40. THE CYSTINE AND METHIONINE IN CERTAIN SPECIES OF GRASSLAND HERBAGE

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It would appear from recent investigations that both of the amino-acids containing sulphur are to be regarded as essential in animal nutrition, so that it is important to have data on the amounts of these acids in animal foodstuffs. Grass is the most important source of protein to the animal, and the object of the present investigation was to determine the cystine and methionine in a few of the more important species of grassland herbage. Most of the work with the grasses has been carried out by Chibnall and his co-workers, who have made fairly comprehensive analyses of the protein preparations of the leaves of many plants.

EXPERIMENTAL

Material examined. Attention was confined to four species, viz. white clover, *Trifolium repens*; cocksfoot (Danish), *Dactylis glomerata*; perennial rye-grass (Ayrshire), *Lolium perenne*; and timothy (Scotch), *Phleum pratense*. Samples were taken at different stages of growth from different places during two seasons. Most of the farms were in late districts and in rather exposed areas, so that there was not much growth of grass before May when the first samples were collected. One farm was situated on deep peat which had recently been reclaimed by drainage and had received liberal dressings of lime and fertilizers. The other farms were on mineral soils varying in texture from light to heavy loams and in pH value from about 5 to 7.5. The readily available potassium and phosphorus varied from very low to moderately good values. A few experiments with fertilizers were incorporated in the plan of the work, but, as is shown below, the results were relatively unimportant. In some cases the samples were obtained from plots of pure species, but usually the herbage was mixed and the individual species were separated by hand. The plants were cut about 1 in. above ground level with a knife and the fresh material was transported to the laboratory in waterproof bags and examined immediately.

Separation of protein. The preparation of a reasonably pure protein from the leaves of plants has engaged the attention of many workers and the question is fully dealt with by Chibnall [1939]. In the present investigation, it was decided to employ the method of extraction using a slightly alkaline buffer solution as suggested by Lugg [1939]. Between 300 and 400 g. of the fresh plant material were disintegrated twice in a mincing machine with about three times that quantity of a solution of borax containing 1.1 g. boron per litre and of pH 9.2. The cell-wall debris was removed by squeezing the pulp in a linen bag and the colloidal extract was filtered through paper on a Büchner funnel. The

pH of the filtrate was brought to about 4.5 by the addition of 4 ml. 2N H_2SO_4 per 100 ml. when the protein settled out quite quickly. Next day the supernatant liquid was siphoned off and the precipitate was washed twice with water. The protein was then separated by centrifuge, washed four times with absolute alcohol and twice with methylated ether and then extracted for 20 hr. in a Soxhlet apparatus with light petroleum (B.P. 40–60°). This final extraction was found to be necessary to remove traces of ether-soluble material which interfered with the subsequent determination of methionine. The protein preparation was finally dried over P_2O_5 in a vacuum.

Protein preparations

Yield and total nitrogen. The percentage of N in the protein preparations was rather irregular. It varied from 10 to nearly 14% for white clover, from about 9 to 14% for cocksfoot, from 6 to 13% for rye-grass and from 8 to about 13% for timothy. The success of the extraction naturally depends upon the thoroughness of disintegration and probably upon the nature of the protoplasm. The clover yielded the most protein, the recovery of the N in the original sample varying from 24 to 43%. Cocksfoot on the other hand yielded the least protein, the preparation accounting for only 15 to 34% of the total N. Within each species, more protein was usually obtained from the young and aftermath samples than from the samples of older material. The results are summarized in Table 1, in which the samples have been grouped according to stage of growth rather than to date of sampling.

