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γ -Amino-*n*-Butyric, Aspartic, Glutamic and Pyrrolidonecarboxylic Acid; Their Determination and Occurrence in Grass during Conservation

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The harvesting of a forage crop is followed by rapid and extensive proteolysis, terminated by the attainment of a high dry matter or a low pH as the material is dried or ensiled respectively. Even in a good silage about 50 % net breakdown of the protein may occur; the proteolysis on drying is smaller, but where drying is slow it may be considerable. Since the fresh crop contains 80–90 % of its nitrogen in the form of protein, large amounts of amino acids are released and it is of interest to know what happens to them. It was evident from preliminary analyses of wilted grass and silage extracts that the amounts of aspartic and glutamic acid, which together account for nearly one-seventh of the protein, fell very far short of the amounts expected from the resulting proteolysis. However, other closely related substances, namely asparagine, glutamine, pyrrolidonecarboxylic acid and γ -amino-*n*-butyric acid, were present in the conservation product, often in considerable quantity, and it was desirable that they should be estimated with some accuracy.

For the aspartic and glutamic acid themselves, a rapid single-stage ion-exchange method based on that of Hirs, Moore & Stein (1954) has been developed. Pyrrolidonecarboxylic acid, for which no simple method appears to exist, was determined on the same sample. The method of Vickery, Pucher, Clark, Chibnall & Westall (1935) appeared to be satisfactory for the amides, particularly when present in quantity, and they can be checked as pyrrolidonecarboxylic acid and aspartic acid. A new and simple method for γ -aminobutyric acid determination has also been evolved. These methods have been applied to proteins, to fresh grass and to

grass kept under various conditions encountered in conservation practice. From the results obtained, some general conclusions have been drawn about the metabolism of these substances during conservation.

METHODS

Material for analysis. In all the analyses given below, extraction has been 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 are probably reduced on milled samples. However, while there is little or no loss of nitrogen on oven-drying, except where volatile-base values are high, as in some silages, changes of nitrogenous and other substances may be considerable and some sort of forced-draught drying is preferable. A much-enhanced soluble-nitrogen figure may be obtained when elevated temperatures are employed without rapid dissipation of moisture, owing to increased rate of enzyme action.

Extraction of material for analysis. We have generally employed hot-water extraction for obtaining protein-free, soluble-N fractions. Boiling water is poured on the fresh sample, which is then macerated or kneaded for a few minutes, and the liquid poured off and cooled rapidly. This is done three times and the combined extracts are concentrated *in vacuo* to the required volume; the solution is then filtered and kept under toluene at 0°. A precipitate may separate subsequently, but this does not appear to contain any nitrogen. The main objection to the method is that the use of heat tends to cause some conversion of glutamine into pyrrolidonecarboxylic acid and ammonia, but the time of heating is short and the small amount of pyrrolidonecarboxylic acid is in any case determined. To obtain a true analysis of these substances, determinations of volatile base, glutamine, glutamic acid and pyrrolidonecarboxylic acid should all be done as soon as possible.

Determination of glutamine and asparagine. These are determined by the methods of Vickery *et al.* (1935), both being measured by the extra ammonia formed on hydrolysis: glutamine for 2 hr. at pH 6.5 and asparagine for 3 hr. in $N-H_2SO_4$, both at 100° .

Determination of glutamic and aspartic acid. Wiltshire (1953) has described a separation by the two-column method of Consden, Gordon & Martin (1948) followed by Van Slyke amino N determination. Hirs *et al.* (1954) have used a Dowex-1 acetate column but report poor recoveries, and Campbell (1956) has investigated the separation of dicarboxylic from other amino acids using acetic acid and resins of varying cross-linking. The method described here 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.

Resin of 90–120 mesh is obtained from the coarser 16–50 particles by breaking down for a few minutes in a ball mill and selecting the appropriate fraction; this does not appear to affect the functioning of the resin. The resin is nominally 3.5% cross-linked. It is washed with $N-NaOH$ in a conical flask, then thrice water-washed, with settling and decanting each time. 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 should be prepared. Regeneration of the used material is carried out in the same way and the useful life appears to be considerable. A 15 cm. depth is poured from the 1% acetic acid suspension into a 1.2 cm. column with a very small cotton-wool plug in the bottom. The column is tapped on the bench 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 N, 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 amino acids from other ninhydrin-reacting substances and from each other can be varied by changes in strength of the eluting acid and by promotion or postponement of such changes. The following procedure was 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 about 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 and a further 80 ml. or so is collected. A fraction-collector may be used, but in most of this work fractions were collected via a siphon delivering 3.2 ml. (± 0.1 ml.) into 6 in. $\times$ $\frac{5}{8}$ 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 closely by the aspartic band. The amino acids are then measured by ninhydrin colorimetry, 2 ml. of the Moore & Stein (1954) 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% ethanol and shaken vigorously for a few seconds; the intensities are then read after about

20 min. 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 fractions 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, 1953) 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.

