when expressed as a percentage of the total nitrogen. This observation is in agreement with those recorded previously for typical storage organs.

3. The two plants contained very large amounts of the imino acid, azetidine-2-carboxylic acid. Nitrogen present in azetidine-2-carboxylic acid represented more than 50 % of the total nitrogen of *Polygonatum* rhizome. The imino acid was also present in high concentrations in the young shoots of both plants, but its concentration in the mature shoots was much lower.

4. The analytical data indicate that the root may form a major site of synthesis of azetidine-2-carboxylic acid in *Convallaria*. It is more difficult to draw conclusions for *Polygonatum* since the very high concentrations present in its rhizome at all stages of growth tended to overshadow smaller changes of concentration elsewhere in the plant.

REFERENCES

- Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Dawson, R. F. (1941). *Science*, **94**, 396.
 Dawson, R. F. (1942). *Amer. J. Bot.* **29**, 813.
 Dent, C. E. (1947). *Biochem. J.* **41**, 240.
 Fowden, L. (1954). *Ann. Bot., N.S.*, **18**, 417.
 Fowden, L. (1956). *Biochem. J.* **64**, 323.
 Fowden, L. & Bryant, M. (1959). *Biochem. J.* **71**, 210.
 Fowden, L. & Steward, F. C. (1957). *Ann. Bot., N.S.*, **21**, 53.
 Fowden, L. & Webb, J. A. (1958). *Ann. Bot., N.S.*, **22**, 73.
 James, W. O. (1950). In *The Alkaloids*, vol. 1, p. 15. Ed. by Manske, R. H. F. & Holmes, H. L. New York: Academic Press Inc.
 Mothes, K. (1928). *Planta*, **5**, 563.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Steward, F. C. & Thompson, J. F. (1954). In *The Proteins*, vol. II A, p. 513. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Nitrogenous Constituents of the Thoracic Muscle of the African Migratory Locust (*Locusta migratoria migratorioides*)

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During recent years a considerable amount of information has become available concerning the non-protein nitrogenous constituents, and especially the amino acids in the haemolymph of various species of insects. Most of this is due to the work of Florkin and his colleagues, who have in the main used microbiological methods for their assays of amino acids (Florkin, 1954). It appears that the haemolymph of insects is characterized by a relatively high amino acid content, and in this respect they differ markedly from crustaceans such as lobsters which, though containing a high concentration of free amino acids in their muscles, have very little in the haemolymph (Kermack, Lees & Wood, 1955). Little or no information appeared to be available about the free amino acid content of insect muscle.

An insect which it seemed convenient to study in this respect was the migratory locust *Locusta migratoria migratorioides*. The insect order Orthoptera, to which the locust belongs, contains many of the larger insects, and a considerable amount of research has been carried out on this order. From the available data, the metabolism of orthopteran muscle appears to be similar to that of mammalian muscle; for example, the occurrence of glycolysis and the presence of the tricarboxylic acid cycle in

the African migratory locust has been demonstrated by Rees (1954) and adenosine triphosphate has been isolated from the muscle of the locust *Gastrimargus musicus* (Calaby, 1951).

In the present work the nature of the non-protein nitrogenous constituents of locust muscle was investigated. Some of the present results have already been briefly presented (Kermack & Stein, 1955).

MATERIALS AND METHODS

Locusts. Fifth-stage nymphs of the African migratory locust, supplied by the Anti-Locust Research Centre, London, were kept in the Laboratory under the conditions advised by the Centre. The locusts, after undergoing their final moult to the adult form, were used as required. After starving overnight they were killed by placing for a few minutes in a jar containing chloroform, then cut open dorsally, the alimentary canal and fat body were removed and the thoracic muscles *in situ* washed free of haemolymph by squeezing 1% (w/v) NaCl from a Pasteur pipette between and underneath the muscle bands. Residual saline on the surface was removed with filter paper and the intact muscle excised.

Muscle extracts. Muscle from about six locusts was homogenized with 5% (w/v) trichloroacetic acid (5 ml.) for 5 min. in a Mickel homogenizer. The suspension was allowed to stand for 15 min. and centrifuged. The muscle residue was extracted a second time in the same way, and

the combined supernatants were made up to 20 ml. with water. Ethanol extracts of muscle were made similarly, ethanol being substituted for 5% trichloroacetic acid.