Table 1. *N and amino-N (after hydrolysis) in fat-free protein preparations from white clover (W.C.), cocksfoot (C.), perennial rye-grass (R.) and timothy (T.)*

Nature of sample	% of N in protein preparation				N in protein preparation as % of total leaf-N				% total N in protein preparation as amino-N after hydrolysis			
	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	10.5	12.7	10.0	8.0	38	29	21	*10	66	61	60	58
	13.8	—	—	—	*6	—	—	—	65	—	—	—
	12.8	—	—	—	37	—	—	—	68	—	—	—
Flowering or heading	10.2	10.6	10.4	10.7	*16	24	24	31	71	60	61	64
	10.0	11.0	12.0	10.8	31	26	22	25	59	57	66	62
	10.9	12.9	9.6	—	28	22	25	—	63	62	68	—
	—	14.1	12.8	—	—	26	24	—	—	63	60	—
Old	†10.2	9.7	6.0	11.1	30	20	25	*20	73	72	53	67
	11.7	9.9	8.6	—	31	17	22	—	68	63	63	—
	13.2	11.6	—	—	24	21	—	—	72	63	—	—
Aftermath	11.1	11.0	†8.1	†12.5	34	26	30	29	66	64	57	57
	11.9	11.2	10.2	8.5	27	21	32	33	68	64	59	57
	—	10.1	11.0	—	—	23	34	—	—	61	59	—
	—	†8.6	10.3	—	—	20	28	—	—	70	57	—
	—	10.9	10.7	—	—	27	27	—	—	62	62	—
	—	10.4	—	—	—	15	—	—	—	64	—	—
Grazed	13.8	9.5	10.8	8.6	43	34	26	31	61	63	58	62
	13.0	11.6	12.5	11.8	32	32	29	34	70	63	63	62
	—	—	13.1	—	—	—	24	—	—	—	58	—
	—	—	12.3	—	—	—	25	—	—	—	62	—
Average	11.8	11.0	10.5	10.3	—	—	—	—	67	63	60	61
Ash content	4.8	6.8	7.6	4.3	—	—	—	—	—	—	—	—

* A portion of protein lost during manipulation.

† Samples subjected to determination of ash.

The figures for the N recovered as protein are affected, of course, by the inevitable losses of material during extraction and consequently give only a general idea of the extent of the extraction. In the case of two samples of rye-grass, the residue from extraction was re-extracted and the protein precipitated and prepared in the usual manner. The amount obtained was very small, being only about a fifth of the first preparation, but there was enough to make determinations of total N and amino-N after hydrolysis. A comparison of these figures for the first and second preparations indicates that there is no striking difference between the protein obtained in the first extraction and that left in the residue and lends support to certain observations made by Chibnall [1939].

Sample	Total N in protein preparation		Amino-N after hydrolysis as % of total N	
	1st extract	2nd extract	1st extract	2nd extract
1	11.7	10.4	63	63
2	11.9	9.8	57	57

Ash. The total ash was determined in only one sample of each species and the results varied from 4.3 to 7.6%.

Amino-N. The total amino-N was determined by the Van Slyke [1915] gasometric method using a micro-apparatus. Some preliminary tests were carried out with glycine to find the time of shaking required to give a maximum yield of amino-N. Glycine gives high but consistent results and a maximum recovery of 111.8% was obtained with 8 min. shaking at about 300 r.p.m.; this figure is similar to that obtained by Levene & Van Slyke [1912], and a period of 8 min. shaking was adopted for further work. Five other amino-acids were examined; a small quantity of each was dissolved in water (a little dil. HCl was used with cystine and tyrosine) and made up to a definite volume from which 2 ml. were taken for each test. The average results are given in Table 2 and compared with those obtained by Van Slyke [1911] for the same compounds.

Table 2. *Average percentage recovery of amino-N*

	Arginine	Cystine	Histidine	Lysine	Tyrosine
Present investigation	102.3	108.5	103.0	86.9	102.4
Van Slyke [1911]	99.8	107.4	98.2	95.4	102.1

It will be observed that the results for arginine, histidine and tyrosine are not far removed from the theoretical values, whilst that for cystine is much higher and that for lysine rather low. The amounts of glycine, cystine and lysine in grass proteins are relatively small, however [Chibnall, 1939], so that the error in the total amino-N estimated by the above procedure is unlikely to be serious.

