 0.45% TN.

Bound amino acids — Not measured.

MOIST WILTING — Weight of grass wilted = 100.0 g. TN = 606.0 mg.

After 72 hrs. the weight was still 100.0 g.

Soluble nitrogen — Weight of sample extracted = 50.0 g. TN = 303.0 mg.

Volume of extract = 100 ml.

1 ml. extract ≡ 6.32 ml. N/70 HCl, hence SN = 41.7% TN.

Volatile base — 5 ml. extract ≡ 1.44 ml. N/70 HCl, hence VB = 1.90% TN.

Total amide — 5 ml. extract ≡ 8.29 ml. N/70 HCl, hence TAN = 18.10% TN.

Glutamine — 5 ml. extract ≡ 2.88 ml. N/70 HCl, hence Glutamine-N = 3.80% TN.

∴ Asparagine-N = 14.30% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0569 mg. 0.94% TN.

Aspartic acid-N = 0.0315 mg. 0.52% TN.

PCA-N = 0.0280 mg. 0.46% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0085 mg. 0.14% TN.

Bound amino acids — 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hrs. The HCl was removed in vacuo, and the volume made to 25 ml. 1 ml. was taken for a De-Acidite column.

Glutamic acid-N = 0.0246 mg. 4.06% TN.

Aspartic acid-N = 0.0485 mg. 8.00% TN.

MOIST-DRY WILTING The remainder of the grass which had moist wilted for 72 hrs. was removed from the container and allowed to dry wilt for a further 72 hrs.

Weight of moist wilted sample after 72 hrs. = 50.0 g.

TN = 303.0 mg.

Weight of sample after 72 hrs. dry wilting = 15.1 g.

Soluble nitrogen The whole sample was extracted, and the volume made to 100 ml.

1 ml. extract ≡ 6.71 ml. N/70 HCl, hence SN = 44.3% TN.

Volatile base 5 ml. extract ≡ 4.93 ml. N/70 HCl, hence VB = 6.50% TN.

Total amide 5 ml. extract ≡ 11.40 ml. N/70 HCl, hence TAN = 17.10% TN.

Glutamine 5 ml. extract ≡ 6.52 ml. N/70 HCl, hence Glutamine-N = 4.20% TN.

∴ Asparagine-N = 12.90% TN

De-Acidite column 2 ml. extract

Glutamic acid-N = 0.0291 mg. 0.48% TN.

Aspartic acid-N = 0.0751 mg. 1.24% TN.

PCA-N = 0.0261 mg. 0.43% TN.

Zeo-Karb column 2 ml. extract

γ-aminobutyric acid-N = 0.0221 mg. 0.36% TN.

Bound amino acids Not measured.

CONSERVATION EXPERIMENT B

The grass used was mixed perennial and Italian rye-grass, first year growth. It was cut at Boghall farm on 28th. May 1957, at a height of 12-18 in.

pH 6.10

Dry matter 22.6%

Total nitrogen 755.0 mg. N per 100 g. fresh grass, hence 3.34% of dry matter.

Soluble nitrogen Weight of grass extracted = 124.6 g. TN = 940.6 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 1.71 ml. N/70 HCl, hence SN = 9.1% TN.

Volatile base 5 ml. extract ≡ 0.14 ml. N/70 HCl, hence VB = 0.30% TN.

Total amide 5 ml. extract ≡ 0.39 ml. N/70 HCl, hence TAN = 1.06% TN.

Glutamine 5 ml. extract ≡ 0.30 ml. N/70 HCl, hence Glutamine-N = 0.68% TN.

∴ Asparagine-N = 0.38% TN.

De-Acidite column 2 ml. extract

Glutamic acid-N = 0.0380 mg. 1.01% TN.

Aspartic acid-N = 0.0132 mg. 0.35% TN.

PCA-N = 0.0026 mg. 0.07% TN.

Zeo-Karb column 2 ml. extract

γ-aminobutyric acid-N = 0.0039 mg. 0.10% TN.

Bound amino acids 25 ml. of extract hydrolysed in boiling 6N-HCl for 24 hrs. The HCl was removed in vacuo, and the volume adjusted to 25 ml. 2ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0527 mg. 1.40% TN.

Aspartic acid-N = 0.0218 mg. 0.58% TN.

AMINO ACIDS IN SILAGE

A number of silos were prepared in half-pint bottles and stored in an incubator at 55°C. Bottles were opened and extracted 9 hr., 24 hr., 48 hr. and 72 hr. after ensiling. Two silos contained 0.2 g. sodium metabisulphite per 50 g. of grass. One of these was opened after five weeks, extracted and analysed as usual.

9 HOUR SILAGE — Weight of grass ensiled = 128.7 g. TN = 971.6 mg.
Weight before extraction = 128.6 g.

pH 6.60

Soluble nitrogen — Weight of silage extracted = 123.1 g. TN = 929.8 mg.
Volume of extract = 500 ml.
2 ml. extract ≡ 5.69 ml. N/70 HCl, hence SN = 30.6% TN.

Volatile base — 5 ml. extract ≡ 0.70 ml. N/70 HCl, hence VB = 1.50% TN.

Total amide — Not measured

Glutamine — 5 ml. extract ≡ 0.79 ml. N/70 HCl, hence Glutamine-N = 0.40% TN.

De-Acidite column — 2 ml. extract
Glutamic acid-N = 0.0145 mg. 0.39% TN.
Aspartic acid-N = 0.0126 mg. 0.34% TN.
PCA-N Trace

Zeo-Karb column — 1 ml. extract
γ-aminobutyric acid-N = 0.0428 mg. 2.30% TN.

Bound amino acids were not measured in this series of silages.

24 HOUR SILAGE — Weight of grass ensiled = 123.8 g. TN = 934.6 mg.

Weight before extraction = 123.6 g.

pH 6.40

Soluble nitrogen — Weight of silage extracted = 123.6 g. TN = 934.6 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 12.23 ml. N/70 HCl, hence SN = 65.1% TN.

Volatile base — 5 ml. extract ≡ 0.76 ml. N/70 HCl, hence VB = 1.62% TN.

Total amide — 5 ml. extract ≡ 1.74 ml. N/70 HCl, hence TAN = 4.18% TN.

Glutamine — 5 ml. extract ≡ 0.92 ml. N/70 HCl, hence Glutamine-N = 0.68% TN.

∴ Asparagine-N = 5.50% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0610 mg. 1.63% TN.

Aspartic acid-N = 0.0363 mg. 0.97% TN.

PCA-N = 0.0049 mg. 0.13% TN.

Zeo-Karb column — 1 ml. extract

γ-aminobutyric acid-N = 0.0529 mg. 2.83% TN.

48 HOUR SILAGE — Weight of grass ensiled = 124.3 g. TN = 938.3 mg.

Weight before extraction = 123.8 g.

pH 6.00

Soluble nitrogen — Weight of silage extracted = 123.8 g. TN = 938.3 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 14.10 ml. N/70 HCl, hence SN = 75.1% TN.

Zeo-Karb column — 1 ml. extract

γ-aminobutyric acid-N = 0.0563 mg. 2.93% TN.

72 HOUR SILAGE — Weight of grass ensiled = 136.0 g. TN = 1027 mg.

Weight before extraction = 135.2 g.

pH 5.50

Soluble nitrogen — Weight of silage extracted = 135.2 g. TN = 1027 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 15.91 ml. N/70 HCl, hence SN = 77.5% TN.

Zeo-Karb column — 1 ml. extract

γ-aminobutyric acid-N = 0.0604 mg. 2.94% TN.

5 WEEK METABISULPHITE SILAGE Weight of grass ensiled = 133.8 g. TN = 1010 mg.

Weight before extraction = 132.7 g.

pH 5.60

Soluble nitrogen Weight of silage extracted = 132.7 g. TN = 1010 mg.

Volume of extract = 500 ml.

1 ml. extract ≡ 8.66 ml. N/70 HCl, hence SN = 85.7% TN.

Volatile base 5 ml. extract ≡ 2.36 ml. N/70 HCl, hence VB = 4.66% TN.

Total amide 5 ml. extract ≡ 3.34 ml. N/70 HCl, hence TAN = 3.88% TN.

Glutamine 10 ml. extract ≡ 4.88 ml. N/70 HCl, hence Glutamine-N = 0.32% TN.

∴ Asparagine-N = 3.56% TN.

De-Acidite column 1 ml. extract

Glutamic acid-N = 0.0606 mg. 5.00% TN.

Aspartic acid-N = 0.0354 mg. 1.75% TN.

Zeo-Karb column 1 ml. extract

γ-aminobutyric acid-N = 0.0600 mg. 2.97% TN.

Bound amino acids 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. The HCl was removed in vacuo, and the volume made to 25 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0344 mg. 4.26% TN.

Aspartic acid-N = 0.0378 mg. 4.68% TN.

AMINO ACIDS IN WILTING GRASS

DRY WILTING Two samples were taken and allowed to wilt with loss of moisture for 24 hr. and 48 hr., respectively.

24 HOUR WILTING Weight of grass taken = 100.0 g. TN = 755.0 mg.

Weight after 24 hr. = 31.7 g.

Soluble nitrogen The whole sample was extracted, and the volume made to 500 ml.

2 ml. extract ≡ 1.77 ml. N/70 HCl, hence SN = 11.7% TN.

Volatile base 10 ml. extract ≡ 0.24 ml. N/70 HCl, hence VB = 0.32% TN.

Total amide 10 ml. extract ≡ 0.72 ml. N/70 HCl, hence TAN = 1.28% TN.

Glutamine 10 ml. extract ≡ 0.47 ml. N/70 HCl, hence Glutamine-N = 0.60% TN.

∴ Asparagine-N = 0.68% TN.

De-Acidite column 2 ml. extract

Glutamic acid-N = 0.0133 mg. 0.44% TN.

Aspartic acid-N = 0.0091 mg. 0.30% TN.

PCA-N Trace

Zeo-Karb column 2 ml. extract

γ-aminobutyric acid = 0.0229 mg. 0.76% TN.

Bound amino acids Not measured.

48 HOUR WILTING — Weight of grass taken = 100.0 g. TN. = 755.0 mg.

Weight after 48 hr. = 29.6 g.

Soluble nitrogen — The whole sample was extracted, and the volume made to 500 ml.

2 ml. extract ≡ 1.78 ml. N/70 HCl, hence SN = 11.8% TN.

Volatile base — 10 ml. extract ≡ 0.26 ml. N/70 HCl, hence VB = 0.35% TN.

Total amide — 10 ml. extract ≡ 0.75 ml. N/70 HCl, hence TAN = 1.32% TN.

Glutamine — 10 ml. extract ≡ 0.49 ml. N/70 HCl, hence Glutamine-N = 0.60% TN.

∴ Asparagine-N = 0.72% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0130 mg. 0.45% TN.

Aspartic acid-N = 0.0091 mg. 0.30% TN.

PCA-N = 0.0036 mg. 0.12% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0220 mg. 0.73% TN.

MOIST WILTING Two samples were taken, and allowed to wilt without loss of moisture for 24 hr. and 48 hr. respectively.

24 HOURS WILTING Weight of grass taken = 100.0 g. TN = 755.0 mg.

Weight after 24 hr. = 97.0 g.

Soluble nitrogen Weight of sample extracted = 50.0 g. TN = 389.0 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 1.44 ml. N/70 HCl, hence SN = 18.5% TN.

Volatile base 10 ml. extract ≡ 0.11 ml. N/70 HCl, hence VB = 0.27% TN.

Total amide 10 ml. extract ≡ 0.67 ml. N/70 HCl, hence TAN = 2.88% TN.

Glutamine 10 ml. extract ≡ 0.42 ml. N/70 HCl, hence Glutamine-N = 1.60% TN.

∴ Asparagine-N = 1.28% TN.

De-Acidite column 2 ml. extract

Glutamic acid-N = 0.0244 mg. 1.57% TN.

Aspartic acid-N = 0.0100 mg. 0.64% TN.

PCA-N = 0.0025 mg. 0.16% TN.

Zeo-Karb column 2 ml. extract

γ-aminobutyric acid-N = 0.0017 mg. 0.11% TN.

Bound amino acids 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. The HCl was removed in vacuo, and the volume made to 25 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0080 mg. 2.57% TN.

Aspartic acid-N Not measured.

48 HOUR WILTING — Weight of grass taken = 100.0 g. TN = 755.0 mg.

Weight after 48 hr. = 95.8 g.

Soluble nitrogen — The whole sample was extracted, and the volume made to 500 ml.

2 ml. extract ≡ 4.23 ml. N/70 HCl, hence SN = 28.0% TN.

Volatile base — 10 ml. extract ≡ 0.30 ml. N/70 HCl, hence VB = 0.40% TN.

Total amide — 10 ml. extract ≡ 2.85 ml. N/70 HCl, hence TAN = 6.74%

Glutamine — 10 ml. extract ≡ 1.40 ml. N/70 HCl, hence Glutamine-N = 2.92% TN.

∴ Asparagine-N = 5.82% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0477 mg. 1.58% TN.

Aspartic acid-N = 0.0185 mg. 0.61% TN.

PCA-N = 0.0085 mg. 0.28% TN.

Zeo-Karb column — 4 ml. extract

γ-aminobutyric acid-N = 0.0033 mg. 0.05% TN.

Bound amino acids — 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. The final volume of hydrolysate was 25 ml. 2 ml. were taken for a De-Acidite column.

Glutamic acid-N = 0.0230 mg. 3.80% TN.

Aspartic acid-N — Not estimated

MOIST-DRY WILTING The remainder of the 24 hr. moist wilt was removed from the container and allowed to wilt for a further 24 hr. with loss of moisture.

Weight of grass after 24 hr. moist wilting = 47.0 g.

TN = 366.0 mg.

Weight of grass after 24 hr. dry wilting = 15.0 g.

Soluble nitrogen The whole sample was extracted, and the volume made to 500 ml.

2 ml. extract ≡ 1.60 ml. N/70 HCl, hence SN = 21.8% TN.

Volatile base 10 ml. extract ≡ 0.26 ml. N/70 HCl, hence VB = 0.71% TN.

Total amide 10 ml. extract ≡ 0.87 ml. N/70 HCl, hence TAN = 5.30%

Glutamine 10 ml. extract ≡ 0.52 ml. N/70 HCl, hence Glutamine-N = 1.40% TN.

∴ Asparagine-N = 1.90% TN.

De-Acidite column 2 ml. extract

Glutamic acid-N = 0.0081 mg. 0.55% TN.

Aspartic acid-N = 0.0067 mg. 0.46% TN.

PCA-N = 0.0009 mg. 0.06% TN.

Zeo-Karb column 2 ml. extract

γ-aminobutyric acid-N = 0.0092 mg. 0.63% TN.

Bound amino acids 5 ml. of extract were hydrolysed in boiling 6N-HCl for 24 hr. Final volume of hydrolysate was 25 ml. 2ml. taken for a De-Acidite column.

Glutamic acid-N = 0.0068 mg. 2.31% TN.

Aspartic acid-N Not measured.

OTHER CONSERVATION EXPERIMENTS

FIELD SILAGE

The sample was from a good quality field silage set up in the autumn of 1956, and opened on 21st. February 1957.

Dry matter — 21.5%

Total nitrogen — 46.5 g. silage were digested in H_2SO_4, and the volume made to 250 ml.

2 ml. digest ≡ 10.99 ml. HCl, hence TN = 274.8 mg.

100 g. silage contains 591.0 mg. TN, i.e. 2.75% of dry matter.

Soluble nitrogen — Weight of silage extracted = 250.2 g. TN = 1479 mg.

Volume of extract = 500 ml.

1 ml. extract ≡ 9.95 ml. N/70 HCl, hence SN = 67.2% TN.

Volatile base — 2 ml. extract ≡ 5.20 ml. N/70 HCl, hence VB = 17.6% TN.

Total amide — 2 ml. extract ≡ 5.51 ml. N/70 HCl, hence TAN = 0.76% TN.

Glutamine — 2 ml. extract ≡ 5.21 ml. N/70 HCl, hence Glutamine is absent.

∴ Asparagine-N = 0.76% TN.

De-Acidite column — 1 ml. extract

Glutamic acid-N = 0.0518 mg. 1.75% TN.

Aspartic acid-N = 0.0860 mg. 2.90% TN.

PCA-N = 0.0202 mg. 0.68% TN.

Zeo-Karb column — 2 ml. of a 1 in 10 dilution of the extract.

γ-aminobutyric acid-N = 0.0208 mg. 3.85% TN.

INCUBATED MICROBE-FREE GRASS

FIRST EXPERIMENT

Microbe-free Timothy grass cut on 10th. March 1956 was packed into test tubes and incubated at 30°. The sample analysed was opened on 4th. January 1957. Other samples opened at the same time showed no evidence of contamination by micro-organisms.

Total nitrogen — After hot-water extraction the residue was digested in H_2SO_4, cooled, and the volume made to 250 ml.

3 ml. digest ≡ 0.34 ml. N/70 HCl, hence N in residue = 5.66 mg.

Soluble nitrogen — Weight of grass extracted = 5.80 g. Volume of extract = 25 ml.

1 ml. extract ≡ 4.04 ml. N/70 HCl, hence SN = 20.01 mg.

∴ TN = 5.66 mg. + 20.01 mg. = 25.67 mg. SN = 78.9% TN.

Volatile base — 1 ml. extract ≡ 0.24 ml. N/70 HCl, hence VB = 4.80% TN.

Total amide — 1 ml. extract ≡ 0.39 ml. N/70 HCl, hence TAN = 5.80% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0569 mg. 5.60% TN.

SECOND EXPERIMENT

Microbe-free Timothy grass cut on 11th. October 1957 was incubated anaerobically at 30°C and extracted on 22nd. October 1957. The grass was still free from bacterial contamination.

Weight of grass extracted = 5.26 g.

Total nitrogen — After extraction the residue was digested in H_2SO_4, cooled, and the volume was made to 100 ml.

3 ml. digest ≡ 0.51 ml. N/35 HCl, hence residue N = 6.80 mg.

Soluble nitrogen — Volume of extract = 20 ml.

1 ml. extract = 1.37 ml. N/35 HCl, hence SN = 10.96 mg.

∴ TN = 17.76 mg. SN = 61.7% TN.

Volatile base — 2 ml. extract ≡ 0.07 ml. N/35 HCl, hence VB = 1.58% TN.

Total amide — 2 ml. extract ≡ 0.20 ml. N/35 HCl, hence TAN = 5.70% TN.

Glutamine — 2 ml. extract ≡ 0.11 ml. N/35 HCl, hence Glutamine-N = 1.8% TN.

∴ Asparagine-N = 3.9% TN.

De-Acidite column — 2 ml. extract

Glutamic acid-N = 0.0080 mg. 0.45% TN.

Aspartic acid-N = 0.0137 mg. 0.77% TN.

PCA-N = 0.0068 mg. 0.38% TN.

Zeo-Karb column — 2 ml. extract

γ-aminobutyric acid-N = 0.0427 mg. 2.40% TN.

DISCUSSION

The analytical results for free dicarboxylic amino acids, amides, pyrrolidonecarboxylic acid and γ-aminobutyric acid are collected in Tables 3-5. For asparagine and glutamine the values given are half the total amide nitrogen. This has been done to assist correlation of changes in amide concentration with changes in the other substances measured. Only the α-amino nitrogen of the amides is therefore considered, the amide group nitrogen having no quantitative bearing on the interconversions studied. All results, except those for dry matter are expressed as percent total nitrogen of the material extracted.