Muscle hydrolysates. The thoracic muscle from one locust was hydrolysed with 6N-HCl in a sealed glass tube at 110° for 16 hr. Larger amounts were prepared by refluxing the muscle of a number of locusts with 6N-HCl for 24 hr. HCl was removed by distillation under reduced pressure.

Perchloric acid hydrolysis of muscle extracts. A volume (10 ml.) of locust extract was evaporated to dryness at 100°; 0.5 ml. of 60% (v/v) perchloric acid was added and the mixture kept at 100° for 1 hr.

Total non-protein nitrogen determinations. Trichloroacetic acid extract (1 ml.) of locust muscle was digested by the method of Chibnall, Rees & Williams (1943). Distillation was carried out in the apparatus described by Markham (1942) and the ammonia collected in 1% (w/v) boric acid containing Conway indicator (Conway, 1950).

α -Amino nitrogen. This was determined by the method of Pope & Stevens (1939).

Uric acid. This was determined by the method of Folin (1934).

Paper chromatography. Whatman no. 1 paper was used for general work, and Whatman no. 4 for quantitative work. For a two-dimensional paper chromatogram, 100–150 μ l. of locust extract, containing 5–10 μ g. of α -amino N, was applied to the paper from a micropipette. Solvents used were phenol–water (4:1, v/v) and butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948). Amino acids were located by spraying the paper with ninhydrin in the usual way. In addition the identities of the following amino acids were checked by spraying chromatograms with the appropriate specific reagent: arginine (Block, LeStrange & Zweig, 1952); histidine and tyrosine (Block, 1949); proline and hydroxyproline (Acher, Fromageot & Jutisz, 1950); methionine, cysteine and cystine (Toennies & Kolb, 1951).

Quantitative determination of amino acids. A modification of the method of Powden (1951) was used. The location of amino acids on the chromatogram by their fluorescence in ultraviolet light, recommended by Powden, was found to be incapable of detecting some of the amino acids present in low concentrations. Amino acids were located by spraying the chromatogram with a weak solution (0.05%, w/v) of ninhydrin in butanol (Landua & Awapara, 1949) and allowing it to dry at room temperature overnight. Ammonia was rigorously excluded from reagents and chromatographic paper throughout the work. The faint spots were cut out from the chromatogram and a paper blank was cut out, of the same area. The spots were placed in a series of small tubes, 1 ml. of water was added, and left overnight. A volume (1 ml.) of the ninhydrin stock solution was then added and the procedure completed as described by Powden (1951).

Proline was estimated approximately by spraying one-dimensional chromatograms of locust extract with isatin reagent (Acher *et al.* 1950), and comparing the size and intensity of the proline spot with standard spots on parallel chromatograms.

Purines. These were investigated with one-dimensional paper chromatography in propan-2-ol–HCl (Wyatt, 1951), and the positions of compounds absorbing in the ultraviolet were located by the photoprint method of Markham & Smith (1949). Quantitative determinations of the purines were carried out by the method of Wyatt (1951).

Phosphate. Phosphate present in purine nucleotides eluted from the chromatograms was determined by the method of Berenblum & Chain (1938) as modified by Long (1943).

RESULTS

Determinations of total non-protein nitrogen

Determinations of non-protein N were carried out on trichloroacetic acid extracts prepared from batches of locusts of different ages, including one group of females and one group of males dissected immediately after the final moult. The non-protein N values of the muscle extracts are given in Table 1.

The non-protein N values range between 300 and 400 mg./100 g. of muscle, and show no obvious correlation with either the age or the sex of the locusts. The variation may be due partly to experimental error and partly to the fact that the figures are drawn from analyses of muscle extracts from different stocks of locusts. Table 2 gives the results of four determinations of non-protein N on extracts from the females of a single stock of locusts. The figures indicate that the non-protein N content of the muscle extracts increased over the first 10 days after the moult and subsequently became relatively constant.

Free amino acids

The amino acids were detected in trichloroacetic and ethanolic extracts of muscle by two-dimensional paper chromatography. The relative intensities of the ninhydrin colour of the various spots are indicated in Table 3.