The grass proteins were hydrolysed with acid; a few preliminary experiments showed that a maximum percentage of amino-N was obtained when about 1 g. of the preparation was boiled with 25 ml. of 3N HCl for 8 hr. The yield of amino-N was not affected by the previous addition of known amounts of amino-acid to the protein preparation. Figures for the proportion of amino-N in the different preparations are included in Table 1. There are no important differences between the protein preparations of the four species with regard to the percentages of total amino-N; the figures for clover vary from 59 to 73, for cocksfoot from 57 to 72, for rye-grass from 53 to 68, and for timothy from 57 to 67. It will be observed, however, that the amount of amino-N tends to increase as the plant becomes ripe—a fact which is particularly noticeable when a comparison is made between samples taken from the same farm. Generally speaking, it may

be said that the amount of amino-N in the hydrolysed samples of all four species reached a maximum during July.

Manurial treatment. The effects of manurial treatment were rather irregular. Lime increased the amino-N in both cocksfoot and rye-grass, but with slag the results obtained for cocksfoot were the reverse of those obtained for rye-grass. A late application of ammonium sulphate, three weeks before cutting, produced a considerable decrease (about 12%) in the amino-N of rye-grass protein, although the total N in the grass was increased by about 40%.

Sulphur-containing amino-acids

The method used in the present investigation was based on the procedure devised by Baernstein [1936] and involved hydrolysis with HI.

Apparatus. A few modifications were made of the apparatus used by Baernstein. The digestion flask was connected to the condenser by a ground joint which seemed to be tight enough without the mercury seal suggested by Kassell & Brand [1938]. The absorbers were connected in series by rubber stoppers and tubing. The first absorber contained 10 ml. of a 1% suspension of red phosphorus in distilled water; the outlet of this tube was packed with glass wool to prevent the phosphorus from being carried over. The second absorber contained 10 ml. of a solution containing 20% CdCl_2 and 20% BaCl_2 to absorb H_2S and SO_2 ; the third contained 10 ml. of saturated HgCl_2 to remove phosphine from the KH_2PO_2 added to the HI; the fourth contained 10 ml. of 10% potassium acetate in glacial acetic acid with six drops of bromine. This solution absorbs the methyl iodide produced from the methionine and the fifth absorber contained 5 ml. of the same solution to ensure complete absorption of the volatile iodide. The HI was purified by double distillation; its specific gravity was 1.7 and 1% KH_2PO_2 was added to prevent formation of free iodine.

Hydrolysis. About 0.5 g. of the protein preparation was placed in the digestion flask (capacity about 50 ml.), which was then filled with nitrogen gas previously passed through a solution of 5% AgNO_3 and concentrated H_2SO_4 . A few small pieces of unglazed porcelain, 10 ml. of HI and about 0.02 g. of KH_2PO_2 were then added and the flask was connected to the condenser, in which the water was maintained at about 50°. The absorption train was then assembled and attached to the condenser. The digestion flask was immersed in an oil bath at 150° and the first three absorbers in water at 50–60°. During hydrolysis, a stream of nitrogen was passed through the apparatus at a constant rate controlled by a pinch cock on the side tube of the digestion flask. Maximum values were obtained when the boiling and aeration were continued for 5–6 hr. for pure protein and for 7–8 hr. for grass protein.

Cystine. At the end of the digestion, the absorption train was disconnected from the condenser, 0.02 g. of KH_2PO_2 was added to the digestion flask and, with suction from a pump at the condenser outlet, the digest was concentrated to about 3 ml., the stream of nitrogen being continued meantime. After the digest had cooled in a current of nitrogen it was made up to 25 ml. with 4% HCl. 10 ml. were pipetted into a conical flask filled with nitrogen and a few ml. of $N/100 \text{ KIO}_3$. HIO_3 were added until the solution was definitely orange in colour. The excess iodine was titrated with $N/100 \text{ Na}_2\text{S}_2\text{O}_3$, using starch as an indicator.