Free γ-aminobutyric acid, dicarboxylic amino acids, amides and pyrrolidonecarboxylic acid in grass during conservation

Table 3 - Conservation Experiment A

Material extracted	Dry Matter %	Soluble N	γ-amino N	Glutamic Acid N	Glutamine N/2	PCA N	Aspartic Acid N	Asparagine N/2
Fresh grass	19.3	9.8	0.20	1.00	0.20	0.15	0.30	0.20
Dry wilt, 72 hr.	58.5	31.9	0.45	0.38	1.60	0.38	0.28	2.55
Moist wilt, 72 hr.	19.3	41.7	0.14	0.94	1.90	0.46	0.52	7.15
Moist, 72 hr., then dry, 72 hr.	74.6	44.3	0.36	0.48	2.10	0.43	1.24	6.45
Silage, 1 week	-	68.0	3.60	1.86	0.20	0.34	2.60	0.56
Silage, 5 weeks	-	69.5	3.38	1.84	0	0.71	2.80	0.60

Table 4 - Conservation Experiment B

Material extracted	Dry Matter %	Soluble N	γ-amino N	Glutamic Acid N	Glutamine N/2	PCA N	Aspartic Acid N	Asparagine N/2
Fresh grass	22.7	9.1	0.10	1.01	0.34	0.07	0.35	0.19
Dry wilt, 24 hr.	71.4	11.7	0.76	0.44	0.30	Tr.	0.30	0.34
Dry wilt, 48 hr.	76.4	11.8	0.73	0.43	0.30	0.12	0.30	0.36
Moist wilt, 24 hr.	23.3	18.5	0.11	1.57	0.80	0.16	0.64	0.64
Moist wilt, 48 hr.	23.6	28.0	0.05	1.58	1.46	0.28	0.61	1.91
Moist, 24 hr. then dry, 24 hr.	73.1	21.8	0.63	0.55	0.70	0.06	0.46	0.95
Silage, 9 hr.	-	30.6	2.30	0.39	0.20	Tr.	0.34	-
Silage, 24 hr.	-	65.1	2.83	1.63	0.34	0.13	0.97	1.75
Silage, 48 hr.	-	75.1	2.93	-	-	-	-	-
Silage, 72 hr.	-	77.5	2.94	-	-	-	-	-
Metabisulphite silage	-	85.7	2.97	3.00	0.16	0.54	1.75	1.78

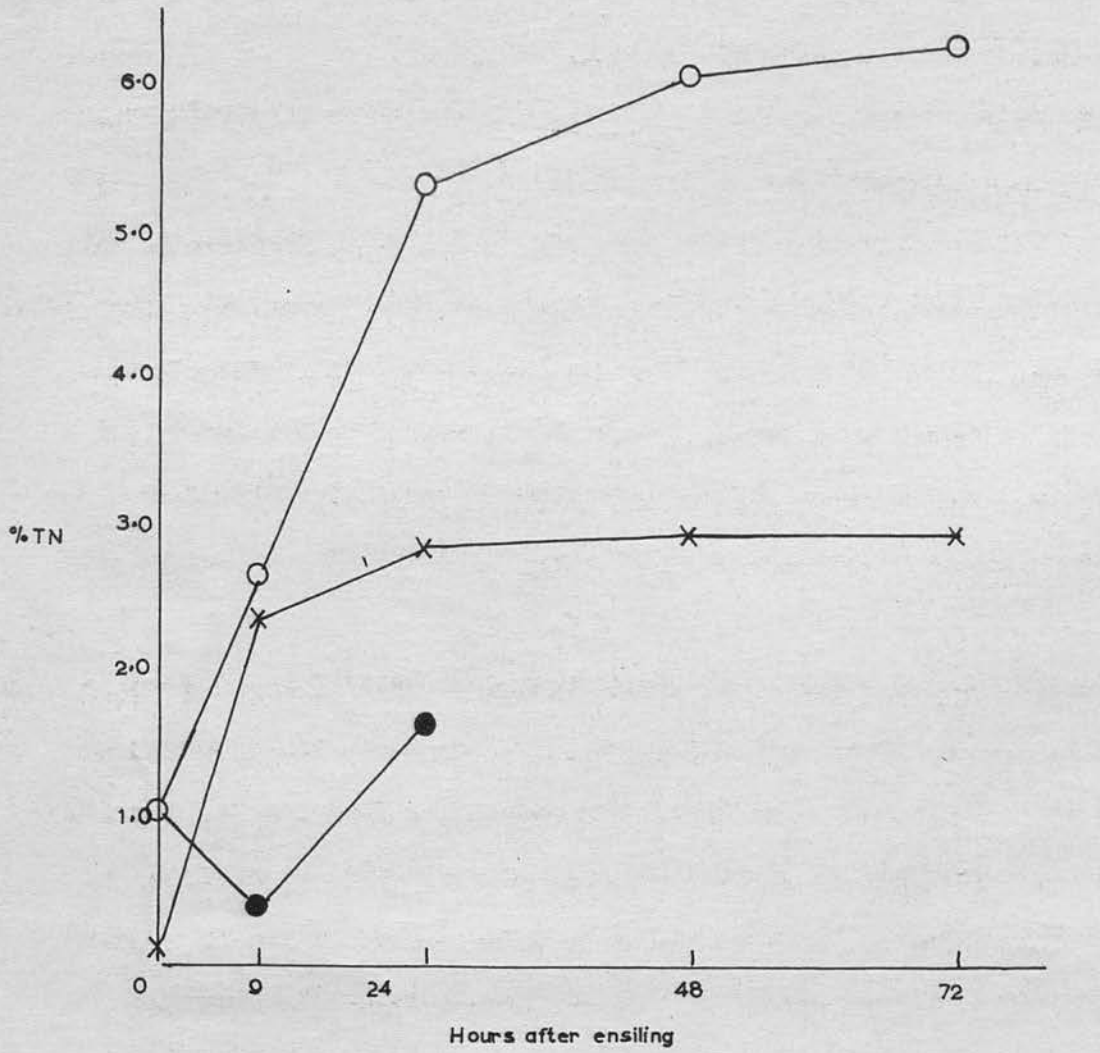
Table 5 - Other Conservation Experiments

Field silage	-	67.2	3.85	1.75	0	0.68	2.90	0.38
Incubated microbe-free grass, 11 days	-	61.7	2.40	0.45	0.82	0.38	0.77	2.03
Incubated microbe-free grass, 10 months	-	78.9	3.60					

$\frac{1}{2}$ Total amide-N = 2.90% TN

FIG 3

FORMATION OF γ-AMINOBUTYRIC ACID IN SILAGE



Formation of glutamine and γ-aminobutyric acid

For silage the most striking feature of the results is the large amount of γ aminobutyric acid formed. The silage in experiment A had reached an equilibrium nitrogen distribution after one week, there being no significant changes in the substances measured after a further four weeks. It was therefore decided to examine nitrogen metabolism in the first few days after ensiling, and this has been done in experiment B. The extensive production of γ-aminobutyric acid was confirmed, and it was further shown that this substance had almost reached its maximum concentration after only 9 hours. The very rapid accumulation of γaminobutyric acid was paralleled by a sharp decrease in the amount of glutamic acid, which thereafter increased due to continued proteolysis. From Fig. 5, we see that in 9 hours, almost all the original glutamic acid, and glutamic acid released in proteolysis was accountable as γ-aminobutyric acid. After nine hours, however, although glutamic acid is still being formed rapidly from the protein, very little more γ aminobutyric acid is produced.

It is evident that glutamic acid is being decarboxylated as a direct result of anaerobiosis in the silage, and that the decarboxylating enzyme is being inactivated before the proteolytic enzymes. The fact that the γ-aminobutyric acid is formed before micro-organisms can have begun to multiply abundantly indicates that the decarboxylation is purely a function of a plant enzyme. This is confirmed by the results with microbe-free "silage" and metabisulphite silage. γ-aminobutyric acid is of ubiquitous occurrence in plants, and many species have also been shown to contain a glutamic acid decarboxylase. Naylor and Tolbert (41) have found that in excised barley leaves kept under anaerobic conditions glutamic acid is rapidly converted to γ-aminobutyric acid, while Sims (21b) has made a similar observation for yeast growing under conditions of oxygen limitation.

A different picture is presented by the wilting experiments. In moist wilting there is no formation of γ-aminobutyric acid; glutamine is however formed, as has been shown in much earlier work. In dry wilting both γ-aminobutyric acid and glutamine may be produced. The proportions of these substances depend on the rate of drying. In experiment A, when the grass dried slowly, there was less γ-aminobutyric acid than in B. B, however was rather an extreme case, as no glutamine was synthesised. The drying was so rapid that equilibrium nitrogen distribution was reached in 24 hours. This dependence of γ-aminobutyric acid and glutamine production on the moisture content of the wilting grass is well shown by the two experiments in which grass which had been moist wilted was then allowed to wilt with loss of moisture. In both cases this had the effect of increasing the amount of γ-aminobutyric acid. The glutamine value remained static, but glutamic acid decreased and is probably the precursor of γ-aminobutyric acid.

In summary, it appears that γ-aminobutyric acid can be formed both in aerobic conditions as in dry wilting, or in largely anaerobic conditions as in silage or incubated microbe-free grass. Glutamine formation appears to limit the formation of γ-aminobutyric acid, so that when conditions are inimical to glutamine production, γ-aminobutyric acid is formed instead. Synthesis of glutamine requires high-energy phosphate from oxidative phosphorylations, or from photosynthesis and could not be expected to occur in anaerobic conditions in the dark. In dry wilting its synthesis may be limited by lack of proteolysis and subsequent deamination.

Two explanations of these observations may be submitted

(a) There are two pathways open to glutamic acid metabolism in excised leaves, depending on the moisture content and available free oxygen.

$$\gamma\text{-aminobutyric acid} \xleftarrow{-CO_2} \text{Glutamic acid} \xrightarrow{+NH_3} \text{Glutamine}$$

(b) γ-aminobutyric acid is always produced from glutamic acid first, and then converted to glutamine. The extent of the second step depends on the environmental conditions, and in the dark, with anaerobiosis, it may be prevented entirely.

$$\text{Glutamic acid} \xrightarrow{-CO_2} \gamma\text{-aminobutyric acid} \xrightarrow[+CO_2]{+NH_3} \text{Glutamine.}$$

Steward, Bidwell and Yemm (42) have found by isotopic techniques that glutamine can be synthesised from γ-aminobutyric acid.

The choice between these two alternatives cannot be made yet, but additional information might be gained if C^{14} labelled glutamic acid was infiltrated into excised leaves, and the appearance of C^{14} in γ-aminobutyric acid and glutamine studied during wilting. Wilting with slow loss of moisture, as in Experiment A, would probably be the best condition, as reasonable amounts of both substances accumulated. It might also be possible to demonstrate synthesis of glutamine from γ-aminobutyric acid if grass which had been incubated anaerobically for a few hours was then wilted in moist aerobic conditions.

As regards (a) it is not clear from the results whether glutamine is formed directly from glutamic acid. Certainly in moist wilting such large amounts of glutamine may be formed that another precursor must be involved. One possibility is α-ketoglutaric acid, as C^{14} from this compound has been shown to appear in glutamine in excised leaves. (27)

Only 0.1 - 0.2% of the total nitrogen of fresh grass was found to be in the form of γ-aminobutyric acid. Although very small amounts may be formed between the time of cutting and the inactivation of the enzymes by boiling water, it seems probable that most of that found occurs as such in fresh grass. The values are lower than those estimated by Synge (22), but in his analyses the sap was exposed to a lengthy dialysis, and it seems likely that much of it may have been formed during this time.

Dicarboxylic amino acid balance in conservation

An attempt has been made to correlate the non-protein glutamic acid and aspartic acid found in silage and wilting grass with the amounts expected from the degree of proteolysis. Kemble and Macpherson (13) showed that the neutral amino acid composition of the residual protein in silage is similar to that of the fresh grass, and assumed that protein breakdown is uniform. Similar figures are not available for the dicarboxylic amino acids, but if they are not released uniformly this will be reflected by different proportions of neutral amino acids compared with the fresh grass protein. It is therefore assumed that the amounts of glutamic acid and aspartic acid released are directly proportional to the degree of proteolysis.

The expected free amino acid in any extract is found from the formula (14)

$$A_e = A_f + K\left(\frac{Sn - Sf}{100}\right)$$

A_e = Expected free amino acid, % T.N.

A_f = Free amino acid N in fresh grass, % T.N.

K = Amino acid N in protein, % protein N.

S_n = S.N. of grass n days after cutting, % T.N.

S_f = S.N. of fresh grass, % T.N.

Two other assumptions are made to simplify the calculation. All the non-extractable nitrogen of the material is assumed to be present as protein. The nitrogen of nucleic acids and pigments is therefore discounted. The absolute values of the substances estimated will be slightly inaccurate, but general trends in relative amino acid distribution can still be demonstrated. Secondly, some protein synthesis probably takes place after the grass has been cut, but this will be of very short duration, and the net result is a loss of true protein.

This calculation calls for accurate values of the dicarboxylic amino acid content of grass proteins. These are usually measured after hydrolysis of

whole plant material, or of extracted proteins. Both methods are liable to serious error either through destruction of amino acids by humin formation, or by extraction of an unrepresentative protein. These two factors probably account for many of the variations noted in the amino acid composition of leaf proteins (43). In the present work the figures used were: glutamic acid 7.62%, and aspartic acid 6.10% of total protein N. These were obtained by analysis of mixed proteins prepared by alkaline extraction and acid precipitation from five grasses, all in the pre-flowering stage, at different Laboratories.

Table 6

The glutamic acid balance in grass conservation

Results expressed as % T.N. of extracted material.

Material extracted	N of free glutamic acid, glutamine (N/2), PCA and γ-aminobutyric acid (from Tables 3 and 4)	N of glutamic acid and γ-aminobutyric acid present after hydrolysis of extract	Glutamic acid N expected from proteolysis
	Experiment A		
Fresh grass	1.55	1.50	-
Moist wilt, 72 hr.	3.44	4.20	4.05
Silage, 1 week	6.00	6.10	6.00
Silage, 5 weeks	5.95	6.30	6.10
	Experiment B		
Fresh grass	1.52	1.50	-
Moist wilt, 24 hr.	2.64	2.68	2.20
Moist wilt, 48 hr.	3.37	3.85	2.96
Moist, 24 hr., then dry, 24 hr.	1.94	2.94	2.49
Metabisulphite silage	6.67	7.23	7.38

Table 6 shows the glutamic acid balance in conservation. The free glutamic acid always falls short of the expected value, but if it is assumed that pyrrolidonecarboxylic acid, γ-aminobutyric acid, and glutamine come directly or indirectly from glutamic acid formed in proteolysis, the balance, when bound glutamic acid is taken into account is generally satisfactory for silage. Differences between the first and second columns of Table 6 are due to bound glutamic acid only, since glutamine and PCA are both converted to glutamic acid by acid hydrolysis. It has been assumed that γ-aminobutyric acid does not increase after acid hydrolysis. The five week silage of experiment A showed a slight decrease in this compound after hydrolysis, and a later moist wilting experiment also contained no bound γ-aminobutyric acid. In the wilting experiments, more glutamic acid, or glutamic acid derivatives, were found than was expected. This may be entirely due to glutamine synthesis from a precursor other than glutamic acid.

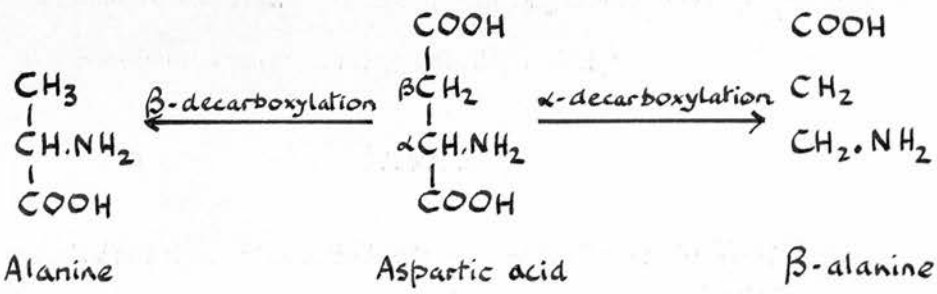
Table 7

The aspartic acid balance in grass conservation

Results expressed as % T.N. of extracted material.

Material extracted	Aspartic acid N and asparagine N/2 free in extract (from tables 3 and 4)	Aspartic acid N present after hydrolysis of extract	Aspartic acid N expected from proteolysis
	Experiment A		
Fresh grass	0.50	0.48	-
Moist wilt, 72 hr.	7.67	8.00	2.41
Silage, 1 week	3.15	3.30	4.00
Silage, 5 weeks	3.40	3.90	4.10
	Experiment B		
Fresh grass	0.54	0.58	-
Metabisulphite silage	3.53	4.68	5.10

Table 7 shows the aspartic acid balance in conservation. In moist wilting, such large amounts of asparagine are formed that some source other than aspartic acid must supply the carbon skeleton for the asparagine molecule. Thus it is impossible to attempt a balance until quantitative knowledge is available on the precursor of asparagine. In anaerobic conditions, as in ordinary and metabisulphite silage, very little asparagine is formed, but the non-protein aspartic acid, including bound aspartic acid, falls short of the expected value. β-alanine, the decarboxylation product of aspartic acid analogous to γ-aminobutyric acid from glutamic acid, was not found in significant amounts, Kemble (8) noted excess alanine, the other decarboxylation product of aspartic acid, in the early stages of ensilage, and in anaerobically incubated microbe-free grass.



Determination of amides

The methods for the amides appear to be reliable when considerable amounts are present, but the value for glutamine is less reliable when the amounts are small, since it is affected by ammonia liberated from other substances in the extract (33).

The estimation of PCA after hydrolysis at pH 6.5 does not provide a satisfactory check on the glutamine value, since small amounts of glutamic acid are also converted to PCA. During hydrolysis at pH 6.5 the aspartic acid and asparagine remain unchanged, and after N-acid hydrolysis the asparagine can be

recovered quantitatively as aspartic acid, thus providing a useful cross-check. These results are shown by an additional experiment on the 72 hr. moist wilt of Experiment A

Table 8

Results expressed as % T.N. of extracted material.

Material extracted	Glutamine N/2	Asparagine N/2	Glutamic acid N	Aspartic acid N	PCA N
Moist wilt, 72 hr.	1.90	7.15	0.94	0.52	0.46
Extract hydrolysed at pH 6.5	-	7.15	0.90	0.52	2.10
Extract hydrolysed with N-acid	-	-	3.10	7.62	1.10

N-acid hydrolysis does not convert PCA completely to glutamic acid, and although the same total of PCA-N and glutamic acid-N is always obtained, the proportions may vary. Most of the glutamine N appears as PCA on hydrolysis at pH 6.5; the slight reduction in glutamic acid has however been noted several times. From this result it might be inferred that ammonia is being formed from substances other than glutamine at pH 6.5.

Table 8 shows a large increase in the sum of PCA-N and glutamic acid-N on acid hydrolysis. This release of bound glutamic acid by N-acid is discussed in the section on bound amino acids.

Occurrence of pyrrolidonecarboxylic acid in grass

The values obtained for PCA in grass and silage extracts are usually very small, but even so they will be overestimates, because of the partial conversion of glutamine during the preparation of hot water extracts. Vickery et al. (33) have studied the stability of glutamine at various temperatures in pH 6.5 solution. From their results it may be estimated that about 20% of the glutamine

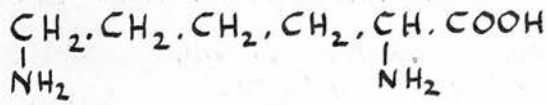
is converted to PCA during extraction. In view of this, and the relative proportions of glutamine and PCA in fresh grass, it seems probable that PCA does not occur in the grasses examined. Some of the silages contain much more PCA, particularly after several weeks, but there has been simultaneous disappearance of glutamine, and the PCA may have been derived from this substance during the long period of storage. Ellfolk and Synge (44) consider that PCA is a very minor constituent of rye-grass juice, having found "much less than 0.86%" of the total nitrogen of press-juices as PCA-N. In the present work PCA-N was 1.53% of the non-protein nitrogen in experiment A and 0.77% in experiment B.