Table 1. Total non-protein nitrogen values of trichloroacetic acid extracts of muscle

Time after final moult (days)	Total non-protein N (mg./100 g. of fresh muscle)	
	Females	Males
0	326	310
15–18	—	398
17–20	350	—
18–22	370	368
21–28	320	340
25–28	327	—
35–38	328	329
35–42	356	355
65	299	315

Table 2. Total non-protein nitrogen values of trichloroacetic acid extracts of muscle from a single stock of female locusts

Time after final moult (days)	Total non-protein N (mg./100 g. of fresh muscle)
0–2	288
11	310
20	336
30	336

Table 3. *Semi-quantitative estimation of free and protein-bound amino acids in locust muscle*

-, Signifies not detectable.

	Trichloroacetic acid extracts of muscle	Ethanollic extracts of muscle	Muscle hydrolysate
α -Alanine	+++	++	++
β -Alanine	-	+	-
Arginine	++++	+	++
Aspartic acid	+	-	+++
Cysteic acid	Trace	-	Trace
Glutamic acid	+	+	+++
Glutamine	++++	+++	-
Glycine	++	+++	++
Histidine	-	+	+
Leucine (including isoleucine)	+++	+	++
Lysine	Trace	+	++
Methionine sulphoxide	-	-	Trace
Phenylalanine	-	+	++++
Proline	++	+	+
Serine	+	+	++
Taurine	++++	+++	-
Threonine	-	+	++
Tyrosine	Trace	+	+
Valine	+	++	++

Table 4. *α -Amino nitrogen values of trichloroacetic acid extracts of muscle*

Time after final moult (days)	α -Amino N (mg./100 g. of fresh muscle)
Females	
0	104
1-2	118
7-10	194
17-20	111
18-22	94
21-28	86
25-28	77
35-38	114
65	77
Males	
0	92
8-11	176
15-18	93.5
18-22	88
24-27	83
21-28	87
36-39	105
65	71

Determinations of total α -amino nitrogen

The results obtained with trichloroacetic acid extracts are given in Table 4. It can be seen from these figures that the α -amino N values differ between the sexes, the value for female locusts being usually higher than for males of the same age. The α -amino N also varies with the age of the insect, rising from a low value immediately after the final moult to a peak and then falls. In females this fall is usually found to occur about 3 weeks after the final moult and may correspond with egg formation. In males the fall occurs about 2 weeks after the final moult and may correspond with

sperm formation. After this fall values remain low for about 2 weeks, during which time mating is at its height (Norris, 1950). Later, the α -amino N rises slightly, but falls off as the locusts become older and more sluggish.

The possibility that the changes in the α -amino N content of tissue extracts were related to the reproductive activity of the locusts was investigated further. A series of extracts were prepared from the females of two batches of locusts, and the state of the ovaries and the time at which egg-laying began were noted. The first batch, from which extracts 35, 36, 37 and 38 were prepared, was found to be parasitized with the nematode worm *Mermis nigrescens* and no egg-laying had occurred. The second batch, from which extracts 40, 41 and 42 were prepared, began egg-laying later than usual, owing to the scarcity of fresh grass for their food. Locusts in extract 40 had ovaries that were not fully developed, whereas in locusts in extracts 41 and 42 the ovaries were well developed, and egg-laying was intense in the period between the making of the two extracts. It would appear from the values given in Table 5 that the α -amino N was high when the eggs were being formed (extract 41) and fell after the eggs had been released (extract 42).

Determinations of individual amino acids

The aim of these investigations was to study the effects, if any, of egg-laying on the amounts of the individual amino acids present. The results of a number of estimations carried out on extracts from female locusts are presented in Table 5. As stated above, extracts 35, 36 and 37 show the effect of increasing age, uncomplicated by egg-formation.

Table 5. *Quantitative estimation of individual amino acids in trichloroacetic acid extracts of muscle of female locusts*Amino acid values are given as mg. of α -amino N/100 g. of muscle.