Methionine. The methionine, which has been converted during the hydrolysis into the lactone of homocysteine, was now determined in the same solution. A few drops of bromothymol blue were added together with one drop of octyl alcohol and a quantity of freshly prepared 0.04 $N \text{ Na}_2\text{S}_4\text{O}_6$ so as to give, with the tetrathionate produced in the cystine titration, an excess of 2.5 ml. 1 ml. of

0.04 *N* $\text{Na}_2\text{S}_4\text{O}_6$ is equivalent to 6 mg. of methionine and the amount of methionine present was already estimated in the volatile iodide determination described below. The flask was fitted with a rubber stopper, carrying two tubes, and filled with nitrogen gas. It was then evacuated and about 2 ml. of concentrated ammonia were drawn in from a burette, inserted through one of the tubes, to make the solution definitely alkaline. The flask was again evacuated and immersed in warm water at 40° for 15 min. It was again filled with nitrogen and the connecting tubes were removed. The digest was acidified with 10 ml. of 5 *N* HCl and the thiosulphate produced during the process of oxidation was titrated with *N*/100 $\text{KIO}_3 \cdot \text{HIO}_3$. A most satisfactory end-point was obtained by adding a slight excess of the biiodate and titrating back with thiosulphate. The methionine was calculated from the iodine required, 1 ml. of *N*/100 $\text{KIO}_3 \cdot \text{HIO}_3$ being equivalent to 0.1407 mg. of methionine-N or 1.49 mg. of methionine.

The methionine was also determined from the volatile iodide absorbed in the fourth and fifth absorbers. The contents of these were rinsed into a 100 ml. volumetric flask containing 25 ml. of 25 % sodium acetate solution. A little formic acid (about 10–12 drops) was added to reduce the bromine, the flask was shaken and the contents made up to the mark. Aliquot portions of 20 or 25 ml. were pipetted out, a little KI and a few drops of 10 % HCl were added and the liberated iodine was titrated with *N*/100 $\text{Na}_2\text{S}_2\text{O}_3$.

Sulphate. The H_2S and SO_2 , produced by the reduction of sulphate, have been absorbed in the second absorber as CdS and BaSO_3 , which may be quantitatively oxidized. The contents of the tube were rinsed into a beaker, a small excess of *N*/100 $\text{KIO}_3 \cdot \text{HIO}_3$ was added followed by a few crystals of KI and 2 ml. of 10 % HCl. After standing for a short time, the excess iodine was titrated with *N*/100 $\text{Na}_2\text{S}_2\text{O}_3$, 1 ml. of the iodate being equivalent to 0.16 mg. of sulphur.

Tests of the method. It was found advisable to keep both the condenser and the first three absorbers warm in order to ensure that the methyl iodide passed to the fourth and fifth absorbers. Lugg [1938, 1] recommended that the digestion of protein be commenced with the addition of 0.02 g. of KH_2PO_4 and that further quantities be added at intervals as long as iodine coloration was obvious. In hydrolysing impure leaf protein, however, the digest is dark orange in colour for reasons other than the presence of iodine, and it was found that the addition of 0.02 g. of KH_2PO_4 at the beginning and end of the hydrolysis was sufficient. Blank determinations were made with the HI at frequent intervals and corresponded to about 0.02–0.04 mg. of cystine-N, 0.03–0.04 mg. of S, 0.01 mg. of methionine-N by volatile iodide and 0.06–0.1 mg. of methionine-N by homocysteine.