The results for bound aspartic acid and glutamic acid are discussed in the section on bound amino acids and peptides.

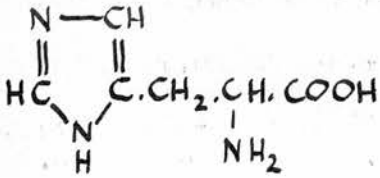
SECTION 2

BASIC AMINO ACIDS IN CONSERVATION

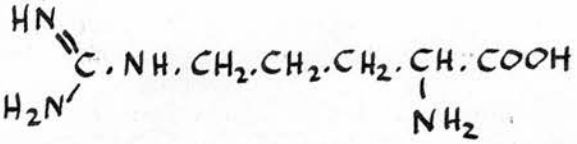
The only basic amino acids which have been identified in plant proteins are lysine, histidine and arginine.



Lysine

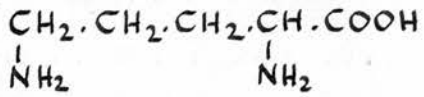


Histidine



Arginine

Steward and Pollard (25) list 11 basic amino acids which have been found in the free or bound form in protein-free preparations from plant tissues. In addition to lysine, histidine and arginine there are:- ornithine, δ-hydroxylysine, canavanine, α,β-diaminopropionic acid, α,γ-diaminobutyric acid, β,ε-diaminocaproic acid, γ-aminobutyric acid, roseoline. Many other non amino acid bases have been identified, such as amines, purines and pyrimidines.



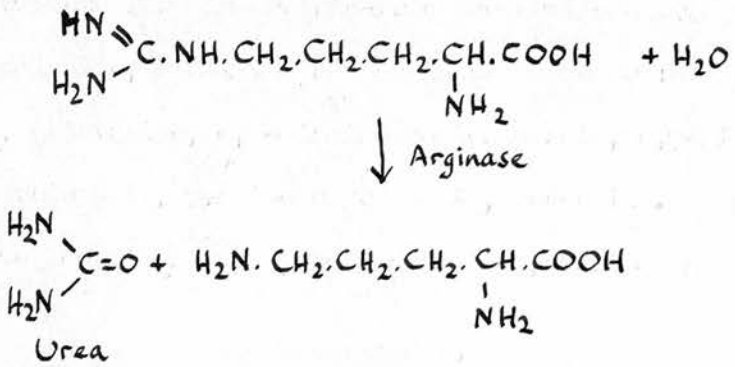
Ornithine

Free histidine, lysine and arginine occur in very small amounts in living plants & are not in the same proportions as in the protein. This has been established quantitatively for perennial rye-grass by Bathurst (24), who also measured non-protein bound amino acids, and found that in this fraction the basic amino acid proportions were closer to the relative amounts in protein.

There are very few precedents for the study of free basic amino acids in excised leaves, and none for the study of these compounds in grass conservation.

Wood and Cruickshank (28) found that arginine was released by proteolysis and then disappeared from excised leaves of Sudan grass and Kikuyu grass. Frankenberg (45) studied proteolysis during the shed curing of tobacco, which is effectively wilting with slow loss of moisture, and found that lysine was completely destroyed, arginine was depleted, and that histidine was present in amounts greater than in the fresh leaves.

Preliminary work by Macpherson showed that histidine, lysine and arginine were all released from protein in silage and wilted grass, and accumulated, but not to the extent expected from the degree of proteolysis. In fact, in silage, the arginine concentration reached a maximum after 2-3 days and then gradually decreased. This maximum coincided with the appearance of ornithine which ultimately became one of the major amino acid constituents of silage. Ornithine may arise from arginine by the action of the enzyme arginase, though neither ornithine nor this enzyme have been detected very often in plants.



Macpherson also detected γ-aminobutyric acid as a basic component in silage, and extended work on this substance has already been reported.

It was decided to measure lysine, histidine, arginine and ornithine in silage and wilted grass, and at the same time to look for any other compounds which might arise from the metabolism of basic amino acids in conservation conditions. H.T. Macpherson carried out parallel determinations of proline and it was hoped to find some correlation between proline accumulation and loss of arginine in dry wilted grass.

The choice of an analytical method fell naturally on the use of ion exchange chromatography for the separation of the amino acids, followed by ninhydrin colorimetry. Since the original publication by Moore and Stein of a complete scheme for separating amino acids on buffered columns of Dowex 50 (30), this has become the foremost technique for comprehensive amino acid analysis of protein hydrolysates and biological fluids.

The resins used are strong cationic exchangers of the sulphonated polystyrene type and should contain 4-8% of the crosslinking agent, divinylbenzene. Equivalent strong cationic exchange resins are Dowex 50, Amberlite IR-120 and Zeo Karb 225.

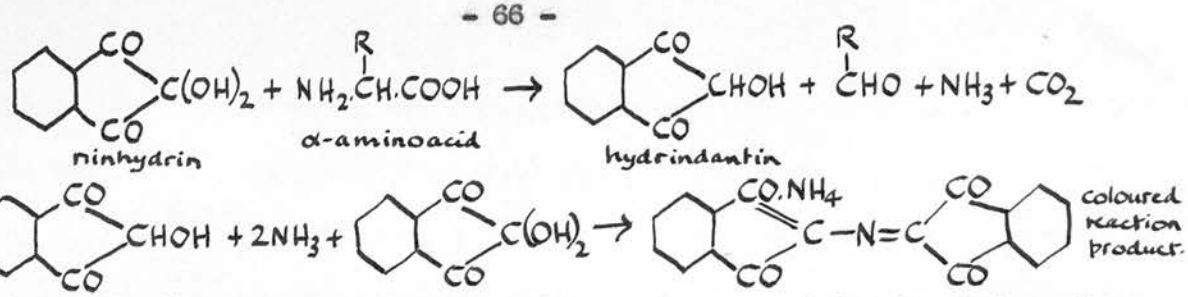
In Moore and Stein's original procedure (39) 100 x 0.9 cm. columns of Dowex 50, 8% crosslinked were used. The resin was initially equilibrated with a buffer at pH 3.4. After the sample had been applied to the column, the amino acids were eluted by a stepwise increase of pH to 11.0, and emerged as sharp, well resolved peaks in order of increasing iso-electric point. At the pH of elution of the basic amino acids recoveries were low, and the 100 cm. columns were used only for dicarboxylic and monocarboxylic-monoamino acids. For the basic amino acids a 15 cm. column was employed and the acidic and neutral amino acids were first eluted in a bulk fraction at pH 5.0. Separation and quantitative recoveries of the basic amino acids were then achieved by elution with buffers at pH 6.75 (0.1 M) and 6.5 (0.2 M). In a refinement of the original scheme all the amino acids in protein hydrolysates are now resolved on a single column of 4% crosslinked resin by a gradient increase of pH from 3.1 to 5.1 and of sodium ion concentration from 0.2 to 1.4 (46).

In the present work it was not intended to analyse for acidic and neutral amino acids, so that the abbreviated column of Moore and Stein for basic amino acids provided a useful technique which could be adapted to suit the problem

at hand. The main difficulty was to separate ornithine and lysine. Hamilton and Anderson, who investigated the ornithine content of gelatin hydrolysates found that Moore and Stein's method does not separate these two homologues and devised a different elution system for the purpose (47). Eastoe has confirmed that the Moore and Stein method does not separate ornithine and lysine (48). Some idea of the similarity of behaviour of ornithine and lysine can be gained from the elution pattern obtained by Moore and Stein's gradient elution method, in which these compounds are barely resolved on a column 150 cm. long.

In Hamilton and Anderson's technique the resin used was Dowex-50, 8% crosslinked. Ornithine and lysine were eluted by pH 5.0, 0.1 M citrate buffer, histidine by pH 7.5 0.1 M phosphate buffer, and finally ammonia and arginine by pH 5.0 0.4 M citrate buffer. When the present work was started the available resin most closely resembling Dowex-50 was Zeo Karb 225, 4½% crosslinked, which has been used throughout. Since at this degree of crosslinking the resin is subject to considerable shrinking when the molarity of the eluent buffer is increased, it was hoped to devise a scheme which used only one molarity. It soon became apparent that the scheme of Hamilton and Anderson could not be reproduced on Zeo-Karb 225 x 4½. The difficulties encountered will be discussed later.

The quantitative use of the ninhydrin reaction for estimating amino acids has been carried to a high degree of precision by Moore and Stein and their coworkers, and is used in the analysis of eluates from buffered ion exchange columns. Although for all α-amino acids the coloured substance produced in the reaction is the same, colour yields per mole of amino acid differ slightly but can be referred to a standard amino acid and are reproducible for specified conditions.



Thus although a large number of amino acids may be present in the mixture the need to run a series of standard solutions for each component is eliminated. The reference amino acid only need be checked for colour with each new batch of ninhydrin solution, and the amounts of other amino acids can be found by reference to this calibration curve and the colour yields.

Specific colour reactions have been employed in detecting ornithine, lysine, histidine and arginine bands from columns, especially in the beginning of the work where they could be used to identify peaks and hence bypass the need for desalting and paper chromatography. The reactions were: the Chinard reaction for ornithine and lysine in which these amino acids react with ninhydrin in acid solution giving red and yellow compounds respectively (49); the Sakaguchi reaction for arginine; and the diazotisation of sulphanilic acid followed by coupling with histidine to give a red compound.

For the paper chromatographic study of basic amino acids a solvent devised by Macpherson was used. Phenol-water (73-27)/Methyl cellosolve/pyridine, 90/9/1, separates ornithine, lysine, histidine, arginine and γ-aminobutyric acid and was useful for studying peaks from ion exchange columns, basic fractions obtained by electrodialysis, or even untreated grass extracts.

Much of the non-protein nitrogen in grass, wilted grass and silage is non-amino acid in nature. An attempt has been made to find how much of the non-amino acid nitrogen is basic in character. Electrodialysis in a three-compartment cell was applied to extracts of fresh grass, wilted grass and silage and the nitrogenous compounds migrating cathode-wise were determined by a micro-Kjeldahl technique. By subtracting from the total catholyte nitrogen,

the nitrogen contributed by ornithine, lysine, histidine, arginine and γ-amino-butyric acid, some measure of the unknown basic compounds was achieved.

EXPERIMENTAL

Solvent for paper chromatography of basic amino acids.

The R_F values of basic amino acids in Phenol-Water (73-27)/Methyl Cellosolve/pyridine, 90/9/1 are as follows:-

Ornithine	0.19
Lysine	0.27
Arginine	0.32
Histidine	0.46
γ-aminobutyric acid	0.60

This solvent is superior to Phenol-Water, and is most useful when applied to cathode fractions from electrodialysis of grass extracts. Aspartic acid runs slower than ornithine, while glutamic acid, glycine, serine, threonine, alanine, tyrosine and valine all have R_F values within the range of the basic amino acids.

Buffers used in column chromatography

Sodium citrate buffers have been used for the pH range 2.5-5.0, and sodium phosphate buffers for higher ranges. Buffers used for elution of amino acids contain 0.1 gm. disodium versenate, 0.5 ml. thiodiglycol and 1.0 ml. benzyl alcohol per 100 ml. (39). The buffers were not boiled before use. After preparation they were shaken with toluene and stored in aspirators fitted with a sulphuric acid trap to prevent access of ammonia.

Preparation of the resin The resin used was Zeo-Karb 225, 4½% crosslinked supplied as spherical particles of 200-400 mesh size. Preliminary treatment was the same as described on page 28) up to the final heating in 2N-NaOH. After decanting the sodium hydroxide solution the resin is rinsed twice with

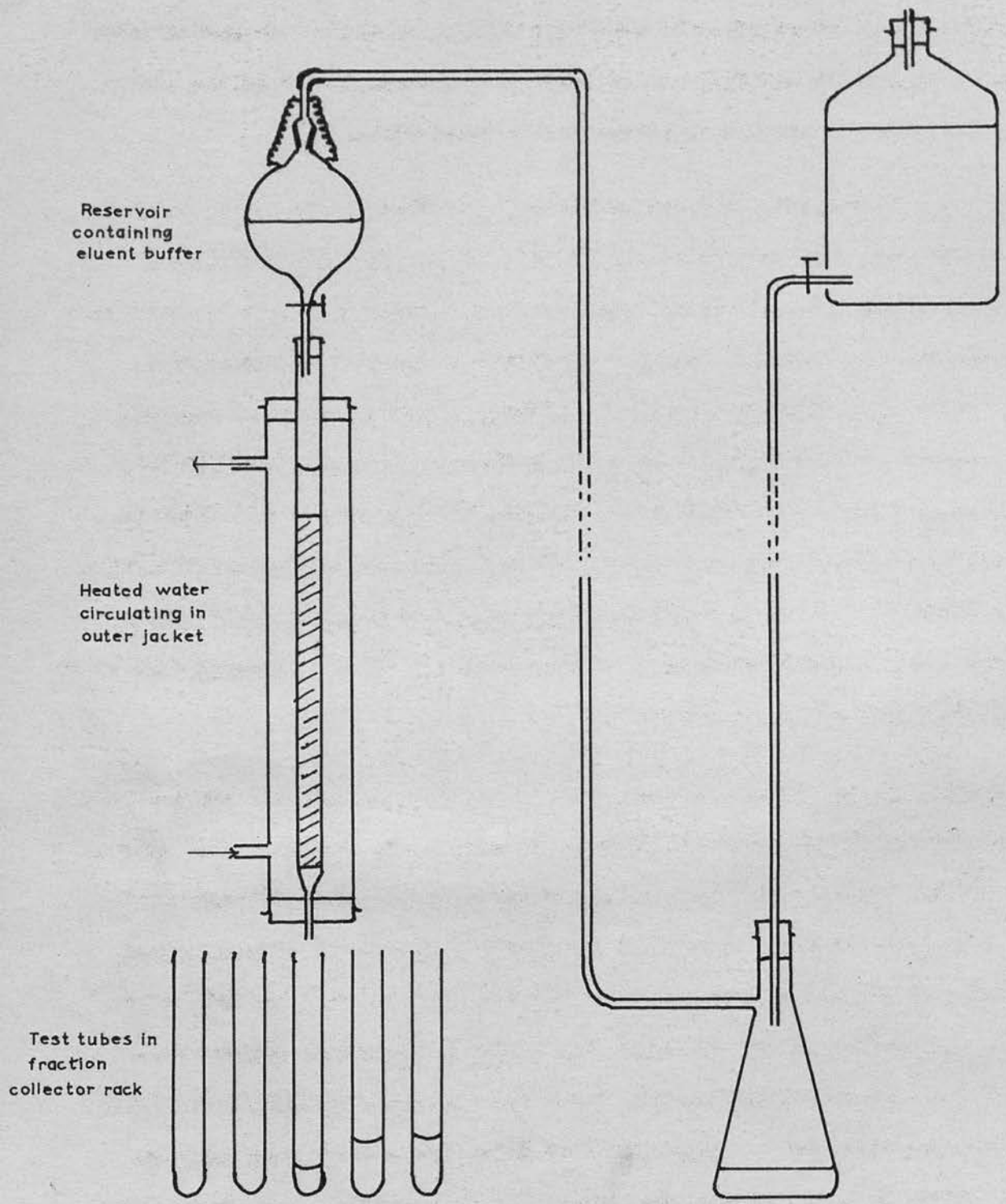


FIG 4
APPARATUS FOR ION EXCHANGE COLUMN CHROMATOGRAPHY OF BASIC AMINO ACIDS

distilled water, transferred to a Buchner filter and then water washed under slight suction until the filtrate is neutral. The resin, now in the sodium form, is ready for use and is stored in the moist state.

Apparatus A diagram of the apparatus used in column operation is shown opposite. The tube containing the resin is 40 cm. long and its internal diameter is 1.2-1.3 cm. Water from a constant temperature bath is circulated through the outer jacket. Owing to the fineness of the resin flow rates under gravity may be as low as 4 ml./hr. through a 20 cm. column, and this is increased to 15-20 ml./hr. under 10 cm. mercury pressure, produced by a water head of about five feet as indicated in the diagram. The column is mounted over a fraction collector working on a drop counting principle, which is preferable to a time interval mechanism because of slight variations in the flow rate, especially in newly packed columns. It is important that all fractions should be of equal volume.

Preparation of the column Enough resin for one column is first suspended in its own volume of the buffer to be used for subsequent elution. The suspension is then heated to 80° on a boiling water bath for about an hour. If this step is omitted the resin will degas after the column has been poured, and when the operating temperature is over 30°, serious cracking will occur. Before forming the column the water circulating in the outer jacket is adjusted to the required temperature. The hot suspension is then poured into the chromatography tube, and the resin is allowed to settle under gravity. It is advisable to remove the supernatant every 15 minutes and replace with fresh suspension, until the correct length of column is obtained. This prevents inclusion of very fine particles which decrease the flow rate.

100 ml. of sodium hydroxide are then passed through the column under pressure. The normality of the NaOH must be the same as the sodium ion con-

centration in the eluent buffers, otherwise contraction or expansion of the resin occurs. Before the column is to be used 50 ml. pH 3.4 sodium citrate buffer of the correct molarity are passed through under pressure.

Column operation 2 ml. of the solution to be analysed at pH 2.5 are then put on the column from a bent-tip pipette. The sample is drained in under gravity and is followed by 2 ml. pH 2.5 citrate buffer. The reservoir containing the eluent buffer is then fitted and elution is started under increased pressure. The fractions collected from the column are usually 2-3 ml., and the exact volume is checked gravimetrically from time to time. The fractions were collected and analysed in test tubes graduated at 10 ml.

The system finally developed was ideal for separating 0.1 to 2.0 μM quantities of the basic amino acids. Acidic amino acids, neutral amino acids, γ-aminobutyric acid, ammonia, ornithine, lysine and histidine are eluted with pH 4.3, 0.15 M sodium citrate buffer, and arginine by pH 5.0, 0.15 M sodium citrate buffer.

Analysis of fractions by ninhydrin colorimetry The ninhydrin reagent is the same as that used in the estimation of dicarboxylic amino acids and γ-aminobutyric acid (Page 31). A fresh solution is prepared for each batch of tubes. Not more than 50 fractions may be analysed at one time as this is the maximum number which can be read on the spectrometer before the colours begin to fade appreciably. The procedure has been standardised as follows:- The column fractions, or aliquots are adjusted to 3 ml. with the appropriate buffer and mixed with 2 ml. ninhydrin reagent. The tubes are heated for 15 minutes in a boiling water bath, cooled to room temperature, and the volume adjusted to 10 ml. with 50% ($^{V}/_{V}$) ethanol. The tubes are then stoppered with neoprene bungs and are shaken vigorously for 5 secs. to oxidise residual hydrindantin,

which is red in colour at the reaction pH. The solutions are then read against a blank prepared from the eluent buffer, on a Unicam S.P. 600 visible spectrometer at 570 mμ. 1 cm. glass cells are used. For all the amino acids studied the graph of colour density against concentration is a straight line up to colour densities of 1.0.