Determination of pyrrolidonecarboxylic acid. By an extension of the above method, pyrrolidonecarboxylic acid can also be determined as glutamic acid. After the fractions required 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 water bath 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.

Determination of γ -aminobutyric acid. Waksman & Bigwood (1955) have described a separation of the acid in 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. Total time occupied in separation and determination is about 5 hr. The resin used is Zeo-Karb 225, 4.5% cross-linked, 200–400 mesh (The Permutit Co. Ltd.). Preliminary treatment followed closely that of Moore & Stein (1951) for Dowex 50, except that selection of particles by sieving was omitted. An amount (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. $\times$ 1.2 cm. poured. Two bed-volumes of 0.2% 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 30 ml./hr. after several regenerations owing to loss of the finer particles; 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 total N 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 -aminobutyric acid band generally appears in fractions 33–43 with 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.

Cellosolve blanks. Some batches of ethyl Cellosolve (2-ethoxyethanol), which we have generally employed instead of methyl Cellosolve (2-methoxyethanol), 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 Cellosolve to remove traces of yellow colour from the resin eluate.

In a typical aminodicarboxylic acid 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.* (1954) but is well separated here, preceded by tyrosine and phenylalanine. In the separation of γ -aminobutyric acid, the first peak contains aspartic and glutamic acid, the second the monoaminomonocarboxylic acids, the third and fourth phenylalanine and tyrosine respectively and the fifth γ -aminobutyric acid. Tryptophan comes off much later with stronger pyridine. Separation of the early peaks can be improved on a longer column. β -Alanine overlaps phenylalanine and δ -aminovaleric acid appears earlier; glucosamine and ethanolamine were not found in the 0.2% pyridine eluate.

Recoveries. Recovery of glutamic acid from the De-Acidite column is $100 \pm 1\%$ and that of aspartic acid $99 \pm 1\%$. 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 in the formic acid eluate. No other ninhydrin-reacting substance was found on chromatography of the glutamic and aspartic acid bands, either before or after hydrolysis.

Recovery of pyrrolidonecarboxylic acid by the method described has been tested on an authentic sample containing 7% of free glutamic acid. The free glutamic acid was determined directly in the sample after 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 chromatograms of the formic acid fractions. The presence of pyrrolidonecarboxylic acid was demonstrated by the appearance of an acid spot with the correct R_F , hydrolysis of which yielded only glutamic acid. The remainder of the chromatograms showed no trace of glutamic acid 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.

Five experiments on two complete amino acid mixtures gave recoveries of 97.5–103.6% (mean 100.1) of the γ -aminobutyric acid. Three experiments in which known amounts were added to a fresh grass extract of small, known γ -aminobutyric acid content gave recoveries of 97.5–101.8% (mean 99.6). Chromatograms of the γ -aminobutyric acid fraction from extracts of plant material, developed in three solvents, showed no other ninhydrin-reacting substance and the fraction did not form a complex with CuCO₃ (Crumpler & Dent, 1949).

Grass and silage

Grass A was a lawn-grass mixture containing no rye-grass. It was cut on 8 October 1956 and was 4–5 in. high; it had already been cut several times during the summer. Nitrogen content was 3.14% of dry matter. Grass B was mixed perennial and Italian ryegrass, first-year growth, cut on 28 May 1956, 12–18 in. high, not in flower. Nitrogen content was 3.34% of dry matter.

Silages from grasses A and B are laboratory silages prepared as described by Kemble (1956) without additions except to the metabisulphite silage, which consists of grass mixed intimately with dry sodium metabisulphite (0.2 g./50 g. of grass): the pH is higher than in ordinary silage, the metabisulphite acting as a bacteriostatic substance and the resulting product exhibits a very high degree of proteolysis with little ammonia formation (Macpherson, Wylam & Ramstad, 1957).

All results quoted in the tables are means of at least two determinations. The experiments with lawn and ryegrass have been selected as typical of numerous other ensilage and wilting experiments, all of which have shown similar trends in amino acid distribution.