Extract no.	35	36	37	38	40	41	42
Days after final moult	0-2	11	20	30	10	35	44
α -Amino N (Pope & Stevens, 1939)	60.5	91	83	89.5	75	98.5	71
Aspartic acid	—	0.1	1.5	—	—	—	—
Glutamic acid	—	1.5	2.7	—	—	1.8	0.5
'Spot 1'	—	3.0	4.2	—	—	3.0	1.0
Glycine	—	12.9	6.4	—	—	28.0	31.0
Alanine	3.5	15.5	15.8	—	—	12.2	4.6
Valine	1.8	2.7	3.8	—	—	1.3	0.5
Leucine (including isoleucine)	15.0	13.4	12.9	—	—	2.8	0.5
Glutamine	17.3	13.9	20.7	—	—	12.1	1.3
Arginine	23.0	20.0	22.8	—	—	15.6	23.8
Serine	—	—	2.6	—	—	—	—
Proline	6.2	7.4	10.2	—	—	18.8	13.2
Taurine N	9.3	15.5	36.4	47	—	30.0	33.5

Table 6. *Uric acid nitrogen content of trichloroacetic acid extracts of muscle*

Time after final moult (days)	Uric acid N (mg./100 g. of muscle)
Females	
0	8.2
5-7	1.5
35-42	<1.3
Males	
0	8.3
35-42	<1.7

isoleucine) all fall to one-third or less of their original value, and proline to about two-thirds of its original value. On the other hand, glycine rises slightly in amount and arginine increases considerably. In both batches of locusts, the amount of taurine increases with the age of the locusts and is unaffected by egg-laying.

Determinations of uric acid

No uric acid was detected in extracts of muscle of male and female locusts of 35-42 days. From the sensitivity of the method used, the maximum amount of uric acid that could have been present was calculated, and is included in Table 6. It would appear from these results that only over the period of moulting does uric acid account for any appreciable amount of extractable non-protein N in muscle extracts.

Adenylic acid

A spot absorbing ultraviolet light, believed to be that of adenylic acid, was detected on chromatograms of locust-muscle extract. The maximum absorption of the eluted spot was at 260 m μ . The R_F value (0.39) of the unknown spot with the propan-2-ol-HCl solvent (Wyatt, 1951) was similar to that of adenylic acid (0.41), and after perchloric acid hydrolysis was identical with that of adenine (0.26).

The unknown spot contained phosphorus. After elution from the chromatogram the amount of adenine was determined spectrophotometrically, and the amount of phosphorus determined. The molecular proportions of adenine to phosphate were 1:1, indicating that the unknown substance was adenylic acid, rather than adenosine diphosphate or adenosine triphosphate. On the assumption that the unknown substance was adenylic acid, the results of quantitative determinations carried out on muscle extracts from several batches of locusts are given in Table 7. It is probable that the

Table 7. *Estimation of adenylic acid in locusts of different ages*

Time after final moult (days)	Adenylic acid N (mg./100 g. of fresh muscle)
0 (males)	45
5-7 (females)	41
19 (males)	56
21-28 (females)	67
35-40 (females)	60

Extracts 41 and 42 show the changes in amino acids before and after oviposition. A ninhydrin-staining spot having the same R_F as glutamic acid in phenol, and a slightly lower R_F than glutamic acid in butan-1-ol-acetic acid-water is referred to in Table 5 as 'spot 1'. Since the spot was not identified, the strength of the spot was compared with that of a glutamic acid standard, and the α -amino N content then calculated as if the unknown were glutamic acid.

The increase in α -amino N between extract 35 and extract 36 corresponds with increases in most of the amino acids, although glycine is a notable exception.

In the muscle tissue from the second batch of locusts, glutamine shows the greatest change; it falls from 12.1 mg. of α -amino N in extract 41 to 1.3 mg. of α -amino N in extract 42. Glutamic acid, 'spot 1', alanine, valine and leucine (including

adenylic acid found in muscle extracts is partially present as adenosine triphosphate in the living insect, but has been hydrolysed at the low pH at which the extracts were prepared and stored.

Muscle hydrolysates

The amino acids identified in muscle hydrolysates are included in Table 3, the intensity of the ninhydrin-staining spots being as indicated. Further, a spot believed to be lanthionine consistently appeared on the chromatograms, its position being identical with that of a sample of lanthionine. Evidence of the identity of this compound with lanthionine has been published previously (Stein, 1955).

Distribution of non-protein nitrogen in tissue extracts

Table 8 is a description of a typical locust-muscle extract. The volatile-amine value included is only an average value, taken from a series of estimations by the method of Conway (1950). It must be emphasized that many of the values quoted in the table are dependent on the age, sex and physiological state of the locusts.