Ornithine was chosen as the standard amino acid for ninhydrin colorimetry, and colour yields with respect to ornithine have been calculated for γ-amino-butyric acid, lysine and histidine. For arginine, which is eluted at pH 5.0, a series of arginine standards is always prepared. The standard amino acid solutions are of course made up in the correct pH buffer.

Determination of colour yields Standard solutions of lysine hydrochloride, ornithine hydrobromide, histidine hydrochloride dihydrate and γ-aminobutyric acid were prepared in pH 4.3 0.15 M sodium citrate buffer. Aliquots of these solutions were taken and made to 5 ml. with pH 4.3 buffer. The ninhydrin colours were developed as described above and optical densities determined against a blank from the same buffer. The ornithine standard graph was then drawn of optical density against micromoles ornithine (μM_o). The optical densities of the other amino acid standards were then converted to μM_o from this graph, and the colour yield determined by dividing μM_o by the μM of amino acid taken.

Table 9

Specimen determination of colour yields of histidine, lysine and γ-amino-butyric acid, with respect to ornithine, at 570 mμ. (ε = extinction)

ORNITHINE		γ-AMINOBUTYRIC ACID			
μM	ε	μM	ε	μM_o	C.Y.
0.049	0.150	0.057	0.132	0.050	0.88
0.097	0.256	0.114	0.269	0.102	0.90
0.146	0.397	0.228	0.520	0.196	0.86
0.242	0.632	0.343	0.775	0.293	0.86
0.339	0.880	0.457	1.008	0.382	0.84

Mean C.Y. = 0.87
Average deviation = 0.02

LYSINE				HISTIDINE			
μM	ε	μM_o	C.Y.	μM	ε	μM_o	C.Y.
0.067	0.174	0.064	0.95	0.057	0.141	0.053	0.93
0.134	0.341	0,125	0.93	0.114	0.280	0.106	0.93
0.201	0.462	0.174	0.87	0.170	0.420	0.158	0.93
0.335	0.805	0.310	0.93	0.284	0.675	0.256	0.90
				0.397	0.920	0.348	0.88

Mean C.Y = 0.92
Average deviation = 0.02

Mean C.Y. = 0.91
Average deviation = 0.02

Table 10

Relative colour yields of basic amino acids with respect to ornithine at 570mμ.

	Number of determinations	Mean colour yield	Range of values	Average deviation from mean colour yield
γ-aminobutyric acid	2	0.86	0.85-0.87	0.01
Lysine	7	0.91	0.89-0.98	0.02
Histidine	7	0.88	0.82-0.92	0.03

Development of the elution method

In preliminary qualitative work the procedure of Hamilton and Anderson was adopted. 15 cm. columns were prepared and equilibrated to pH 3.4, 0.1M citrate buffer. The first samples examined were solutions of single amino acids, containing usually 0.1-2.0 μM. With pH 5.0, 0.1M sodium citrate γ-aminobutyric acid emerged first, followed by ornithine, lysine and ammonia, which were eluted at the same position, with histidine closely behind. Arginine emerged much later. This behaviour was repeated with a mixture of all six components. In contrast Hamilton and Anderson found that ornithine and lysine were separated with pH 5.0, 0.1M buffer, that histidine required pH 7.5, 0.1M phosphate buffer, and that NH_3 did not appear until the molarity of the buffer was increased four times.

The above results are better understood on a study of the elution pattern in the gradient elution method of Moore and Stein (39). In their scheme the highest pH used is 5.1; the ammonia, ornithine, lysine and histidine peaks are quite close together, and emerge in that order at a pH between 4.2 and 4.4. In addition Moore and Stein use 150 cm. columns, although the molarity of the buffer in this region of the chromatogram is about 1.0. A pH of 5.0 would therefore be expected to move these four substances together on a 15 cm. column. However, the high buffer concentration required to remove the basic amino acids on the long columns afforded some hope that with lower concentrations and a pH of around 4.3 a separation would be achieved on shorter columns.

In finding the best elution pH it was convenient to use a mixture of basic amino acids and ammonia of millimolar concentration of each component. 0.5 ml. samples were analysed, and since the ninhydrin colour yields of these substances are similar, the number of components in any peak was readily determined. If the identity of a peak was in doubt it would be checked by the use of the

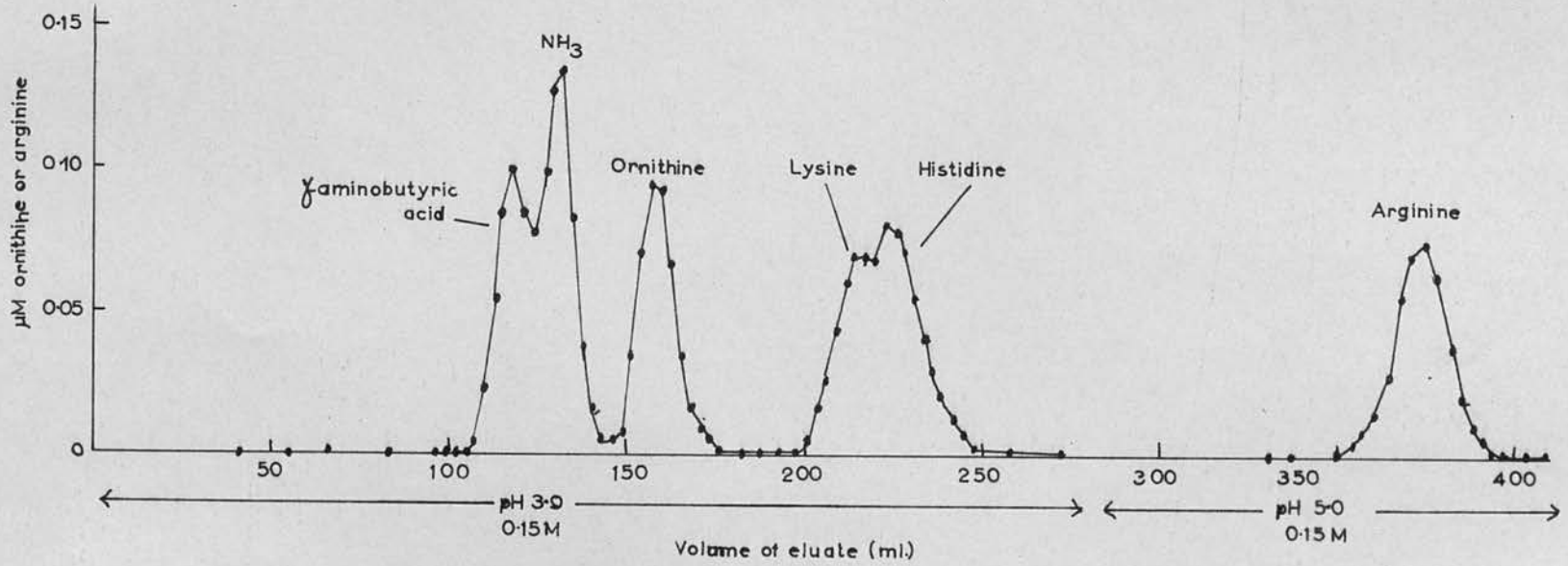
FIG 5

SEPARATION OF BASIC AMINO ACIDS ON 20cm. COLUMNS OF ZEO-KARB 225 AT 50°C

EFFECT OF pH ON THE ELUTION PATTERN

Approx 0·5μM of each amino acid

(a) pH 3·9



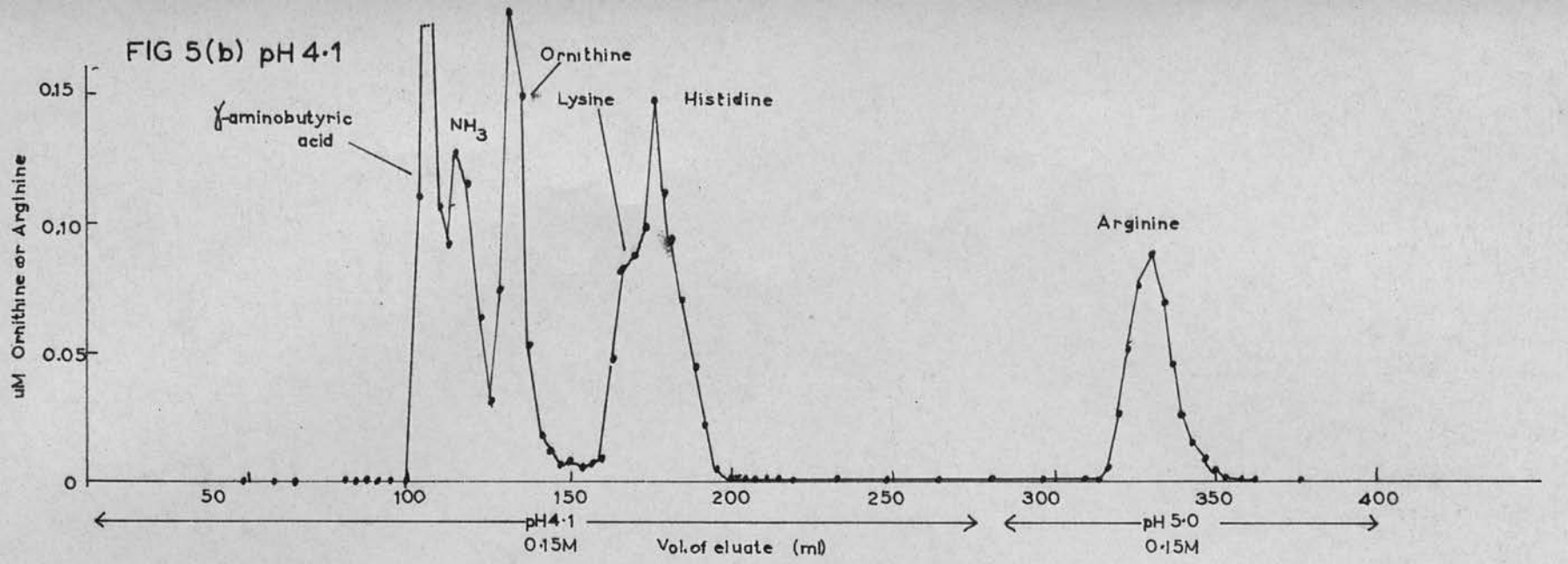


FIG 5(b) pH 4·1
Ornithine
Lysine
Histidine
γ-aminobutyric acid
NH3
Arginine
uM Ornithine or Arginine
0.15
0.10
0.05
0
50
100
150
200
250
300
350
400
pH 4·1
0·15M
Vol. of eluate (ml)
pH 5·0
0·15M

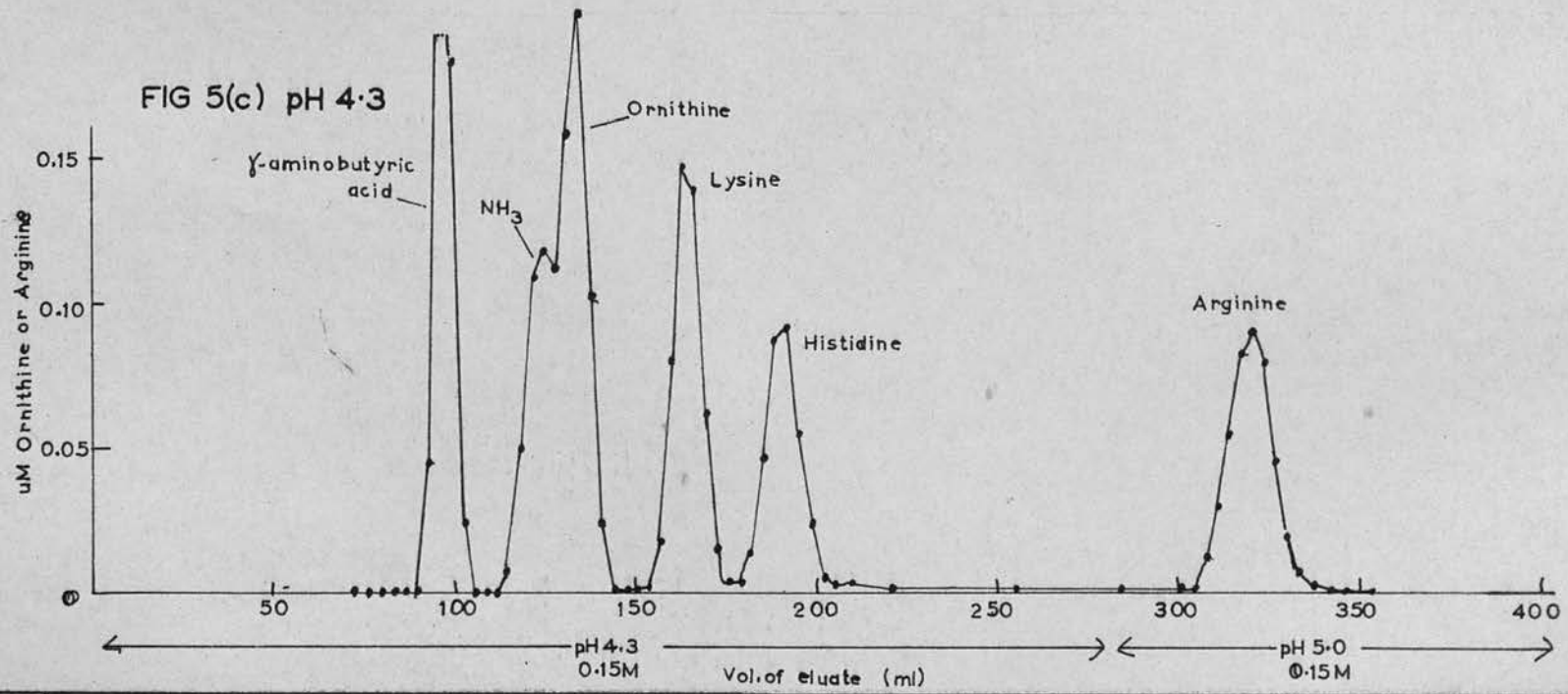


FIG 5(c) pH 4·3
Ornithine
γ-aminobutyric acid
NH3
Lysine
Histidine
Arginine
uM Ornithine or Arginine
0.15
0.10
0.05
0
50
100
150
200
250
300
350
400
pH 4·3
0·15M
Vol. of eluate (ml)
pH 5·0
0·15M

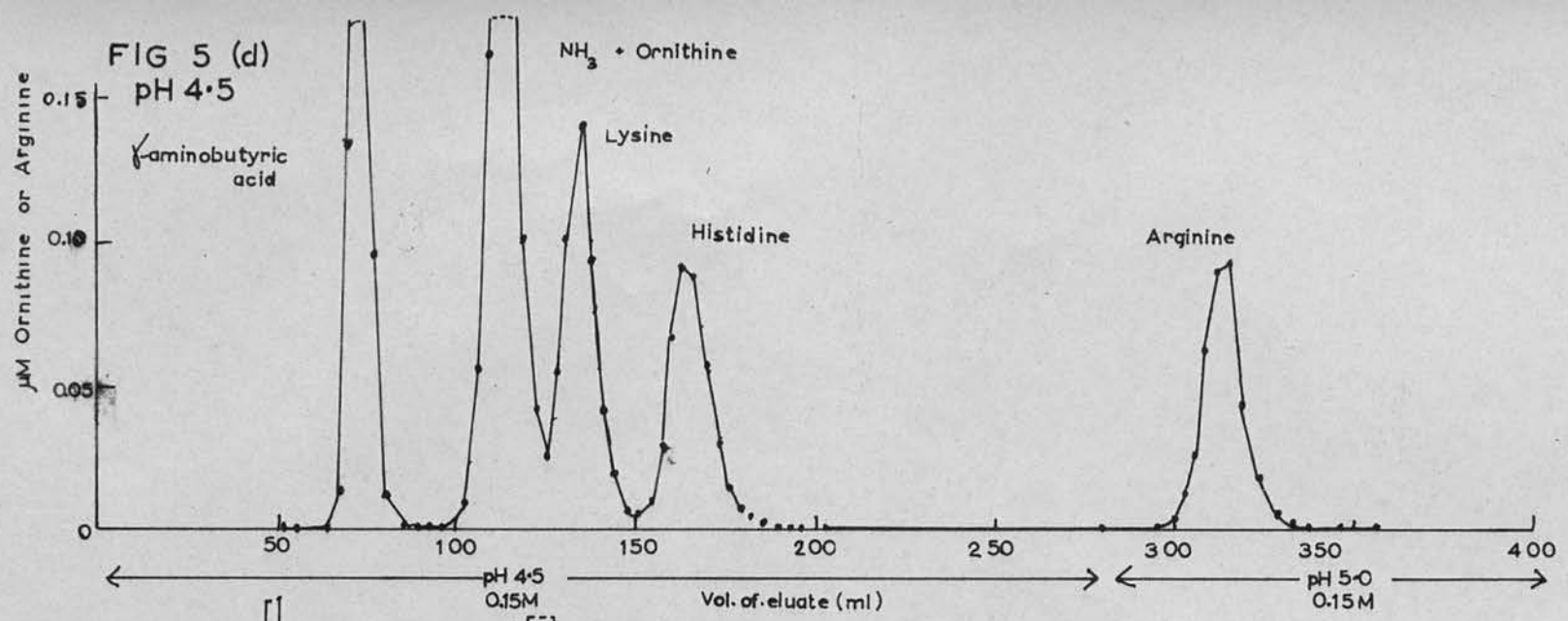


FIG 5 (d)
pH 4·5
NH_3 + Ornithine
Lysine
γ-aminobutyric acid
Histidine
Arginine
µM Ornithine or Arginine
0.15
0.10
0.05
0
50
100
150
200
250
300
350
400
pH 4·5
0.15M
Vol. of eluate (ml)
pH 5·0
0.15M