In the examination of the method with a sample of edestin, containing 15.58 % N, with and without the phosphorus absorber, and with both cold and warm conditions for the condenser and the first three absorbers, there was not much difference between the figures either for cystine or for methionine by homocysteine, but the percentage of methionine by volatile iodide depended upon the conditions employed. The volatile iodide appeared to be detained when the conditions were cold and it seemed to be advisable to introduce the absorber containing red phosphorus. The percentage distribution of the sulphur in the edestin was found to correspond to 1.09 % cystine-N and 1.24 % methionine-N as compared with figures, obtained by other workers, of 0.89–1.31 for cystine-N and of 1.08–1.35 for methionine-N.

A number of preliminary trials were made of the recovery of cystine and methionine alone or mixed or added to edestin, the analyses being carried out

under the conditions already described. The recovery of cystine averaged 97.5% when examined alone, 99.3% in presence of methionine and 98.8% in presence of methionine and edestin. The recovery of methionine examined alone averaged 95.5% by the volatile iodide method and 91.1% by the homocysteine method, and the recovery was not affected by the presence of cystine or cystine and edestin.

With regard to the recovery of pure substances, Baernstein [1936] found values of 101.5% for cystine and 99.4 and 99.6% for methionine by the volatile iodide and homocysteine methods respectively. In the present investigation the recovery of cystine was satisfactory but the recovery of methionine was low. This, according to Kassell & Brand [1938], is probably due to the formation of methyl mercaptan. It is noteworthy that a small quantity of cystine, amounting on an average to 5.2% of the methionine-N, was found in the estimation of methionine, suggesting that a small proportion of the homocysteine was titrated as cystine. When the analysis of a mixture of cystine and methionine was carried out, the recovery of cystine was therefore higher than when cystine was examined alone, while the recovery of methionine was not affected by the presence of cystine. The percentage recoveries were in good agreement with those found by Kassell & Brand [1938], who obtained 97.8% for cystine and 93.7 and 89.3% for methionine by the volatile iodide and homocysteine methods respectively.

Since the grass proteins contain both cystine and methionine, the correction factors used in the subsequent analysis were 1.007 for cystine and 1.047 or 1.098 for methionine according to the method of estimation. In the examination of the grass proteins, however, the yield of methionine was always about 25% higher by the methyl iodide method than by the homocysteine method even with the above correction factors. It must be assumed that the protein preparations were not entirely free from impurities which could yield volatile iodide or could render methionine-sulphur insoluble.

Results

General. The results are all reported (Table 3) on a nitrogen basis, but do not include the figures for ten samples collected from manurial experiments. The distribution of S (Table 4) was calculated on the assumption that cystine and methionine were the only two S-containing amino-acids in the proteins and that the remainder of the S was in the form of sulphate. The figures for the sulphate-S were not corrected for the small amounts of H_2S liberated from cystine and methionine and corresponding to 1.25 and 0.89% respectively of the cystine-S and methionine-S. The figures for methionine by the homocysteine method are given in Table 4.

The four species do not reveal noteworthy differences in their contents of cystine and methionine. The former varies from 0.90 to 1.40% for white clover, 0.91 to 1.46% for cocksfoot, 0.96 to 1.26% for rye-grass and 0.82 to 1.32% for timothy. The corresponding figures for methionine-N by homocysteine are 0.70 to 1.20%, 0.73 to 1.24%, 0.91 to 1.10% and 0.74 to 1.29%. In an examination of ten samples of leaf proteins by the same method, Lugg [1938, 1] found that the cystine varied from 1.1 to 1.5% and the methionine from 1.2 to 1.6% and that rather higher values were obtained by his differential oxidation method. Our figures are, therefore, somewhat lower than those obtained by Lugg. It will be observed that the legume, white clover, contained about the same amounts of the amino-acids as the grasses.