DISCUSSION

Each muscle extract used in this work was prepared from a number of locusts, all of the same age and sex. In this way, individual variations were averaged, and the effects of age, sex and physiological conditions could be studied by comparison of different groups. A criticism of the present work might be that the muscle extracts were possibly contaminated by haemolymph contained within the intercellular spaces, since no estimation of the volume of the intercellular spaces was made. Some observations which we have made on the amino acid content of locust haemolymph suggest, in agreement with the results of Kilby & Neville (1957, personal communication), that the α -amino N content is of the order of 100 mg./

100 ml. This is in agreement with the recent work of Patterson (1956), which indicates that the hemimetabolous group of insects, to which the locust belongs, has not as high a concentration of amino acids in the haemolymph as the holometabolous group, various representatives of which have been studied by Florkin (1954). Thus the concentration of amino acids in locust haemolymph is about the same as that we have found in locust tissue, and it is not possible to explain the concentration of amino acids found in the muscle on the assumption that the muscles themselves have low concentrations of amino acids and owe their relatively high concentration to contamination by haemolymph. Chromatographic investigations of the amino acids confirm this conclusion, for they show that asparagine and tyrosine are present in haemolymph, but not in muscle extracts, whereas taurine, alanine, glutamic acid and aspartic acid are present in muscle extracts, but not in haemolymph (authors' unpublished work). Thus it seems probable that there was no gross contamination of the extracts by haemolymph.

The qualitative investigations of the amino acids in locust-muscle hydrolysates suggest that locust-muscle protein is made up of a number of α -amino acids that are chromatographically indistinguishable from those found in other hydrolysed proteins. In addition, lanthionine has been detected in muscle hydrolysates, and it is probable that this amino acid exists preformed in the muscle protein of the locust, and is not merely produced as an artifact during hydrolysis (Stein, 1955).

All of the amino acids detected in hydrolysates of locust muscle also occurred in protein-free muscle extracts, except lanthionine and methionine sulphoxide. (The latter only appeared in traces and may be an artifact formed by oxidation of methionine during hydrolysis.) In addition, muscle extracts contained taurine and β -alanine. The relative proportions of the amino acids in muscle hydrolysates and in muscle extracts were quite different.

The total α -amino N content of muscle extracts was observed to vary with the physiological state of the insect. In males, a decrease in the α -amino N was observed about 2 weeks after the final moult, and possibly corresponded with the release of sperm. In females, the fall in α -amino N occurred about 3 weeks after the final moult, and corresponded with the onset of egg-laying. Cheu (1952) has found that at the time of egg-laying approximately 144 mg. of protein is lost from the locust with each egg-pod. According to Cheu, this represents about one-quarter of the total protein built up by the insect from the time of the final moult to the onset of oviposition. Since a mature female *Locusta migratoria*, fed on a normal diet, will lay

Table 8. *Distribution of nitrogen in trichloroacetic acid extracts of muscle from female locusts 11 days after final moult*

	N (mg./100 g. of fresh muscle)
Total N	310
α -Amino N	91
Taurine N	16
Arginine guanidyl N	68
Glutamine amide N	14
Adenylic acid N	45
Uric acid N	1
Volatile-amine N	20
Total	255

five to six egg-pods in the course of its life at the rate of one egg-pod about every 5 days (Norris, 1950), evidently there must be a rapid rate of protein synthesis throughout the reproductive period. Since the locust stops feeding just before oviposition, the level of amino acids in the tissues might be expected to fall during oviposition.

With the exception of taurine in some extracts, arginine was the most abundant amino acid. Part of it at least was presumably derived from arginine phosphate present in the intact muscle and hydrolysed during the preparation of the extract. This may be related to the fact that the amount of arginine present in the extracts was not affected by the general decrease in the level of amino acids at the time of egg-laying. Glutamine was present in considerable quantity in muscle extracts; in the absence of egg-laying the glutamine level remained fairly constant, whereas at the time of oviposition it fell to 11% of its original value. This suggests that glutamine acts as a nitrogen store which is used in the formation of the egg-pods. It can be seen from Table 5 that although the amount of glutamine in locust-muscle extracts was reduced at the time of egg-laying, the amount of glutamic acid was not correspondingly increased. It may be inferred that the glutamine which disappeared was not simply deamidated, but was incorporated into protein or otherwise metabolized. Kilby & Neville (1956) have demonstrated the presence of a glutamic dehydrogenase in the fat body, and of glutamate-alanine and glutamate-aspartate transaminases in the fat body, Malpighian tubules and gut wall of the desert locust. It is probable that these enzymes are also present in the African migratory locust, and may account for the disappearance of the glutamic acid produced by the glutamine.