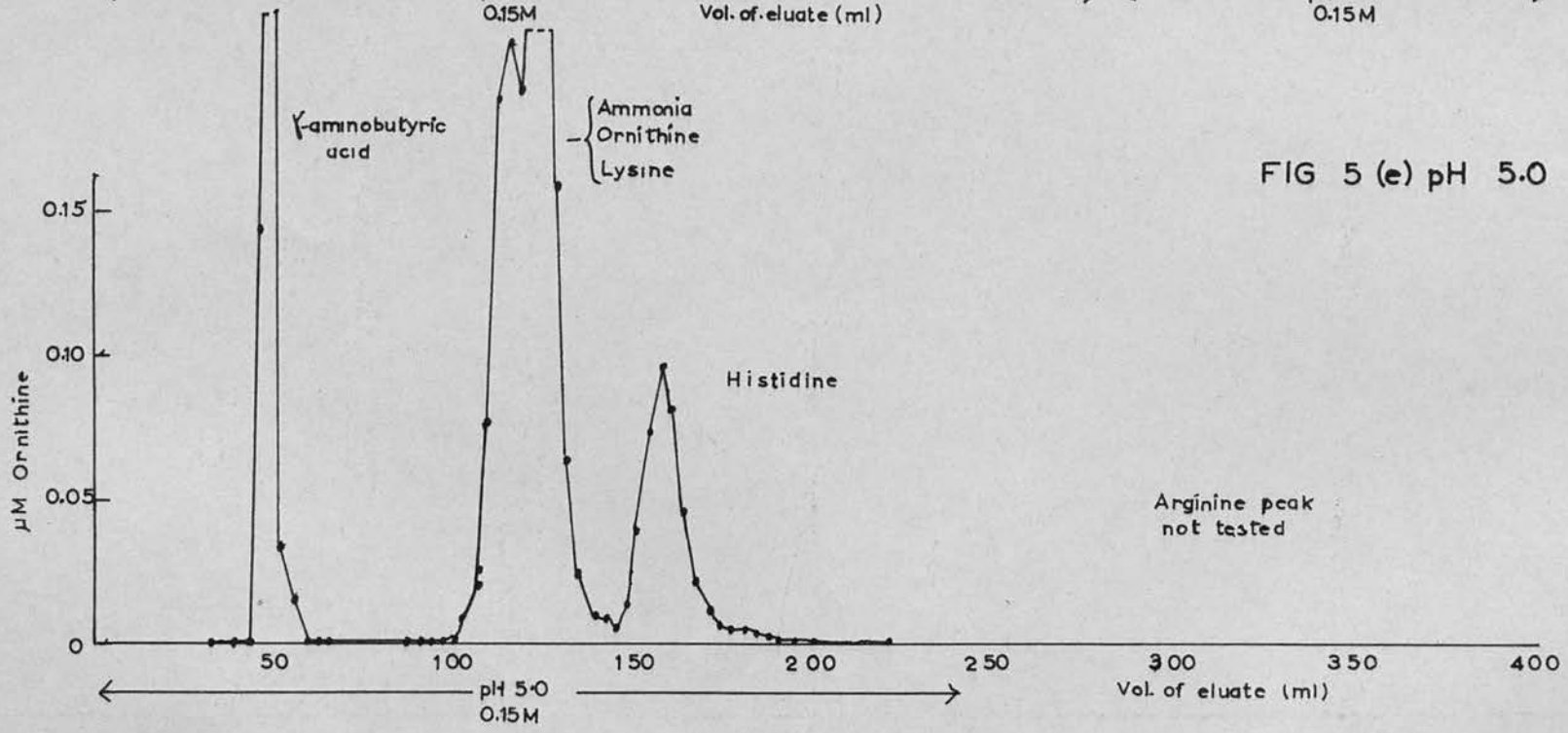
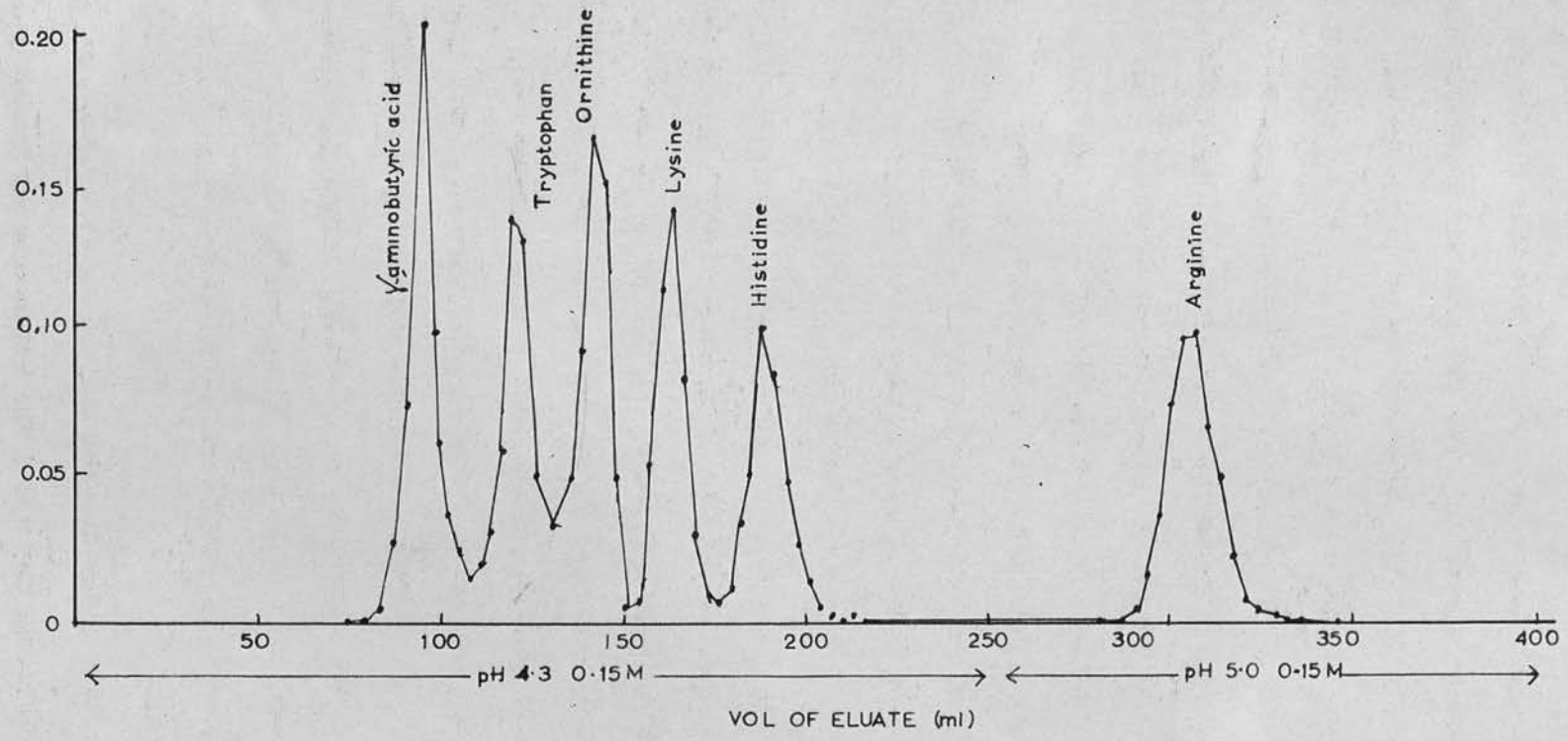

γ-aminobutyric acid
Ammonia
Ornithine
Lysine
Histidine
Arginine peak not tested
µM Ornithine
0.15
0.10
0.05
0
50
100
150
200
250
300
350
400
pH 5·0
0.15M
Vol. of eluate (ml)

FIG 5 (e) pH 5.0

FIG 6

SEPARATION OF TRYPTOPHAN AND BASIC AMINO ACIDS ON ZEO-KARB 225
20cm column Approx 0·5 μM of each amino acid



specific colour reactions mentioned on page 66.

In these experiments the columns were all 20 cm. long and the temperature of operation was 50°C. The columns were initially equilibrated to pH 3.4, 0.15M buffer and this molarity was used throughout. After emergence of histidine the arginine was removed as a sharp peak by changing the pH to 5.0, with quantitative recovery. If the lower pH was continued for arginine it formed a broad diffuse peak and recoveries were erratic. The results obtained are best demonstrated by the elution patterns (Fig. 5).

As expected from the results of Moore and Stein, the best separation was at pH 4.3. Ornithine and ammonia were however not separated at this pH. This difficulty has been surmounted by the Chinard reaction, which can be used for the quantitative measurement of ornithine (49, 50). Ammonia does not interfere with the determination in concentrations up to 4 times that of ornithine.

Finally the elution of tryptophan by pH 4.3 buffer was examined. This amino acid frequently shows unpredictable behaviour in buffered column chromatography, and for this reason was omitted from earlier samples until a separation of basic amino acids had been worked out. At pH 4.3 tryptophan is eluted just ahead of the ornithine-ammonia peak (Fig. 6).

When the elution scheme was applied to grass extracts and protein hydrolysates, the separation of ornithine, lysine and histidine was never as good as with standard mixtures. This however was still the best pH, and some of the columns were made 25 cm. long instead of 20 cm. in order to improve separations.

The very large amounts of ammonia often found in grass and silage extracts and hydrolysates can spoil the resolution of lysine and histidine completely. In such cases, the bulk of the ammonia was removed from the sample before chromatography by adjusting the pH to 10.5 and heating on a boiling water bath

for 5 mins. under a stream of air. The pH was then re-adjusted to 2.5 before applying to the column.

Summary of Method

The sample is adjusted to pH 2.5 and drained into the column, which has been equilibrated with pH 3.4, 0.15M citrate buffer. The sample is followed by 2 ml. pH 2.5 buffer, and then elution is begun with pH 4.3, 0.15M citrate buffer. 2 ml. or 3 ml. fractions are collected. For 20 cm. columns, the eluent is changed to pH 5.0, 0.15M citrate buffer after 220 ml., and for 25 cm. columns, after 250 ml. pH 4.3 buffer have passed through. The temperature of operation is 50°C throughout.

Determination of ornithine by the Chinard reaction.

Ninhydrin reagent. 625 mg. ninhydrin are dissolved in 10 ml. 6M-phosphoric acid and 15 ml. glacial acetic acid.

1 ml. aliquots are taken from the fractions containing the ornithine band, and are mixed with 2 ml. glacial acetic acid and 2 ml. ninhydrin reagent in test tubes graduated at 10 ml. The tubes are heated at 100°C for one hour, cooled to room temperature, and the volume made to 10 ml. with glacial acetic acid. The colour densities are then read on a Unicam S.P. 600 spectrometer, at 500 mμ. A series of standards is run concurrently. Under the reaction conditions lysine gives a yellow colour, which at 500 mμ shows about 10% of the light absorption of an equimolecular amount of ornithine. These two compounds are usually well resolved, and no error arises in this respect.

Table 11

Effect of ammonia on the Chinard reaction

Ornithine only μM	ε(1 cm.) 500 mμ	0.5 μM $(NH_4)_2SO_4$ per tube + ornithine μM	ε(1 cm.) 500 mμ
0.121	0.185	0.121	0.193
0.242	0.375	0.242	0.380
0.364	0.576	0.364	0.576
0.485	0.756	0.485	0.756

In many hydrolysates and extracts containing up to 25% ammonia-N, ammonia made no contribution to the colour density.

Determination of other amino acids

This is done by ninhydrin colorimetry at pH 5.5, as already described. For the fractions containing the ornithine band, 1 ml. aliquots were tested.

After a column has been used it may be regenerated by passing through 100 ml. of 0.3N sodium hydroxide, followed by 50 ml. of pH 3.4 0.15M sodium citrate buffer.

Recoveries of basic amino acids from standard mixtures

The recoveries tabulated below were obtained at different pH values between 4 and 5, but always at a buffer molarity of 0.15. In spite of the sensitivity of the actual elution pattern to pH it is felt that recoveries are unlikely to vary due to changes within this pH range. The standard mixtures used were of varying complexity, and except for ornithine in presence of ammonia, recoveries were determined on pure amino acid peaks. When ammonia was present, ornithine was determined by the Chinard reaction.

Table 12

Amino acid	Number of experiments	Mean Recovery %	Average deviation from mean recovery %	Range of recoveries %
γ-aminobutyric acid	12	100.5	2.2	94.6-106.3
Ornithine	12	99.1	4.2	93.4-106.5
Lysine	11	97.6	3.1	94.4-104.6
Histidine	14	94.3	4.9	86.0-102.8
Arginine	7	96.2	4.7	91.0-102.9

In view of the rather erratic recoveries, the amounts of amino acids found in natural products have not been corrected for low recovery.

APPLICATION OF ION-EXCHANGE CHROMATOGRAPHY TO NATURAL PRODUCTS

Basic amino acids in insulin

Zinc insulin supplied by Boots Pure Drug Co. Ltd. was used.

N constant = 13.70%. 107.2 mg. (14.70 mg.N) were hydrolysed in boiling 6N-HCl for 24 hr. The final volume of the hydrolysate was 50 ml.

2 ml. hydrolysate were taken for amino acid analysis.

Lysine-N	=	0.0176 mg.	3.00%TN
Histidine-N	=	0.0479 mg.	8.15%TN
Arginine-N	=	0.0346 mg.	5.89%TN

Assuming a molecular weight of 12,000 and N content of 15.54% for zinc-free insulin the numbers of basic amino acid residues per molecule of insulin are: lysine 2.0, histidine 3.62, arginine 1.96. From the work of Sanger and Tuppy (51) and Sanger and Thompson (52) these figures are 2, 4 and 2 respectively.

Basic amino acids in a mixed grass protein

A mixed leaf protein donated by Dr. G.R. Tristram was analysed. It had been prepared from pre-flowering Italian rye-grass. N content = 10.96%, not corrected for moisture or ash. 212.8 mg. (23.32 mg.N) of protein were hydrolysed in boiling 6N-HCl for 24 hours. The HCl was removed *in vacuo*, the hydrolysate was made to 50 ml., and finally filtered to remove humin.

Hydrolysate N. 2 ml. hydrolysate ≡ 2.28 ml. N/35 HCl, hence hydrolysate N = 22.80 mg. ∴ Humin N = 0.53 mg., i.e. 2.27% TN

3 ml. of filtered hydrolysate were taken for column chromatography.

Lysine-N	=	0.0655 mg.	7.00% TN
Histidine-N	=	0.0318 mg.	3.39% TN
Arginine-N	=	0.1028 mg.	11.00% TN

The object of this experiment was to test the analytical method. The literature shows a wide variety of analytical results for the basic amino acid content of leaf protein, and the results above are within the range of the more generally accepted values. The figure for arginine is probably low, in view of the impurity of the protein sample, and Tristram's finding that arginine is the most susceptible amino acid to destruction when protein is hydrolysed in presence of carbohydrate impurities (53).

CONSERVATION EXPERIMENT C

BASIC AMINO ACIDS IN WILTING GRASS

The pure perennial rye-grass used was received from the Bush Farm on 9th. June 1958, about 1 hr. after cutting. A portion of the grass was extracted immediately, and two samples were set aside for dry and moist wilting. Both were extracted after 48 hrs.

FRESH GRASS

Dry matter 14.7%

Total nitrogen 100 g. fresh grass contain 480.8 mg. TN, i.e. TN = 3.28% d.m.

Soluble nitrogen Two samples were extracted, and volumes were made to 250 ml.

a) Weight of grass extracted = 131.0 g. TN = 629.8 mg.

2 ml. extract ≡ 1.13 ml. N/35 HCl, hence SN = 8.94% TN.

b) Weight of grass extracted = 136.6 g. TN = 656.6 mg.

2 ml. extract ≡ 1.21 ml. N/35 HCl, hence SN = 9.25% TN.

Average soluble nitrogen = 9.10% TN.

Basic amino acids 2 ml. of 10 to 1 concentrate of extract (b)

	mg. N	% TN
γ-aminobutyric acid	0.0210	0.04
ornithine	Absent	0
lysine	0.0315	0.06
histidine	Trace	
arginine	Trace	

48 HOUR DRY WILT — Weight of grass taken = 97.7 g. TN = 469.7 mg.

Weight of grass after 48 hrs = 40.2 g.

Soluble nitrogen — The whole sample was extracted, and the volume made to 500 ml.

5 ml. extract ≡ 2.84 ml. N/35 HCl, hence SN = 24.2% TN.

Volatile base — 5 ml. extract ≡ 0.19 ml. N/35 HCl, hence VB = 1.60% TN.

Total amide — 5 ml. extract ≡ 0.67 ml. N/35 HCl, hence TAN = 8.17% TN.

Basic amino acids — Two columns were run, each using 2 ml. of a 25 — 10 concentrate of the extract.

	Column 1		Column 2		Average
	mg. N	% TN	mg. N	% TN	% TN
γ-aminobutyric acid	0.0123	0.26	0.0108	0.23	0.25
ornithine	Absent		Absent		0
lysine	0.0199	0.42	0.0243	0.52	0.47
histidine	0.0112	0.24	0.0087	0.18	0.21
arginine	—		0.0098	0.21	0.21

48 HOUR MOIST WILT Weight of grass taken = 96.0 g. TN = 461.5 mg.

Weight of grass after 48 hr. = 93.6 g.

Soluble nitrogen The whole sample was extracted, the volume was made to 500 ml.

2 ml. extract ≡ 1.36 ml. N/35 HCl, hence SN = 29.4% TN.

Volatile base 5 ml. extract ≡ 0.26 ml. N/35 HCl, hence VB = 2.25% TN.

Total amide 5 ml. extract ≡ 0.77 ml. N/35 HCl, hence TAN = 8.83% TN.

Basic amino acids Two columns were run, using sample volumes of 4 ml. and 5 ml. respectively.

	Column 1 (4ml)		Column 2 (5ml)		Average
	mg. N	% TN	mg. N	% TN	% TN
γ-aminobutyric acid	0.0021	0.06	0.0028	0.06	0.06
ornithine	Absent		Absent		0
lysine	0.0221	0.62	---		0.62
histidine	0.0034	0.09	0.0059	0.13	0.11
arginine	0.0216	0.58	0.0202	0.44	0.51

CONSERVATION EXPERIMENT D

BASIC AMINO ACIDS IN SILAGE

The grass used was pure perennial rye-grass cut at Bush Farm on 30th July 1958 in the early flowering stage. Several small scale silages were set up and were kept in an incubator at 35°C. Bottles were opened and extracted after 2, 5, 12, and 55 days. This grass was also used for a large scale (1 ton) series of silos prepared at Bush Estate, and opened after 55 days. An extract from one of these silages has been analysed for comparison with the 55 day laboratory silage.

FRESH GRASS

Dry matter 15.8%

Total nitrogen 100 g. grass contain 314.1 mg. TN. i.e. TN = 1.99% d.m.

Soluble nitrogen Weight of grass extracted = 150.8 g. TN = 473.4 mg.

Volume of extract = 500 ml.

5 ml. extract ≡ 1.12 ml. N/35 HCl, hence SN = 9.72% TN.

Volatile base 10 ml. extract ≡ 0.11 ml. N/35 HCl, hence VB = 0.48% TN.

Basic amino acids 2 ml. of a 4 to 1 concentrate of extract.

	mg. N	% TN
γ-aminobutyric acid	0.0114	0.15
ornithine	Absent	0
lysine	0.0053	0.07
histidine	Trace	
arginine	Trace	

2 DAY SILAGE — Weight of grass ensiled = 195.2 g. TN = 615.0 mg.

Weight before extraction = 194.1 g.

pH 4.10

Soluble nitrogen — Weight of silage extracted = 181.3 g. TN = 572.7 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 2.85 ml. N/35 HCl, hence SN = 49.8% TN.

Volatile base — 5 ml. extract ≡ 0.89 ml. N/35 HCl, hence VB = 6.2% TN.

Basic amino acids — 2 ml. extract

	mg. N	% TN
γ-aminobutyric acid	0.0649	2.84
ornithine	0.0495	2.15
lysine	0.0246	1.07
histidine	0.0074	0.32
arginine	Trace	

5 DAY SILAGE — Weight of grass ensiled = 200.1 g. TN = 628.4 mg.

Weight before extraction = 198.7 g.

pH 3.96

Soluble nitrogen — Weight of silage extracted = 183.2 g. TN = 579.3 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 3.18 ml. N/35 HCl, hence SN = 54.8% TN.

Volatile base — 5 ml. extract ≡ 1.16 ml. N/35 HCl, hence VB = 8.0% TN.

Basic amino acids — Not measured.

12 DAY SILAGE — Weight of grass ensiled = 209.0 g. TN = 656.3 mg.

Weight before extraction = 207.5 g.

pH 3.87

Soluble nitrogen — Weight of silage extracted = 190.1 g. TN = 601.2 mg.

Volume of extract = 500 ml.

2 ml. extract ≡ 3.54 ml. N/35 HCl, hence SN = 58.8% TN.

Volatile base — 5 ml. extract ≡ 1.31 ml. N/35 HCl, hence VB = 8.7% TN.

Basic amino acids — Two columns were run, on 2 ml. extract each.

	Column 1		Column 2		Average
	mg. N	% TN	mg. N	% TN	% TN
γ-aminobutyric acid	0.0782	5.20	0.0820	5.36	5.28
ornithine	0.0667	2.73	0.0650	2.66	2.70
lysine	0.0342	1.40	0.0364	1.49	1.45
histidine	0.0100	0.41	0.0124	0.51	0.46
arginine	Absent		—		0

55 DAY SILAGE — Weight of grass ensiled = 213.1 g. TN = 669.2 mg.

Weight before extraction = 211.4 g.

pH 3.90

Soluble nitrogen — Weight of silage extracted 175.5 g. TN = 555.5 mg.