RESULTS AND DISCUSSION

Formation of γ -aminobutyric acid in silage. The most striking feature of the results shown in Table 1 is the rapidity with which γ -aminobutyric acid is formed, and this is paralleled by a correspondingly rapid fall in the amount of free glutamic acid present. The initial increase in γ -aminobutyric acid is out of all proportion to the increase in the soluble nitrogen and it seems likely that much of it is produced from the free glutamic acid present, since this drops very sharply. Thereafter the rate of production falls off and the glutamic acid, enhanced by continuing proteolysis, increases again.

Formation of γ -aminobutyric acid during wilting. Wilting was carried out as described by Kemble & Macpherson (1954). We have distinguished arbitrarily between 'dry-wilting', where moisture is lost fairly rapidly, and 'moist-wilting', where the moisture content is sustained. Both processes are largely aerobic. It can be seen from Table 1 that in

Table 1. *Free γ -aminobutyric acid, dicarboxylic acids, amides and pyrrolidonecarboxylic acid in grass during conservation*

Results except those for dry matter, are expressed as percentage of total nitrogen of the original material. PCA, Pyrrolidonecarboxylic acid.

Extracted from	Dry matter (%)	Soluble N	γ -Amino N	Glutamic acid N	Glutamine N/2	PCA N	Aspartic acid N	Asparagine N/2
Old lawn grass A								
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	0.55
Silage, 5 weeks	—	69.5	3.40	1.84	0	0.71	2.80	0.60
Young perennial ryegrass B								
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	Trace	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	Trace	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
Other samples analysed								
Mature 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

'moist-wilting' there is no formation of γ -aminobutyric acid; glutamine, however, is formed. In dry-wilting, on the other hand, there is considerable formation of γ -aminobutyric acid, but none of glutamine. In the dry wilt of grass A, where drying was slower, the formation of γ -aminobutyric acid was somewhat less and that of glutamine higher than in dry wilts of grass B. When the grass was first moist-wilted and then dried, γ -aminobutyric acid was formed during drying. In another experiment, a microbe-free grass was incubated under mercury seal for 11 days and gave considerable γ -aminobutyric acid and a little glutamine. The grass was shown to be free from bacterial contamination throughout the experiment, the pH did not drop and the conditions were largely those of an anaerobic moist-wilting.

From these experiments it appears that γ -aminobutyric acid can be formed under aerobic conditions as in dry-wilting or under virtually anaerobic conditions as in laboratory silages and in the incubated microbe-free grass, where glutamine is not normally produced. Glutamine formation appears to limit the formation of γ -aminobutyric acid. In moist-wilting, where glutamine formation may be considerable, γ -aminobutyric acid is either not formed or, if it is produced, is immediately converted into glutamine (cf. Steward, Bidwell & Yemm, 1956). Dry-wilting conditions, due to lack

of proteolysis and subsequent deamination, are probably inimical to amide formation.

In the experiments with the combination of moist- and dry-wilting, the γ -aminobutyric acid seems to be derived from glutamic acid, not from the glutamine already formed, and may be a precursor of glutamine.

We have found only 0.1–0.2% of the total nitrogen of fresh grass to be in the form of γ -aminobutyric acid. Although very small amounts may be formed between the time of cutting and 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 (1951), but in his analysis the grass sap was exposed to a lengthy dialysis and ionophoresis and it seems likely that much of it may have been formed during this period.

Amino dicarboxylic acid balance in conservation. We have attempted to correlate the glutamic and aspartic acid found with the amounts expected from the degree of proteolysis. Kemble & Macpherson (1954) showed that protein breakdown during conservation appeared to be uniform, i.e. the residual protein is of similar amino acid composition to the original, and that there is no preferential liberation of any of the acids. Given the amino acid composition of the plant protein therefore we can calculate how much of an amino acid

we should expect to find in the extracts, knowing the value in an extract of the fresh material and the percentage proteolysis. This, of course, is an oversimplification since some synthesis undoubtedly goes on even in the dying cells, but the net effect is a general proteolysis. Table 2 shows the contents of glutamic acid and aspartic acid of a few representative grass proteins: these samples have been obtained from various Laboratories and all were prepared from mature pre-flowering growth of pure strains by the general method of mild alkaline extraction and acid precipitation. The proteins showed almost identical amino dicarboxylic acid composition and the small differences indicated are probably not significant.