In many muscle extracts, taurine was the most abundant amino acid, and the amount increased with the increasing age of each batch of locusts. Taurine also occurs in lobster muscle in amounts comparable with those found in locust muscle (Kermack *et al.* 1955), and is present in the muscle of *Mytilus edulis* (Baldwin, 1952).

The uric acid content of locust-muscle extracts was greatest immediately after the final moult. This may be due to the temporary cessation of the excretory processes at ecdysis, when uric acid formation is probably increased. The amount present declined with the increasing age of the locusts, and none could be detected 35 days after the final moult.

The adenylic acid detected in locust-muscle extracts possibly in part represented adenosine triphosphate in the intact muscle which had been hydrolysed as a result of the acid conditions under which the extracts were prepared and stored. The

amount of adenylic acid in locust muscle was of the same order of magnitude as the amount (present in the form of adenosine triphosphate) in mammalian skeletal muscle. The rise in concentration of adenylic acid during the first weeks of the locusts' adult life would be presumably correlated with their gradually increasing muscular power.

SUMMARY

1. Total non-protein nitrogen values of trichloroacetic acid extracts of locust muscle varied from 290 to 400 mg./100 g. of muscle and rose with the increasing age of the locusts. About 80% of the total non-protein nitrogen was accounted for as amino acid nitrogen, purines and volatile amines.

2. α -Amino nitrogen values of trichloroacetic acid extracts of muscle varied from 60 to 190 mg./100 g. of fresh muscle. The values were low in the extracts made from the muscle of locusts shortly after emergence from the final moult, but rose to a peak before the onset of mating.

3. In trichloroacetic acid extracts of muscle the amount of taurine increased with the increasing age of the locusts. Glutamine was abundant before egg-laying and fell to one-tenth of its original value after this time. Glutamic acid, alanine, valine, leucine (including isoleucine) and an unidentified ninhydrin-staining substance all fell to about one-third of their original amounts at the time of egg-laying, whereas arginine and glycine rose slightly.

4. Uric acid accounted for 8 mg. of nitrogen/100 g. of muscle in newly moulted adult locusts. The value fell to 1.5 mg. 6 days later, and none could be detected after 14 days.

5. Adenylic acid accounted for 20–60 mg. of nitrogen/100 g. of fresh muscle, and was presumably present as adenosine triphosphate in the intact muscle.

6. Seventeen amino acids were detected in acid hydrolysates of locust-muscle protein, including the unusual amino acid lanthionine.

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REFERENCES

- Acher, R., Fromageot, C. & Jutisz, M. (1950). *Biochim. biophys. Acta*, **5**, 81.
Baldwin, E. (1952). *Dynamic Aspects of Biochemistry*, 2nd ed. Cambridge: University Press.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Block, R. J. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 337.
Block, R. J., LeStrange, R. & Zweig, G. (1952). *Paper Chromatography*, 1st ed. New York: Academic Press Inc.
Calaby, J. H. (1951). *Arch. Biochem. Biophys.* **31**, 294.

- Cheu, S. P. (1952). *Bull. ent. Res.* **43**, 101.
- Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
- Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed. London: Crosby Lockwood and Son Ltd.
- Florkin, M. (1954). *Bull. Soc. zool. Fr.* **79**, 369.
- Folin, O. (1934). *J. biol. Chem.* **106**, 311.
- Fowden, L. (1951). *Biochem. J.* **48**, 327.
- Kermack, W. O., Lees, H. & Wood, J. D. (1955). *Biochem. J.* **60**, 424.
- Kermack, W. O. & Stein, J. (1955). *Biochem. J.* **61**, xvi.
- Kilby, B. A. & Neville, E. (1956). *Biochim. biophys. Acta*, **19**, 389.
- Landua, A. J. & Awapara, J. (1949). *Science*, **109**, 385.
- Long, C. (1943). *Biochem. J.* **37**, 215.
- Markham, R. (1942). *Biochem. J.* **36**, 790.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Norris, M. J. (1950). *Anti-Locust Bull.* **6**.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Patterson, D. P. (1956). Ph.D. Thesis: University of London.
- Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
- Rees, K. R. (1954). *Biochem. J.* **58**, 196.
- Stein, J. (1955). *Chem. & Ind.* **27**, 774.
- Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

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.