Volume of extract = 250 ml.

1 ml. extract ≡ 3.34 ml. N/35 HCl, hence SN = 60.1 % TN.

Volatile base — 2 ml. extract ≡ 1.06 ml. N/35 HCl, hence VB = 9.6 % TN.

Basic amino acids — Three columns were run on 1 ml. aliquots of the extract

	Column 1		Column 2		Column 3		Average
	mg. N	% TN	mg. N	% TN	mg. N	% TN	% TN
γ-aminobutyric acid	0.0885	3.97	0.0901	4.04	0.0892	4.01	4.01
ornithine	—		0.0565	2.55	—		2.55
lysine	0.0424	1.91	—		0.0363	1.63	1.77
histidine	0.0191	0.86	0.0196	0.88	0.0125	0.56	0.77
arginine	—		0.0048	0.26	—		0.26

55 DAY LARGE SCALE SILAGE

Dry matter 16.9%

pH 5.70

Total nitrogen 100 g. silage contains 309.0 mg. TN i.e. TN = 1.85% d.m.

Soluble nitrogen Weight of silage extracted = 100.0 g. TN = 309.0 mg.

Volume of extract = 250 ml.

1 ml. extract ≡ 1.83 ml. N/35 HCl, hence SN = 59.2 % TN.

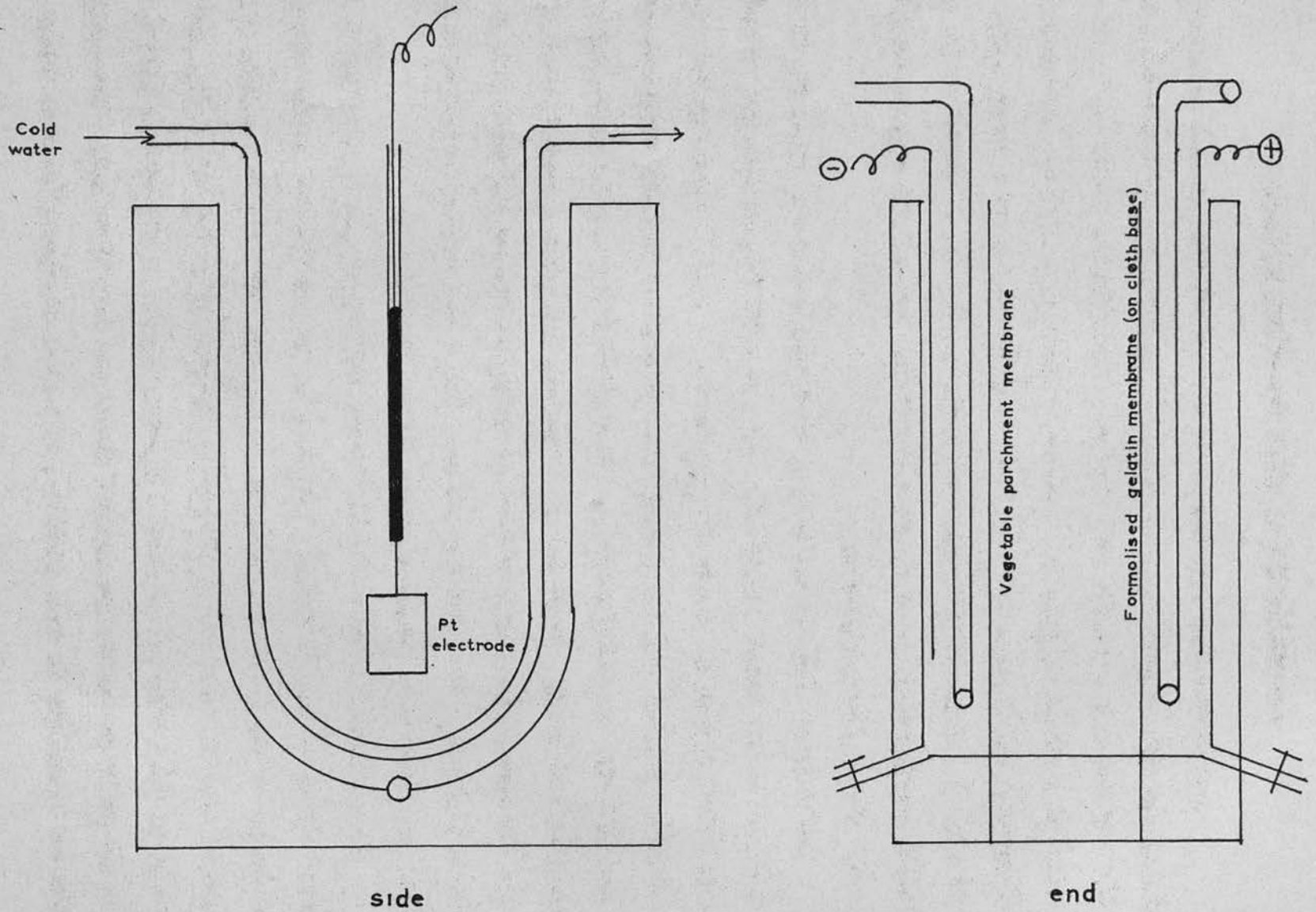
Volatile base 5 ml. extract ≡ 1.24 ml. N/35 HCl, hence VB = 8·0% TN.

Basic amino acids 1 ml. extract.

	mg. N	% TN
γ-aminobutyric acid	0.0366	2.96
ornithine	0.0196	1.58
lysine	0.0329	2.66
histidine	0.0143	1.16
arginine	0.0071	0.58

FIG 7

ELECTRODIALYSIS APPARATUS



ELECTRODIALYSIS OF GRASS AND SILAGE EXTRACTS

A three compartment cell was used, and was the same as that described by Macpherson (54) (Figure 7). It is made of perspex, with a formolised gelatin membrane dividing the anode from the centre compartment, which is, in turn, separated from the cathode by a vegetable parchment membrane. The centre compartment has a capacity of about 250 ml., and the outside compartments 150 ml. The platinum electrodes and cooling coils are contained in the external compartments, and the centre compartment solution is stirred continuously during electrodialysis.

The extract under investigation, containing 5 - 10 mg. nitrogen, at pH 6, is placed in the centre compartment, and the outer compartments are filled with distilled water. Under the influence of a direct current at 250 volts migration of acidic and basic substances takes place into the anode and cathode compartments. As the migration nears completion the current falls, and should reach 20 - 40 mAmp. If an excess of material is present, then diffusion back into the centre compartment attains equilibrium with the migration out, at a higher current. When this happens portions of the external compartment solutions are removed and replaced with distilled water.

On the first run the pH of the centre compartment may fall to 2.5 to 3.5, thus causing neutral compounds to migrate to the cathode, and acidic compounds to remain in the centre compartment. After the first run, the solutions are removed from the cell, the catholyte is adjusted to pH 5-6 with H_2SO_4, and replaced in the centre compartment. Electrodialysis is repeated as before, and now the pH of the centre compartment should not fall below 4.0. The catholyte from the second run is then subjected to a final electrodialysis to ensure

complete removal of neutral amino acids. This procedure isolates the basic amino acids in the final catholyte. The neutral fraction from the first run is adjusted to pH 5-6 with dilute aqueous ammonia, and electrodialysed again to remove acidic amino acids into the anolyte. The various acidic, basic and neutral fractions are then combined and reduced to a suitable volume in vacuo. The catholytes are evaporated at pH 10.5 to remove ammonia. Nitrogen contents of the fractions are determined by the micro-Kjeldahl technique.

This procedure has been applied to grass and silage extracts and their hydrolysates.

The cathode solutions were also used to determine histidine and arginine by the colorimetric methods of Macpherson (54).

Determination of Histidine

An aliquot of the catholyte containing 0.005 - 0.05 mg. of histidine-N is transferred to a 25 ml. graduated flask, and the volume is made 5 ml. 1 ml. of 1% ($^{W}/_{V}$) sulphanilic acid in N-HCl and 1 ml. of 5% ($^{W}/_{V}$) sodium nitrite are added, the solution is mixed, and is left for 5 min. 3 ml. of 10% ($^{W}/_{V}$) anhydrous sodium carbonate are then pipetted into the flask, which is shaken vigourously for 10 sec., and then 10 ml. of 20% ($^{V}/_{V}$) ethanol are added. The flask is shaken while cooling under the tap, and the volume is then made to 25 ml. The colour produced is then read against a water blank in a Unicam colorimeter with a green Ilford 404 filter, and compared with a series of standards prepared at the same time.

It was known that certain inorganic ions, especially the heavy metals, can complex with histidine, and cause low recoveries when this method is used. Macpherson found this to be the case with hydrolysates of zinc insulin, and also showed that quantitative recoveries of histidine were obtainable if disodium versenate was added to the catholyte before the colour reaction was

performed. Disodium versenate (disodium ethylene-diaminetetraacetate) complexes metal ions in preference to the histidine. When versenate was added to the catholytes from grass extracts, higher histidine values were obtained.

Determination of Arginine

An aliquot of the catholyte containing 0.01 - 0.1 mg. of arginine-N is transferred to a 25 ml. graduated flask, and diluted to 5 ml. 1 ml. of 10% ($^{W}/_{V}$) potassium hydroxide is added, followed by 2 ml. of 0.1% ($^{W}/_{V}$) α-naphthol in 50% ($^{V}/_{V}$) ethanol and 1 ml. 5% ($^{W}/_{V}$) urea. 2 ml. of potassium hypobromite solution (2 g. Br_2 in 100 ml. 5% KOH) are then added from a rapid delivery pipette. The flask is shaken vigourously at once, made to volume, and left for 15 minutes to ensure complete development of the colour. The colour is then read against a water blank using the same filter as for the histidine determination, and compared with a series of standard arginine colours.

Addition of versenate was found to have no effect on the colour developed from catholytes of grass extracts.

Hydrolysis of extracts

Extracts were hydrolysed for 24 hours in boiling 6N-HCl at a nitrogen concentration of about 15 mg./litre. This very high dilution was used to minimise destruction of amino acids by humin formation. The HCl was removed by evaporation in vacuo three times, and the residue was then dissolved in water, made to volume and filtered. Humin-N was determined by the difference between the extract N taken, and the N content of the filtered hydrolysate.

Results from Electrodialysis of Grass Extracts and Hydrolysates

The extracts used were obtained in conservation experiments C and D

48 HOUR DRY WILT Volume of extract taken = 35 ml. SN = 7.95 mg. (= 32.82 mg. TN)

	mg.	% TN
Basic - N	0.58	1.76
Acidic - N	1.65	5.03
Neutral - N	5.41	16.48
Ammonia - N	0.52	1.58

SN accounted for = 102.8%

Hydrolysate Volume of extract hydrolysed = 75 ml. SN = 17.00 mg. (= 70.4 mg. TN)

Volume of hydrolysate = 50 ml.

25 ml. of hydrolysate were taken for electrodialysis. This is equivalent to 8.50 mg. extract SN and 35.2 mg. TN.

	mg.	% TN
Basic - N	0.69	1.96
Acidic - N	1.80	5.12
Neutral - N	3.36	9.55
Ammonia - N	2.38	6.76
Humin - N	0.20	0.57

SN accounted for = 99.1%

FRESH GRASS (EXP. D)

Volume of extract taken = 50 ml. SN = 4.60 mg. (=47.3 mg. TN)

	mg.	% TN
Basic - N	0.34	0.72
Acidic - N	1.50	3.17
Neutral - N	1.50	3.17
Ammonia - N	0.23	0.49

SN accounted for = 77.6%

2 DAY SILAGE

Volume of extract taken = 15 ml. SN = 8.56 (= 17.20 mg. TN)

For histidine, the 1st. value refers to determination without versene, the 2nd. with versene.

	mg.	% TN
Basic - N	2.14	12.4
Histidine - N	0.163,0.252	0.95,1.47
Arginine - N	0.093	0.54
Acidic - N	0.99	5.8
Neutral - N	4.00	23.3
Ammonia - N	1.06	6.2

SN accounted for = 95.6%

Hydrolysate

Volume of extract hydrolysed = 60 ml. SN = 34.25 mg. (= 68.8 mg. TN)

Volume of hydrolysate = 50 ml.

10 ml. of hydrolysate were taken for electrodialysis. This is equivalent to 6.85 mg. extract SN and 13.76 mg. TN.

	mg.	% TN
Basic - N	1.54	11.2
Histidine - N	0.142,0.198	1.03,1.44
Arginine - N	0.122	0.89
Acidic - N	0.49	3.56
Neutral - N	2.83	20.6
Ammonia - N	1.46	10.6
Humin - N	0.10	0.73

SN accounted for = 93.6%

5 DAY SILAGE

Volume of extract taken = 15 ml. SN = 9.53 mg. (= 17.39 mg. TN)

	mg.	% TN
Basic - N	2.23	12.8
Histidine - N	0.200,0.270	1.15,1.55
Arginine - N	0.047	0.27
Acidic - N	1.20	6.9
Neutral - N	4.39	25.5
Ammonia - N	1.40	8.0

SN accounted for = 96.8%

Hydrolysate

Volume of extract hydrolysed = 50 ml. SN = 31.75 mg. (= 57.92 mg TN)

Volume of hydrolysate = 50 ml.

15 ml. hydrolysate were taken for electrodialysis. This is equivalent to 9.53 mg. of extract SN, and 17.39 mg. TN.

	mg.	% TN
Basic - N	1.79	10.3
Histidine - N	0.176,0.216	1.01,1.18
Arginine - N	0.68	0.39
Acidic - N	0.83	4.8
Neutral - N	3.91	22.5
Ammonia - N	2.06	11.8
Humin - N	0.20	1.2

SN accounted for = 92.2%

12 DAY SILAGE — Volume of extract taken = 10 ml. SN = 7.18 mg. (= 12.2 mg. TN)

	mg.	% TN
Basic - N	1.84	15.1
Histidine - N	0.170, —	1.33, —
Arginine - N	—	—
Acidic - N	0.66	5.4
Neutral - N	3.48	28.5
Ammonia - N	1.07	8.8

SN accounted for = 99.5%

Hydrolysate — Volume of extract hydrolysed = 50 ml. SN = 35.90 mg. (= 61.0 mg. TN)

Volume of hydrolysate = 50 ml.

10 ml. of hydrolysate were taken for electrodialysis. This is equivalent to 7.18 mg. of extract SN, and 12.2 mg. TN.

	mg.	% TN
Basic - N	1.92	15.7
Histidine - N	0.198,0.256	1.62,2.10
Arginine - N	0.089	0.73
Acidic - N	0.31	2.5
Neutral - N	2.83	23.2
Ammonia - N	1.52	12.5
Humin - N	0.34	2.8

SN accounted for = 96.4%

55 DAY SILAGE — Volume of extract taken = 5 ml. SN = 6.66 mg. (= 11.08 mg. TN)

	mg.	% TN
Basic - N	1.80	16.3
Histidine - N	0.169, —	1.53, —
Arginine - N	0.089	0.79
Acidic - N	0.63	5.7
Neutral - N	3.14	28.4
Ammonia - N	1.06	9.6

SN accounted for = 99.5%

Hydrolysate — Volume of extract hydrolysed = 25 ml. SN = 53.30 mg. (= 55.40 mg TN)

Volume of hydrolysate = 50 ml.

10 ml. of hydrolysate were taken for electrodialysis. This is equivalent to 6.66 mg. of extract SN and 11.08 mg. TN.

	mg.	% TN
Basic - N	1.75	15.8
Histidine - N	0.190,0.242	1.71,2.18
Arginine - N	0.084	0.76
Acidic - N	0.52	4.7
Neutral - N	2.75	24.8
Ammonia - N	1.44	13.0
Humin - N	0.14	1.3

SN accounted for = 99.1%

DISCUSSION

The results obtained by the application of ion exchange chromatography to grass and silage extracts are contained in Table 13. For lysine, histidine and arginine the table also includes the amounts of these amino acids to be expected from the degree of proteolysis.

Basic amino acids in grass leaf proteins have been measured by Tristram (55), Smith and Agiza (56), and Armstrong (57), among others. A very wide range of results has been reported. Much of the trouble arises from destruction of amino acids during hydrolysis. From a study of the hydrolytic techniques used by these workers it seems that the most likely approximate proportions of basic amino acids in grass leaves are: arginine 14.0%, histidine 4.0% and lysine 7.0% of total protein nitrogen, all values being plus or minus 0.5%. The histidine figure quoted by Tristram is about 2.4%, but as Lugg and Weller have shown (58), this is probably 50% too low, because of defects in the gravimetric method of analysis. The above figures have been used in determining amounts of free basic amino acids expected in silage and wilting grass. For the method of calculating expected amounts see page 56.

Table 13

The Basic Amino Acids in Grass Conservation

Material extracted	Soluble N	γ-amino N	Ornithine N	Lysine N		Histidine N		Arginine N	
				Found	Exp.	Found	Exp.	Found	Exp.
Conservation Experiment C									
Fresh grass	9.1	0.04	0	0.06	-	Tr	-	Tr	-
Dry wilt, 48 hr.	24.2	0.25	0	0.47	1.12	0.21	0.60	0.21	2.12
Moist wilt, 48 hr.	29.4	0.06	0	0.62	1.48	0.11	0.81	0.51	2.84
Conservation Experiment D									
Fresh grass	9.7	0.15	0	0.07	-	Tr	-	Tr	-
Silage, 2 days	49.8	2.84	2.15	1.07	2.92	0.32	1.63	Tr	5.70
Silage, 12 days	58.8	3.28	2.70	1.45	3.51	0.46	1.97	0	6.88
Silage, 55 days	60.1	4.01	2.55	1.77	3.53	0.77	2.01	0.26	7.05
Large scale silage, 55 days	59.2	2.96	1.58	2.66	-*	1.16	-	0.58	-

* For the large scale silage expected values cannot be calculated because of loss of effluent.

γ-aminobutyric acid. The results obtained are similar to those from conservation experiments A and B, (See Tables 3 and 4, pp. 51-52), although γ-aminobutyric acid represents a higher proportion of the total nitrogen in the silage of experiment D. γ-aminobutyric acid is again less in moist wilting than in dry wilting.

Ornithine and Arginine

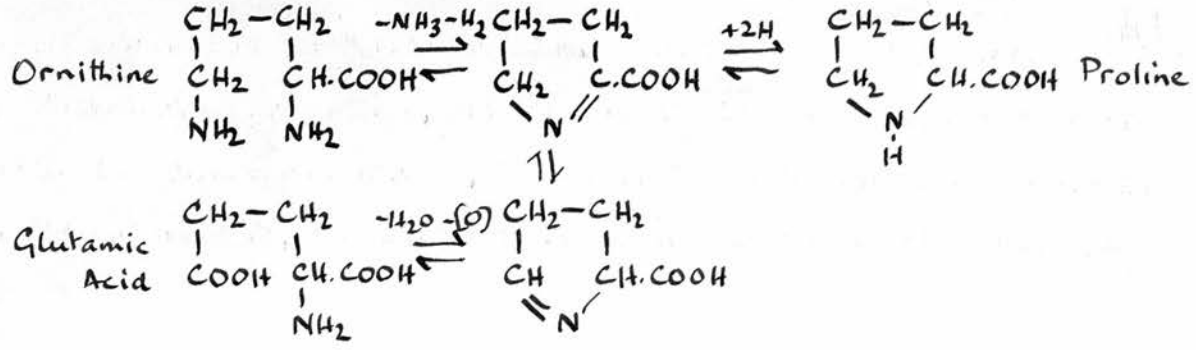
The free arginine found in both silage and wilting grass falls well below the expected values. In silage only trace amounts are detectable in column chromatography. Application of colorimetry to cathode fractions from electrodialysis gave higher apparent arginine contents, which should have been measurable in the ion exchange column method. Addition of 0.505 μM arginine hydrochloride to an aliquot from the 12 day silage extract, which apparently contained no arginine, gave a recovery of 0.515 μM, the peak appearing at the usual position from the column. It seems that other Sakaguchi positive substances must be present in silage.