Table 3. *Cystine and methionine in grass protein preparations*

Nature of sample	% of total N in protein preparation as cystine- and/or cysteine-N				% of total N in protein preparation as methionine-N							
					By volatile iodide				By homocysteine			
	W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
Young	1.18	1.28	1.04	0.86	1.22	1.59	1.27	1.04	1.04	1.17	1.04	0.78
	1.14	—	—	—	1.26	—	—	—	1.02	—	—	—
	1.03	—	—	—	1.30	—	—	—	0.94	—	—	—
Flowering or heading	1.33	1.37	1.16	1.13	1.40	1.52	1.30	1.17	1.15	1.12	1.10	0.91
	1.32	0.91	1.16	1.32	1.50	1.29	1.50	1.59	1.20	0.98	1.06	1.29
	1.40	1.32	1.26	—	1.46	1.52	1.43	—	1.14	1.20	1.10	—
Old	—	1.25	1.16	—	—	1.30	1.43	—	—	0.84	0.91	—
	1.35	1.46	1.12	1.24	1.38	1.30	1.52	1.53	1.13	1.22	1.01	1.19
	1.20	1.42	1.21	—	1.42	1.46	1.55	—	1.11	1.24	1.10	—
Aftermath	1.36	1.42	—	—	1.48	1.54	—	—	1.20	1.21	—	—
	1.01	1.01	0.96	1.20	1.20	1.21	1.40	1.38	0.90	0.73	1.05	1.12
	1.10	1.25	1.03	0.82	1.23	1.40	1.28	1.34	0.98	1.08	0.97	0.74
Grazed	—	1.05	0.98	—	—	1.41	1.34	—	—	0.84	0.89	—
	—	1.34	1.08	—	—	1.28	1.14	—	—	1.07	0.95	—
	—	1.16	1.03	—	—	1.50	1.20	—	—	1.09	0.94	—
Average	—	1.44	—	—	—	1.46	—	—	—	1.15	—	—
	0.90	1.13	1.10	0.85	1.29	1.35	1.18	1.40	0.70	1.03	1.00	0.97
	1.04	1.12	1.15	1.25	1.32	1.30	1.25	1.26	1.00	1.03	1.05	1.06
Average	—	—	1.22	—	—	—	1.08	—	—	—	0.95	—
	—	—	1.02	—	—	—	1.22	—	—	—	0.94	—
	1.18	1.25	1.11	1.08	1.34	1.40	1.31	1.34	1.04	1.06	1.00	1.01

Stage of growth. Lugg [1938, 2] could not find any association between the composition of extracted protein and the age of the leaves, but the results of this investigation show that stage of growth does affect both cystine and methionine. In general, the young and grazed samples contain less cystine and methionine than the older samples, the maximum figures being reached at about the stage of flowering or heading. This holds good for all four species with the single exception that the cystine-N in a sample of cocksfoot at flowering stage taken in July is the lowest figure for all the cocksfoot samples.

When the actual dates of sampling were considered it was obvious that the figures for clover reached a maximum in July and then decreased, whereas with the grasses the maximum in July was followed by a decline in August or September and another rise in September or October. This was the case for both cystine and methionine and this difference in seasonal variations between the clover and grasses may be explained by the fact that the growing period of the former is somewhat restricted, whereas the grasses continue to grow throughout the season.

The numbers of samples in the various age groups are too small to permit many positive assertions, but the figures have been submitted to statistical analysis and, where the number of degrees of freedom permitted, the significance of the differences of means has been tested by the usual *t*-technique—*t* being taken at 2.5. The laboratory standard errors (amounting to about $\pm 2.5\%$ for cystine and $\pm 3.5\%$ for methionine by homocysteine) were duly considered and significant differences were found to exist in the following cases:

Cystine. White clover. Flowering v. young or aftermath or grazed; old v. aftermath or grazed.

Cocksfoot. Old v. aftermath or grazed.

Rye-grass. Aftermath v. flowering or old.

Timothy. No significant differences.

Methionine. *White clover.* Young v. flowering; aftermath v. flowering or old.

Cocksfoot. Old v. aftermath.

Rye-grass and timothy. No significant differences emerge.