The amount of free glutamic acid present in the extracts falls far short of the expected amounts, but if we assume the closely related γ -aminobutyric

acid, pyrrolidonecarboxylic acid and glutamine to have come directly or indirectly from glutamic acid residues, the balance, if bound glutamic acid is taken into account, is generally satisfactory in silage (Table 3). Bound glutamic acid is determined by hydrolysis of the extracts. More than the expected amount is found when much glutamine is present, as in the moist wilts, and it is probable that the glutamine is derived at least partly from some carbon skeleton other than that of glutamic acid.

In aerobic wilting, particularly under conditions of sustained moisture content, very large amounts of asparagine are formed, much more than can arise from aspartic residues of the protein and any such balance would be invalid. During anaerobic incubation, however, as in silage, there is little asparagine formation and under these circumstances we have examined the aspartic acid balance. In the examples shown in Table 4, except in the moist wilt, the aspartic acid found even after hydrolysis is less than the value calculated from the degree of proteolysis, particularly in the week-old silage. We have not found significant amounts of β -alanine, the decarboxylation product analogous to γ -aminobutyric acid from glutamic acid, but Kemble (1956) noted that, in the earlier stages of ensilage and in anaerobic incubation of microbe-free grass, excess of alanine was present: this may account for some of the missing aspartic acid.

Table 2. *Amino dicarboxylic acids in grass proteins*

Source	Total N (% of dry protein)*	Glutamic acid N (% of total N)	Aspartic acid N (% of total N)
Perennial ryegrass	12.8	7.64	6.02
Italian ryegrass	12.5	7.65	6.06
Cocksfoot	14.2	7.70	6.03
Timothy grass (1)	12.6	7.60	6.20
Timothy grass (2)	11.2	7.50	6.20

* Not corrected for ash.

Table 3. *Glutamic acid balance in grass conservation*

Results are expressed as percentage of total nitrogen of the material. PCA, Pyrrolidonecarboxylic acid.

Material extracted	N of free glutamic acid, glutamine, PCA and γ -aminobutyric acid (from Tables 1 and 2)	N of glutamic and γ -aminobutyric acid present after hydrolysis of extract	Glutamic acid N expected from proteolysis
Fresh grass A	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
Fresh grass B	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 4. *Aspartic acid balance in grass conservation*

Results are expressed as percentage of total nitrogen of the material.

Material extracted	Aspartic acid N and asparagine N/2 free in extract (from Tables 1 and 2)	Aspartic acid N present after hydrolysis of extract	Aspartic acid N expected from proteolysis
Fresh grass A	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
Fresh grass B	0.54	0.58	—
Metabisulphite silage	3.53	4.68	5.10

Table 5. *Determination of the amides*

Results are expressed as percentage of total nitrogen of grass A. PCA, Pyrrolidonecarboxylic acid.

Extract from	Glutamine N/2	Asparagine N/2	Glutamic acid N	Aspartic acid N	PCA
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

Amides and pyrrolidonecarboxylic acid. 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 extracts (Chibnall & Westall, 1932; Vickery *et al.* 1935). Even the pyrrolidonecarboxylic acid determination by itself does not provide a satisfactory check since a little of the glutamic acid also appears to be converted. The asparagine and aspartic acid remain unchanged during the hydrolysis of glutamine and asparagine is recovered quantitatively as aspartic acid on hydrolysis with N-acid (Table 5). In the hydrolysis with N-acid the same total glutamic acid + pyrrolidonecarboxylic acid is always obtained, but the final proportions of the two acids may vary. Most of the glutamine nitrogen appears in the pyrrolidonecarboxylic acid fraction on hydrolysis at pH 6.5, the conditions for glutamine determination; there is, however, a significant reduction in the glutamic acid present and this has been noted on several occasions. The aspartic acid nitrogen remains unchanged. Under the conditions for asparagine determination (hydrolysis by N-sulphuric acid at 100°) the asparagine appears as the additional aspartic acid; the considerable increase in total glutamic acid from this hydrolysis is discussed below.

The values obtained for pyrrolidonecarboxylic acid in grass and silage extracts are usually very small, but even so they will be over-estimates because of the partial conversion of glutamine during the preparation of the hot-water extracts. From the figures quoted by Vickery *et al.* (1935), we estimate this conversion to be about 20 % of the glutamine present under our conditions of extraction. In view of this and the proportions of pyrrolidonecarboxylic acid and glutamine found in grass (Table 1), we are of the opinion that pyrrolidonecarboxylic acid probably does not occur as such in the grasses examined. Figures for pyrrolidonecarboxylic acid in some of the silages are higher, particularly after some weeks, but there is simultaneous disappearance of glutamine and part of the pyrrolidonecarboxylic acid could arise from this; there may also have been some conversion of glutamic acid. The amount of pyrrolidonecarboxylic acid nitrogen in fresh grass B is 0.77 % of the soluble

nitrogen, and the figure given by Ellfolk & Synge (1955) is estimated as 'much less than 0.86 %'.