The most obvious product of arginine metabolism in silage is ornithine, which can arise by the action of the enzyme arginase, urea being split off. Apart from preliminary work by Macpherson, this is apparently the first time that ornithine has been reported in silage, and its appearance as a major nitrogenous constituent makes this finding even more interesting. Since ornithine contains two nitrogen atoms and arginine four, the amount of arginine needed to produce ornithine can be obtained by doubling the ornithine values in Table 13. When this is done, we see that formation of ornithine may account for most of the "lost" arginine. Ornithine has not yet been isolated and characterised from silage, but its identity is shown by its position of elution in column chromatography, and the fact that it gives a red colour in the Chinard reaction, the wavelength of maximum light absorption being the

same as for a standard sample of ornithine. This wavelength is 500 mμ, although a wavelength of 515 mμ has been reported by Work (50) and Chinard (49). Ornithine was also found by paper chromatography of catholytes in phenyl-water/methyl cellosolve/pyridine.

Formation of ornithine from arginine in silage is apparently a function of bacteria. Microbe-free Timothy grass, which had been incubated anaerobically for 24 hours was extracted, as well as a fresh sample of the same grass. In 24 hours the soluble nitrogen had increased from 17.9% to 45.7% of total nitrogen. The fresh grass extract was not analysed for ornithine and arginine, but the incubated grass was found to contain no ornithine. The arginine found was 3.40% TN; more free arginine than has been found in any other preparation of excised leaves. If the fresh microbe-free grass contained only trace amounts of free arginine, the expected value in the incubated sample would be about 3.9%. Thus most of the arginine released from protein remains unmetabolised in the anaerobic sterile conditions. Early experiments by Macpherson with sap silages showed that disappearance of added arginine was paralleled by active microbial growth. In presence of toluene, when growth of bacteria was inhibited, arginine did not appear to be destroyed.

Ornithine was not found in either dry or moist wilted grass, but arginine values were again below the expected. In the 48 hour dry wilt the free proline content was 3.45% TN, compared with an expected value of 0.63% TN. Since proline can arise from arginine through ornithine, this may be one route of arginine breakdown.



However, since only one nitrogen atom of arginine can contribute to forming a proline molecule, it seems that if arginine is a precursor, it is not the only one.

No such speculations can be made about the fate of arginine in moist wilting, although indirect metabolism to glutamine is not impossible. The free proline content of the moist wilt was 0.20% TN, compared with an expected value of 0.70%.

Lysine

For all three conservation experiments, the free lysine content falls well below the expected amount.

Histidine

The main point of interest about histidine in silage is that entirely different results are obtained by the two analytical methods used. However, it is felt that the figures obtained by column chromatography do represent the true free histidine content of the silage. These values are very small and are supported by the failure to find a histidine spot in chromatography of silage catholytes. It must be concluded that substances other than histidine are reacting in the colorimetric method of estimation.

1 ml. of catholyte from the 12 day silage, containing 0.184 mg. N gave an apparent histidine-N content of 0.0189 mg. by the colorimetric method. When 1 ml. of the same catholyte was submitted to column chromatography the "true" histidine-N found was 0.0050 mg. After completing the column it was washed with 0.3N-NaOH, and fractions were collected from this eluate and tested by the ninhydrin reaction at pH 5.5 and the histidine colorimetric reaction. By the histidine reaction the NaOH eluate contained an apparent histidine-N of 0.0138 mg., which is equal to the difference between the apparent histidine on the whole catholyte, and the "true" histidine. In addition the elution

with NaOH produced three ninhydrin positive peaks, two of which gave a histidine positive reaction. HISTAMINE ? TYRAMINE ?

The amounts of histidine were generally so small as to make determination inaccurate by column chromatography. In addition resolution between lysine and histidine was not always complete and in wilted grass extracts the position was further complicated by the appearance of a ninhydrin positive peak just behind histidine. The identity of this peak is not known, but it disappeared on acid hydrolysis of the extract.

The apparent histidine measured by colorimetric analysis of silage catholytes is only slightly lower than the expected histidine value, and it may be that histidine released from protein is metabolised to substances which retain the imidazole ring and would give a red colour in the histidine-sulphanilic acid coupling reaction.

It is recognised that the analytical method, as applied here, is far from satisfactory, and it is unfortunate that so much time was spent in its development that the number of extracts which could be analysed was strictly limited.

"Unknown" basic nitrogenous compounds

In Table 14 the total amounts of non-volatile basic nitrogen in extracts, found from electrodialysis, are compared with the sums of the nitrogen in basic constituents measured by column chromatography.

Table 14

Material extracted	Non-volatile basic N	N of γ-aminobutyric acid, ornithine, lysine, histidine and arginine
	% TN	% TN
Fresh grass (Expt. D)	0.72	0.22+
Dry wilt, 48 hr.	1.76	1.20
Silage, 2 days	12.4	6.4
Silage, 12 days	15.1	7.9
Silage, 55 days	16.3	9.1

Only half of the basic nitrogen in silage extracts is accounted for by the basic amino acids measured. Very little can be said about other basic nitrogenous constituents, although the "histidine reacting" compounds presumably contribute some of the "unknown" nitrogen. Adenine has been found in grass extracts (59) and would migrate cationically. Table 14 shows that research is needed into basic nitrogenous compounds in silage, with particular reference to amines formed by decarboxylation of amino acids, which may be toxic.

FOUND LATER: HISTAMINE, TYRAMINE, TRYPTAMINE i.c.
PUTRESCINE, CADAVERINE (often v. large amts)

v. MACPHERSON H.T. J. Sci Fd Agric 1961 13 29
v " & VIGANTE P ibid. 1966 17 124

SECTION 3

PEPTIDES
(BOUND AMINO ACIDS)

Recent general reviews on naturally occurring peptides have been compiled by Synge in 1949 (60) and 1953 (61) and by Fromageot in 1953 (62). Synge's most recent review (1955 - 63) contains many references to experimental methods as well as information of known peptides in higher plants. A paper by Synge and Wood in 1958 (23) reviewed briefly the latest additions to the literature on this subject. Sanger (64) has collected information about isolative procedures.

Peptides, as distinct from proteins, are generally classified as polymers of amino acid units linked through peptide bonds, having molecular weights of not more than 10,000. The extra condition is sometimes applied, where the molecular weight exceeds 10,000 of lack of variety in amino acid composition. None of the known natural peptides exhibit the phenomena of denaturation or heat coagulation.

The criteria by which we distinguish peptides and proteins are empirical and are based on the means used for separating these compounds. This arises from the inadequacy of our knowledge of the chemistry of non-protein bound amino acids found in living tissue. In many studies of this subject the presence of a "peptide fraction" is merely suggested by the liberation of amino acids by acid hydrolysis. Such findings are especially useful if specific amino acids are shown to appear on hydrolysis, but the presence of peptide is by no means proved. Many examples are known of amino acids being coupled with other chemical types. Until the exact chemical nature is known it is safest to refer to compounds which release amino acids on hydrolysis as "bound amino acid compounds".

The first problem in isolating a non-protein bound amino acid fraction from biological material is to ensure complete removal of protein. Ultrafiltration and dialysis are probably the most exact methods of achieving this separation. It is quite well established that no compounds having molecular weights over 10,000 can pass through cellophane membranes. In the ether-water extraction procedures of Chibnall (27) the plant juices are virtually freed from protein by ultrafiltration through the cell wall. Synge has used this technique, and has found that much of the nitrogen in the juices is dialysable through cellophane (22). One limitation of this technique is that the rate of diffusion decreases with increasing molecular size, and for compounds of molecular weight over 1000, one cannot rely on complete separation from protein.

Heat coagulation is widely used for obtaining protein-free extracts of plant material. Macpherson (10) has shown that of the nitrogen in boiling water extracts of grass and silage 92-98% is rapidly dialysable through cellophane.

Extraction into aqueous ethanol has often been used to isolate non-protein nitrogenous fractions, in particular amino acids. Where the nitrogenous constituents are subsequently to be studied by paper chromatography, a great advantage is the low solubility of many inorganic salts in alcohol. Bathurst (24) prepared 80% ethanol extracts of freeze dried grass, and found them to be devoid of protein and peptide. Kemble and Macpherson (14) extracted grass with boiling water, and showed that after addition of ethanol to 80% and removal of the precipitates, the supernatant contained no bound amino acids. They did not, however, examine the composition of the small amount of nitrogenous compounds precipitated by alcohol. The limited solubility of certain amino acids in ethanol would almost certainly lead to some precipitation under the conditions employed. Synge has stressed that for

each individual case temperature, pH and alcohol concentrations should always be rigorously controlled, and that the distribution of amino acids and peptides between the soluble and insoluble fractions should be understood.

Very few comparative studies have been made of the efficiency of the wide range of techniques for separating protein from non-protein nitrogenous compounds. (See however Bisset - 65 and Neuberger and Sanger - 66).

Synge has continually emphasised the need for research into adsorption of amino acids and peptides on to the surface of protein molecules. Under any conditions we use for preparing protein-free extracts it is quite possible that peptides containing basic, acidic, aromatic or even the higher aliphatic amino acids will be strongly attached to the protein. This could be one reason why the bound amino acids found in ryegrass by Synge are predominantly of low molecular weight, and seldom include any of the groups mentioned above.

Much information can be gained by determining total α-carboxyl nitrogen or individual free amino acids before and after acid hydrolysis of deproteinised extracts. Van Slyke's ninhydrin-CO_2 (67), or his nitrous acid method (68), are most commonly used to determine "peptide-N" in this way. The former method is the more useful, being specific for α-amino acids and proline, whereas the nitrous acid method gives high yields with glycine and lysine. The ninhydrin method must be used with care in measuring peptide N, because only one of the nitrogen atoms in basic amino acids is counted, and aspartic acid yields 2 molecules of CO_2 per molecule. Synge has found the ninhydrin-CO_2 method useful in checking bound amino acid content of various fractions during his isolative procedures for peptides. He also discovered that when applied to grass diffusates very high blanks were obtained, and that even when conditions were precisely controlled, results were unreliable. (22)

Although there are several references to peptide-N values in plant

material few workers have made the natural extension of the work and estimated the increase in individual amino acids after hydrolysis. Synge was able to do this semi-quantitatively by paper chromatography,[22] and later quantitatively by ion exchange column chromatography.[23] Bathurst employed microbiological techniques with his hydrolysed peptide fractions.[24]

The main barrier to detecting peptides on paper chromatograms are the relatively large amounts of free amino acids and the fact that the ninhydrin colour reaction becomes less sensitive for peptides as the degree of polymerisation increases. Cyclic peptides do not give the ninhydrin reaction at all, and the same applies to many non-peptide forms of bound amino acids. The colour reaction of Rydon and Smith (69) and of Reindel and Hoppe (70), which are highly sensitive for all compounds containing imino and amino groups should prove most useful, especially when applied to fractions from which the bulk of free amino acids has been removed. Markowitz and Steinberg have recently developed a technique for freeing peptides from amino acids by destruction of the free amino acids with ninhydrin, followed by removal of excess ninhydrin with hydrogen peroxide (71).

Certain artefacts of the extraction procedures can yield amino acids on acid hydrolysis. The Maillard reaction between sugars and amines can proceed in concentrated solutions at high temperatures and therefore is likely to occur in stored or dried tissue. The reaction takes place with loss of amino nitrogen, and on acid hydrolysis there is at least a partial regeneration of amines and amino acids. Asparagine, which yields 1 molecule of CO_2 per molecule in the Van Slyke method, is hydrolysed to aspartic acid, which yields 2 molecules CO_2 per molecule. Both glutamine and glutamic acid yield only one mole CO_2 per molecule. Pyrrolidonecarboxylic acid which can be produced from glutamine during extraction is converted to glutamic acid on hydrolysis, thus

providing another source of increase in α-carboxyl nitrogen. Glycine is among the products of acid hydrolysis of purines.

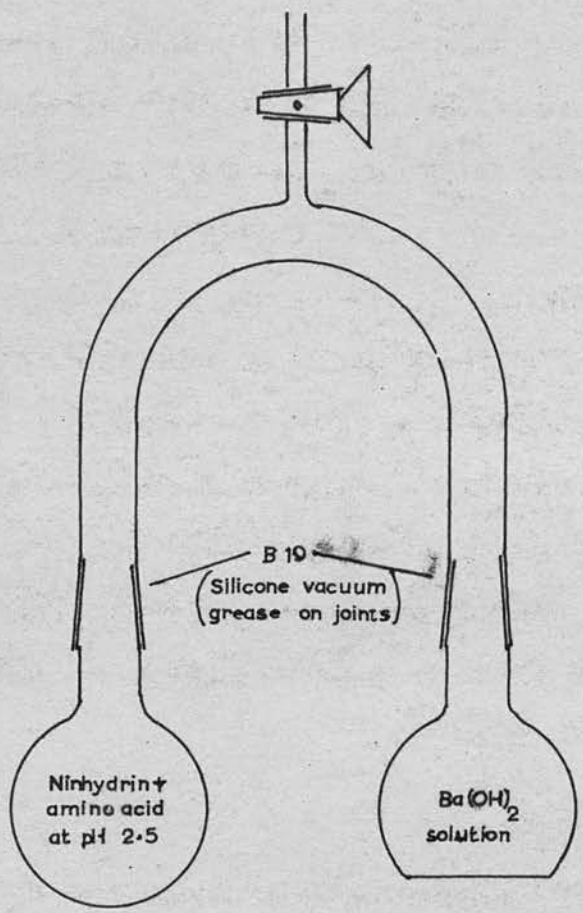
From the vast amount of research into the nitrogenous constituents of higher plants it is clear that peptides are never present in concentrations comparable to the free amino acids. When a search has been made, however, peptides or other forms of bound amino acids have usually been found.

It is interesting to note how often non-amino acid compounds are closely associated with amino acids in fractions rich in bound amino acids. For example, Synge and Wood (23) found that 5 - 15% of the nitrogen of rye-grass diffusates was present in bound amino acid compounds which were strongly anionic. In these the amino acids existed as N-acyl derivations, the acyl radicals being either polyphenolic or glycosidic. There was also evidence of N-oxalyl groups. Only a very small proportion of the bound amino acid compounds were true peptides. In Bathurst's survey of the free and non-protein bound amino acids in rye-grass it was noted that the amino acid composition of the fractions differed, that of the peptide fraction being intermediate between the free amino acid and protein composition.

In the present work the aims of studying peptides were firstly, to attempt to account for the apparent depletion of free amino acids after proteolysis, and secondly, to initiate work on possible intermediates of protein breakdown in excised leaves. The results of Kemble and Macpherson (14) indicated that more peptide would be found in wilted grass than in silage. This was deduced from the smaller amount of nitrogen precipitated from silage extracts by alcohol, and the fact that in the early stages of wilting, the release of α-carboxyl nitrogen proceeded at a slower rate than the general increase in soluble nitrogen. The latter observation was the most promising, because as will be seen later, any evidence which depends entirely on differences in

α-carboxyl nitrogen before and after hydrolysis is of little value when dealing with small quantities of peptides.

FIG 8



U-TUBE USED IN α-CARBOXYL-N DETERMINATION BY THE VAN SLYKE CO_2-NINHYDRIN METHOD

EXPERIMENTAL AND DISCUSSION

The experiments described here deal with the general occurrence of bound amino acids in grass and silage, and were completed before the work on acidic and basic amino acids was begun.

The general method was to study first the increase in α-carboxyl nitrogen caused by hydrolysis of hot water extracts of the grass samples. Precipitation by 75% ethanol was examined as a means of obtaining a peptide rich fraction. Chromatograms of the whole extracts and of fractions from alcohol precipitation were compared with chromatograms from hydrolysates of the extracts and fractions. For measuring α-carboxyl nitrogen two methods were used:

a) The Van-Slyke CO_2-ninhydrin titrimetric method. (67)

b) Chloramine-T decarboxylation, CO_2 measured manometrically. (72)

Hydrolysates generally gave fairly reproducible results by both methods. For the extracts, however, erratic results were obtained, especially by the CO_2-ninhydrin method. The blanks on grass extracts by this method were never as high as reported by Synge (22).

Van-Slyke CO_2-ninhydrin method

A part of the extract or hydrolysate to be analysed is adjusted to pH 2.5 and heated for 30 minutes in a covered beaker at 100°C. The solution is then evaporated under a stream of air for 10 mins., cooled, and made to volume. This procedure reduces blanks considerably. An aliquot of the solution at pH 2.5 is then transferred to a 25 ml. round-bottomed flask, and solid ninhydrin is added (50 mg. per ml. of extract). $\frac{N}{35}$ barium hydroxide containing 12% barium chloride is pipetted into a second flask and both flasks are immediately attached to a U-tube (see Fig. 9), which is evacuated for 30 secs.

The tap is then closed, and the apparatus immersed in boiling water for 15 mins. The U-tube arm containing the barium hydroxide is immersed in cold water, and the contents of the other flask are distilled into the barium hydroxide. The apparatus is shaken throughout the distillation, which occupies about 1 min. The apparatus is cooled to room temperature with constant shaking to ensure quantitative absorption of CO_2 into the barium hydroxide. CO_2-free air is then let into the U-tube, the barium hydroxide flask is removed, and 5 ml. toluene added, to prevent absorption of atmospheric CO_2. The residual barium hydroxide is then titrated against N/35 hydrochloric acid using phenolphthalein as indicator. The α-carboxyl nitrogen in the extract is calculated from the difference between this titration and the titration from a blank prepared without addition of ninhydrin. This method can be used for estimating up to 2 mg. of α-carboxyl nitrogen.

Chloramine-T decarboxylation

Preliminary treatment with pH 2.5 buffer is the same as for CO_2-ninhydrin determination. The method of estimating α-carboxyl nitrogen is the same as described by Kemble and Macpherson for individual amino acids (73), except that the reaction time is 2 hours, to allow for complete decarboxylation of proline. An aliquot of the pH 2.5 solution is pipetted into a Warburg flask, and 0.2 ml. formalin is added to complex amino-groups and prevent ammonia evolution. 1 ml. of 12% chloramine-T solution is then added to the sidearm of the flask, which is attached to the manometer. After equilibration of the apparatus at 30°C the contents of the flask are mixed, and the decarboxylation proceeds at 30°C for 2 hours. The CO_2 produced is measured by the increase in pressure, and the pressure increase from a blank prepared without chloramine-T is deducted. This method was used to determine up to 0.2 mg. of α-carboxyl nitrogen.

Alcohol precipitation

The nitrogen concentration of the extract to be treated was adjusted to about 1.5 mg./ml., and then 3 volumes of absolute alcohol were added. After standing overnight at room temperature the precipitate was centrifuged off and washed with 75% ethanol. The supernatants were combined, evaporated to remove alcohol, and made to volume. The precipitates were extracted with cold water, and the extracts were centrifuged and adjusted to a suitable volume. Generally there was some residue from this water extraction, which was also insoluble in hot water. It never contained more than a trace of nitrogen.