Bound glutamic and aspartic acid. In general, fresh grass extracts showed little or no increase in the acids on hydrolysis. Under conditions of wilting, however, particularly where proteolysis is extensive, up to 1 % of the total nitrogen may be present as bound glutamic acid nitrogen, equivalent to about 5 % of the soluble nitrogen. Only slight increases were found in ordinary silage, but in grass preserved with metabisulphite 0.6 and 1 % respectively of the total nitrogen were present as bound glutamic and aspartic acid nitrogen (Tables 3 and 4).

The most interesting feature of these experiments is that all the bound glutamic acid found in wilted grass is liberated under the comparatively mild conditions used for the hydrolysis of asparagine, and no more is found on boiling with 6N-hydrochloric acid for 24 hr.; there may be slight destruction with the stronger acid in spite of the very large dilution of sample since N-acid usually gives significantly higher figures than 6N-acid. So far we have insufficient evidence to decide the structure of the bound glutamic acid, which may exist as glutamic, glutamine or pyrrolidonecarboxylic acid peptides (cf. Melville, 1935; Dekker, Stone & Fruton, 1949). A large part at least of the bound glutamic acid is, however, recoverable in the early fractions from the De-Acidite column and is not therefore acidic in nature.

SUMMARY

1. Simple quantitative methods, employing ion-exchange resins, are described for the determination of γ -amino-n-butyric, aspartic, glutamic and pyrrolidonecarboxylic acid in plant material.

2. The formation of the considerable amount of γ -aminobutyric acid found in silage and microbe-free grass is examined with respect to the glutamic acid and glutamine present. It seems to be derived from the glutamic acid and accumulates very rapidly under conditions unfavourable for the formation of glutamine.

3. Balances have been worked out comparing the glutamic and aspartic acid found with the amounts expected from the proteolysis involved. All the glutamic acid appears to be recoverable,

though a considerable part of it may exist as readily hydrolysable bound glutamic acid of as-yet undetermined structure.

4. The amounts of pyrrolidonecarboxylic and γ -aminobutyric acid found in extracts of fresh grass are very small, and pyrrolidonecarboxylic acid probably does not exist as such in the grass, the little found being formed during the extraction.

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Studies on Biosynthesis of Cholesterol

8. THE EFFECT OF Mg^{2+} AND Mn^{2+} IONS AND OF ADENOSINE TRIPHOSPHATE ON ENZYMIC ACTIVITY OF MICROSOMES IN STEROL BIOSYNTHESIS FROM MEVALONIC ACID*

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It was shown in previous studies that for the synthesis of squalene and cholesterol from mevalonic acid by cell-free preparations of rat liver a fraction of soluble protein and microsomes were needed and that adenosine triphosphate and reduced pyridine nucleotides were essential cofactors in this enzyme system (Popják, Gosselin, Gore & Gould, 1958). It was further demonstrated that during aerobic conditions, when mostly cholesterol was synthesized, a reducing agent, such as glutathione or ascorbic acid, was needed to activate the system.

The soluble enzymes could be preserved very satisfactorily, after precipitation with ammonium sulphate at -15° , but the microsomes progressively lost their activity when kept frozen at -15° or at -79° . Consequently in our previous work microsomes were prepared freshly for each experiment.

* Part 7: Cornforth, Cornforth, Pelter, Horning & Popják (1959).

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The present paper describes a study of the 'ageing' process of the microsomes and of the conditions leading to the restoration of the ability of old preparations of microsomes to catalyse the synthesis of sterol from mevalonic acid in the presence of dialysed soluble enzymes and of the appropriate coenzymes. It was possible to reactivate fully preparations of microsomes that were kept frozen at -15° for 4 months and which had lost, apparently, 90% of their activity. A brief account of the results has already been presented (Gosselin & Popják, 1958).

METHODS

The methods and materials used in the present investigation were essentially those previously described (Popják *et al.* 1958).

Testing of enzymic activity of microsomes. The criterion of enzymic activity of the microsomes was their ability to catalyse the synthesis of digitonin-precipitable sterols from [$2-^{14}C$]mevalonate when combined with dialysed soluble liver enzymes in the presence of adenosine triphosphate