Hydrolysis of extracts

This was done in boiling 6N-HCl for 24 hours. The concentration of nitrogen in the hydrolysates was never more than 0.5 mg./ml. After hydrolysis the HCl was removed *in vacuo* and the hydrolysate was taken to dryness three times. The residue was then extracted with water, the humin was filtered off through sintered glass, and the hydrolysate made to a suitable volume.

FIRST EXPERIMENT

The full results of this experiment have been reported by Macpherson, Wylam and Ramstad (11). The extracts examined were from four laboratory-scale silages prepared on 9th May 1955 from perennial rye-grass, and opened six months later. The silages were:-

C - Fresh grass, control

M - " " , + solid sodium metabisulphite (0.2 g. per 50 g. grass)

I - " " , + inoculum of Lactobacilli

MI - " " , + inoculum + metabisulphite.

Table 15

α-carboxyl nitrogen (αCN) in laboratory silage (% TN)

Material extracted	αCN by CO_2-ninhydrin on extract	αCN by chloramine-T on extract	αCN by chloramine-T on hydrolysate	Bound αCN (from columns 2 and 3)
C	45.2 47.0	52.8 53.8	64.1 65.0	11.2
M	67.0 68.5	59.9	66.4	6.5
I	40.0 40.8	42.5	53.7	11.2
MI	60.6 63.5	-	69.8	-

These results show wide differences between the α-carboxyl nitrogen values obtained by the two procedures. For each method, however, the results are quite reproducible, the errors being of the same order. Even these inaccuracies may impose an error of up to 10% on the figure for bound amino acid nitrogen. It is difficult to explain the discrepancies between the α-carboxyl nitrogen determined by CO_2-ninhydrin and chloramine-T, because amino acids are known to give quantitative yields of CO_2 by each method (72, 73). The

chloramine-T method was more satisfactory for hydrolysates, because very small traces of HCl can upset the titration in the Van-Slyke technique. The chloramine-T method was generally used in later work.

SECOND EXPERIMENT

Samples from nine-month field silages made from the same grass were received and extracted on 10th January 1956. The silages were:-

FC - Fresh grass, control

FM - " " , + metabisulphite

WC - Grass wilted for 18 hr., control

WM - " " " " " , + metabisulphite

Hot water extracts were prepared on 250 g. samples of the silages, and the volume was made to 250 ml. α-carboxyl nitrogen was determined by chloramine-T, as concordant results could not be obtained with ninhydrin, probably because of volatile acids in the silage, and residual HCl in the hydrolysates.

Table 16

General analyses of field silages

Silage	pH	TN in sample extracted mg.	SN (% TN)	VB (% TN)	TAN (% TN)	αCN (% TN) before hydrolysis	αCN (% TN) after hydrolysis	Bound αCN (% TN)
FC	3.88	1528	65.4	8.9	1.0	30.2	34.0	+3.8
FM	4.56	1349	66.2	7.1	0.5	33.7	30.3	-3.4
WC	3.93	1450	75.1	10.8	0.7	36.8	36.8	0
WM	5.59	1612	76.5	4.8	-	42.8	41.4	-1.4

Table 17

75% Alcohol precipitation of silage extracts

Silage	N taken	N Soluble in EtOH	αCN soluble in EtOH before hydrolysis		αCN soluble in EtOH after hydrolysis		αCN insoluble in EtOH	αCN in EtOH insol. fraction after hydrolysis
	mg.	mg.	mg.	% TN	mg.	% TN	mg.	mg.
FC	15.20	14.12	5.88	25.2	7.15	30.2	0.33	0.49
FM	15.12	13.90	6.90	30.3	7.04	30.8	0.59	0.50
WC	15.26	14.44	6.74	33.2	6.72	33.1	0.42	0.47
WM	15.00	13.86	7.80	39.2	7.06	36.0	0.24	0.34

Paper chromatography in n-Propanol-water, 80/20 and Phenol-water, 73/27, of the fractions insoluble in 75% ethanol showed that the main constituents were aspartic acid and glutamic acid. Several other constituents were observed and most had R_F values similar to known amino acids. No spots were shown to disappear on acid hydrolysis, and amounts were usually too small to detect any increases in the various components, except for FC, in which all the amino acids increased slightly after hydrolysis. Detection of the spots by the chlorine-starch iodide method of Rydon and Smith gave the same pattern as detection by ninhydrin.

The amounts of bound amino acids in these silages were obviously extremely small, but since FC consistently showed increases in amino acid nitrogen, both quantitatively and by chromatography, a second study was made of this silage, on a large scale.

Three volumes of ethanol were added to 40 ml. of FC extract, containing 61.0 mg. of nitrogen.

N precipitated = 2.22 mg.

N in supernatant = 56.6 mg. ∴ recovery = 96.5%

αCN in precipitate before hydrolysis = 1.585 mg.

" " " after hydrolysis = 1.933 mg.

αCN in supernatant before hydrolysis = 24.37 mg.

" " " after hydrolysis = 29.19 mg.

The alcohol precipitate again contained large amounts of aspartic acid and glutamic acid. The bulk of the bound amino acid N has obviously remained in the supernatant from alcohol precipitation. A part of the increase in α-carboxyl nitrogen following hydrolysis will be due to aspartic acid produced from asparagine and glutamic acid from pyrrolidonecarboxylic acid. However, even if all the amide in solution ($\frac{1}{2}$ TAN = 0.48 mg.) was asparagine, hydrolysis of this would only cause an increase in α-carboxyl nitrogen of 0.48 mg.

A fraction containing pyrrolidonecarboxylic acid was obtained by passing the fraction soluble in 75% ethanol through a 10 cm. x 1 cm. column of Zeo Karb 225 (H^+). The column was eluted with water, and the first 30 ml. of eluate collected. This fraction gave a negative ninhydrin reaction, and on paper chromatography in n-Propanol-water 80/20, a spot was observed which travelled the same distance as standard pyrrolidonecarboxylic acid. Several other acid substances were also present. After hydrolysis and rechromatography glutamic acid and a small amount of glycine appeared. A second pyrrolidonecarboxylic acid fraction was obtained, and the α-carboxyl nitrogen found to be zero. After hydrolysis the increase in α-carboxyl nitrogen represented 2.94 mg. of the nitrogen in the original 40 ml. of extract. Since the bound α-carboxyl nitrogen in the alcohol soluble fraction was 4.92, it is seen that 60% of this is accountable for as neutral or quite strongly anionic compounds. In view of later results, it is unlikely that the whole increase is due to glutamic

acid from pyrrolidonecarboxylic acid, although the chromatographic evidence would tend to support this view.

THIRD EXPERIMENT - BOUND AMINO ACIDS IN WILTING GRASS

Perennial rye-grass was cut at Bush Farm on 25th May 1956. Some of the grass was extracted immediately, and a second sample was moist wilted for three days. General analytical results were as follows:

(all figures are % TN of material extracted)

	SN	VB	TAN	αCN (Ninhydrin)
Fresh grass	8.3	0.37	2.84	5.4
3 day wilt	52.4	2.33	8.44	18.8

Alcohol precipitation 50 ml. of extract (71.3 mg. N) were mixed with 150 ml. of absolute alcohol.

N soluble in 75% EtOH = 63.6 mg. αCN = 33.0

N insoluble in 75% EtOH = 9.7 mg. αCN = 5.6

Half of the alcohol-insoluble fraction was hydrolysed, the α-carboxyl nitrogen increasing from 2.8 to 3.4 mg.

Paper chromatographic study of the alcohol-insoluble fraction

An aliquot of this fraction, containing 0.2 mg. N was deposited in a band near the top of a 9 inch broad strip of 3 MM chromatographic paper. The chromatogram was developed for 24 hours in Phenol-water 73/27, the phenol evaporated off at 50°C, and the paper streaked lengthwise with ninhydrin solution to detect the bands of amino acids or peptides. About seven bands of ninhydrin-positive material were found, with R_F values extending between 0.20 0.85, but the boundaries were not well defined. The chromatogram was cut into

six strips which were eluted with 20% ethanol into 5 ml. crucibles. After evaporating the eluates to dryness the residue was dissolved in 100 µl 6N-HCl. These solutions were drawn into lengths of 1 mm. bore capillary tubing which were then sealed at both ends, and placed in an oven at 105°C. After 24 hours the tubes were removed from the oven, cooled, and broken open. The hydrolysates were ejected into 5 ml. crucibles and taken to dryness twice on a boiling water bath. The crucibles were transferred to a vacuum desiccator, and residual HCl was removed overnight by evaporation over solid sodium hydroxide. The residues were dissolved in water and spotted on 3 MM paper. The chromatograms were again developed in phenol-water and then sprayed with ninhydrin solution.

Original chromatogram		Chromatogram of hydrolysates
Strip no.	R_F range	R_F values of spots
1	0 -0.33	0.22, 0.34
2	0.33-0.45	0.36, 0.89
3	0.45-0.55	0.36
4	0.55-0.65	0.18, 0.31
5	0.65-0.75	0.32, 0.47
6	0.75-1.00	0.29, 0.55

Pure aspartic and glutamic acid gave R_F values of 0.20 and 0.32. The chromatograms showed that the alcohol insoluble fraction contained aspartic acid and glutamic acid as the major ninhydrin reacting constituents, but the presence of peptide is strongly indicated by the disappearance of faster running ninhydrin reacting substances, which produce much slower running amino acids on hydrolysis.

FOURTH EXPERIMENT - BOUND AMINO ACIDS IN WILTING GRASS

Perennial rye-grass was cut at Bush Farm on 16th July 1956. It was in the early flowering stage, and the flower heads were removed before sampling. A portion of the fresh grass was extracted, and some was set aside to wilt under moist conditions for three days. General analytical results were as follows:- (all figures are % TN of material extracted).

	SN	VB	TAN	αCN (ninhydrin) before hydrolysis	αCN (ninhydrin) after hydrolysis
Fresh grass	15.8	0.51	2.18	5.11	23.4
3 day wilt	45.4	1.69	17.6*	5.75	30.2

* Asparagine-N = 14.8% TN, glutamine-N = 2.6% TN

For the wilted grass, asparagine α-carboxyl nitrogen is 7.4% TN. Since aspartic acid gives 2 molecules of CO_2 per molecule in the ninhydrin reaction the aspartic acid which has been derived from asparagine by hydrolysis will appear to account for 14.8% TN, giving an increase in measured α-carboxyl nitrogen from 23.4 to 30.6% TN. In fact the α-carboxyl nitrogen increased to 30.2% TN. It must be assumed that the whole increase in α-carboxyl nitrogen is due to asparagine hydrolysis, provided that there is no amino acid destruction. The ammonia content of the hydrolysate was 20% higher than expected from the volatile base and total amide figures, indicating that some destruction had taken place.

It seemed therefore, that in wilting grass, as in silage, the amounts of bound amino acids must be very small.

Attempt to concentrate peptides by use of an ion-exchange resin

An aliquot of the wilted grass extract containing 16.81 mg.N was put on a 20 cm. x 2 cm. column of De-Acidite FF (Acetate), and the column was washed

with 0.1% acetic acid until the eluate was ninhydrin negative (100 ml. eluate). In this way a neutral + base fraction was obtained free of glutamic acid, aspartic acid, and other acidic compounds.

N content of neutral + base fraction (N + B) = 10.8 mg.

Asparagine in N + B = 5.08 mg.N Glutamine in N + B = 1.11 mg N

Asparagine in original extract = 5.48 mg.N Glutamine in original extract = 1.04 mg N

α-carboxyl nitrogen in N + B = 5.44 mg

α-carboxyl nitrogen in N + B after hydrolysis = 9.23 mg

∴ increase = 3.79 mg

Expected increase due to asparagine = 2.54 mg N

Thus of the increase in α-carboxyl nitrogen, 1.25 mg. is not accounted for by asparagine hydrolysis. The volatile base N of the hydrolysate represented 3.75 mg. of the original N, compared with the expected 3.09 mg. from amide breakdown, so that destruction of amino acids had also occurred.

This amount of bound amino acid nitrogen should have been detectable in the earlier analyses, and the result shows that α-carboxyl nitrogen determination is not accurate enough for determination of small amounts of peptides in grass extracts. Some degree of fractionation is essential if useful measurement is to be made of peptide-N.

Alcohol precipitation gives only a vague separation between bound and free amino acids, with much free amino acid material appearing in the precipitates. In addition, alcohol precipitates large amounts of carbohydrate material, which can cause serious destruction of amino acids in subsequent hydrolysis.

It was therefore felt that useful information would be gained more rapidly if individual free amino acids were determined in grass extracts before and after hydrolysis. Analytical results from such experiments have been reported in Sections 1 and 2 and will now be discussed.

Bound Dicarboxylic Amino Acids

See Table 6, page 57, Table 7, page 58, and Table 8, page 60.

The fresh grass extracts which were analysed showed little or no increase in glutamic and aspartic acid on hydrolysis, and the same applies to the two ordinary silages of experiment A. In moist wilting however, up to 1% of the total nitrogen was present in bound glutamic acid, representing about 5% of the non-protein nitrogen. Apart from aspartic acid from asparagine, little bound aspartic acid was found in moist wilting. In the metabisulphite silage examined bound glutamic acid and aspartic acid represented 0.6% and 1.0% of the total nitrogen.

A comparison of Tables 6 and 8 reveals the very interesting fact that all the bound glutamic acid of the 72 hour moist wilt was released by N-acid hydrolysis, the sum of glutamic acid N and pyrrolidonecarboxylic acid N after N-acid hydrolysis being equal to the glutamic acid N after 6N-acid hydrolysis.

As yet there is no evidence as to the nature of the bound amino acid compounds containing glutamic acid. A few peptides have been isolated and characterised from sea-weeds and contain glutamic acid or glutamine as the only amino acid constituent (74, 75). The glutamic acid in these peptides is linked through the α-carboxyl group, but the stability of these peptide links is not discussed. However, glutamyl or glutaminyl peptides in which the glutamic acid residue is linked through the γ-carboxyl group are known to be exceptionally labile (76, 77). Glutathione, γ-glutamylcysteinylglycine, is hydrolysed to glutamic acid and cysteinylglycine in neutral solution at low temperatures. However, peptide links as unstable as this would probably be broken under the conditions used in extraction of grass.

Current work by H.T. Macpherson in this laboratory indicates that there may be several glutamic acid containing compounds which are labile in N-acid.

Some at least are neutral compounds, since they are eluted with the basic and neutral amino acids from De-Acidite columns in the acetate form.

Bound Basic Amino Acids

Column chromatography was not applied to grass or silage hydrolysates, but histidine and arginine were determined colorimetrically on catholytes from the electrodialysis of silage extracts and hydrolysates; (conservation experiment D).

Table 17

Silage	"Histidine N" (% TN)		"Arginine N" (% TN)	
	before hydrolysis	after hydrolysis	before hydrolysis	after hydrolysis
2 days	1.47	1.44	0.54	0.89
5 days	1.55	1.18	0.27	0.39
12 days	-	2.10	-	0.73
55 days	-	2.18	0.79	0.76

These values are in themselves not true histidine and arginine contents, and although there are no significant increases after hydrolysis, this cannot be taken as indicating absence of bound histidine and arginine, for peptides may also give the colorimetric reactions.

The research described in this thesis is relevant to some problems of crop conservation, to the nitrogen nutrition of ruminants and to the metabolism of nitrogenous compounds in detached leaves. Although the work was not taken beyond its primary objective, which was to measure the peptides and basic and acidic amino acids in leaves under different conservation conditions, the analytical results have invariably raised new problems, and suggested lines for future studies. This is particularly true for the nitrogen metabolism of leaves; how far it may apply to ruminant nutrition cannot be said, because of our inadequate knowledge of the importance of amino acid composition in ruminant feeding-stuffs. The analyses of silages have again shown that much remains to be discovered about the relative importance of plant and bacterial enzyme action in determining the nitrogen distribution of the final product.

SUMMARY

1. Glutamic acid, aspartic acid, glutamine, asparagine, pyrrolidonecarboxylic acid and γ-aminobutyric acid have been determined in grass kept under various conservation conditions, at regular intervals after cutting. The conditions studied were wilting with or without loss of moisture (dry or moist wilting) and silage. Samples of field silage, metabisulphite silage and anaerobically incubated microbe-free grass were also analysed.

2. An ion-exchange chromatographic method has been devised for the determination of γ-aminobutyric acid in plant material.

In silage and incubated microbe-free grass γ-aminobutyric acid accumulates very rapidly, and appears to be derived by decarboxylation of glutamic acid, which shows a parallel decrease. The decarboxylation is a function of plant enzymes. In moist wilting no γ-aminobutyric acid is formed, but glutamine is produced. In dry wilting both glutamine and γ-aminobutyric acid are formed, and the relative amounts depend on the rate of drying. It is concluded that γ-aminobutyric acid is formed under conditions unfavourable to production of glutamine.

The amounts of γ-aminobutyric acid in fresh grass were small, and it is not a major amino acid constituent.

3. Experimental details are given for the determination of glutamic acid, aspartic acid and pyrrolidonecarboxylic acid in plant material, by an ion-exchange method.

In all conservation conditions studied free glutamic acid and

and aspartic acid fell below the amounts expected from the degree of proteolysis. In silage the expected glutamic acid was accountable as glutamic acid, γ-aminobutyric acid, glutamine, pyrrolidonecarboxylic acid and chemically bound glutamic acid. The aspartic acid found after 6N-acid hydrolysis of silage extracts was less than the expected value. In wilting, these balances could not be attempted because of synthesis of amides from precursors other than glutamic acid or aspartic acid formed in proteolysis.

Pyrrolidonecarboxylic acid was found in most of the materials analysed, but the quantities were such that it may have been formed as an artefact from glutamine during the extraction procedure.

4. A method has been derived for the determination of γ-aminobutyric acid, ornithine, lysine, histidine and arginine in plant extracts.

These basic amino acids have been determined in fresh grass, silage and wilted grass. Lysine, histidine and arginine all undergo extensive metabolism after release by proteolysis; free arginine is almost completely destroyed in silage. In silage ornithine is produced and may account for most of the arginine. Ornithine appears to be a product of bacterial action, since incubated microbe-free grass contained no ornithine, but almost the expected amount of arginine. Ornithine was never found in fresh or wilted grass. The arginine of dry wilted grass may be metabolised through ornithine to proline, which accumulates in large amounts, but arginine cannot be the only precursor. There is no evidence as to the fate of arginine in moist wilted grass.

5. Grass and silage extracts and their hydrolysates have been

electrodialysed , and the amounts of nitrogen present in the basic, neutral and acidic fractions determined.

In silage, basic nitrogenous compounds make up a much larger proportion of the soluble nitrogen than in fresh or wilted grass. These proportions do not change appreciably after hydrolysis of the silage extracts. Only half of the basic nitrogenous compounds are accountable as γ-aminobutyric acid, ornithine, lysine, histidine and arginine in silage.

Histidine and arginine were estimated colorimetrically in catholytes from electrodialysis. The results obtained were much higher than those found by ion-exchange chromatography. Other closely related compounds, which give the histidine and arginine reactions must be present in silage.

6. Bound amino acids in conservations products have been estimated by the general method of determining α-carboxyl nitrogen before and after hydrolysis, and did not appear to exist in significant amounts. The limitations of such general methods are discussed.

Estimation of specific amino acids before and after hydrolysis contradicted the results obtained in the general bound amino acid survey. Bound glutamic acid and aspartic acid were found in largest amounts in wilted grass and metabisulphite silage. The bound glutamic acid compounds were more labile than the bound aspartic acid compounds, since the glutamic acid was released by N-acid hydrolysis, and the aspartic acid by 6N-acid hydrolysis.

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