There seems to be little doubt, therefore, that both cystine and methionine rise to significant maxima when the plants have reached or passed the heading stage and that they are present in smaller amounts in the aftermath or grazed samples. This is supported by the results in Table 4, which show how the sulphur, other than that present in cystine or methionine, is almost invariably lower in the flowering or old samples than in the others. It is also of interest to note that there is a fairly strong relation between cystine and methionine, the latter on an average being about nine-tenths of the former.

Table 4. *Percentage distribution of S in protein preparations (methionine by homocysteine)*

Nature of sample	White clover			Cocksfoot			Rye-grass			Timothy		
	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest	Cyst.	Meth.	Rest
Young	43.4	38.3	18.3	41.9	38.2	19.9	36.3	36.3	27.4	38.8	35.2	28.0
	36.6	34.0	29.4	—	—	—	—	—	—	—	—	—
	41.1	37.4	21.5	—	—	—	—	—	—	—	—	—
Flowering or heading	47.9	41.4	10.7	45.4	37.2	17.4	47.1	43.0	9.9	49.7	40.0	10.4
	45.6	41.4	13.0	40.5	43.6	16.0	46.8	42.8	10.4	43.8	43.1	13.1
	47.6	38.8	13.7	43.3	39.4	17.4	47.7	41.7	10.6	—	—	—
Old	—	—	—	54.7	36.7	8.6	51.8	40.6	7.6	—	—	—
	48.9	40.9	10.1	48.9	40.9	10.2	47.7	43.0	9.3	46.7	44.8	8.6
	46.5	43.0	10.5	45.9	40.0	14.1	47.7	43.4	9.0	—	—	—
Aftermath	46.6	41.1	12.3	47.2	40.3	12.5	—	—	—	—	—	—
	37.3	33.3	29.4	45.9	33.1	21.0	39.4	43.1	17.6	43.4	40.5	16.1
	39.3	35.0	25.6	48.1	41.5	10.4	43.2	41.3	15.4	39.3	35.5	25.2
Grazed	—	—	—	44.4	35.6	20.0	40.5	36.8	22.8	—	—	—
	—	—	—	49.7	39.7	10.6	41.2	36.2	22.6	—	—	—
	—	—	—	42.5	39.9	17.6	44.1	40.2	15.7	—	—	—
Average	—	—	—	49.0	39.2	11.9	—	—	—	—	—	—
	40.7	31.6	27.7	41.4	37.8	20.8	43.1	39.1	17.8	37.4	42.6	20.0
	40.5	39.0	20.5	41.5	38.2	20.3	42.6	38.9	18.4	44.7	37.9	17.5
Average	—	—	—	—	—	—	50.9	39.6	9.5	—	—	—
	—	—	—	—	—	—	42.7	39.4	17.9	—	—	—
	43.2	38.1	18.7	45.6	38.8	15.5	44.6	40.3	15.1	43.0	40.0	16.9

There is an association between the total amino-N and the S-containing amino-acids in the proteins. The actual correlation coefficient for cystine + methionine and total amino-acids is +0.45, which is quite significant for sixty-three samples.

Environment. Although a substantial amount of evidence is available to show that the mineral matter in herbage is determined to a large degree by soil conditions, it is generally recognized that the composition of the organic matter is affected mainly by stage of growth. It was impossible to obtain samples at exactly the same stage of growth from the different sites in order to compare the influence of local soil and climatic conditions; from the data collected, however, any effects of locality were not apparent for any of the species concerned.

As has been pointed out elsewhere [Smith & Wang, 1941], grazing exerts a very important influence on the composition of grass, both the carotene and total N being considerably increased. This investigation shows that grass kept short by grazing contains cystine and methionine in amounts similar to those for young grass or aftermath. The effects of manuring were observed in only a few

cases and were rather irregular and relatively unimportant. Lime produced a slight increase in the total amino-acids of the proteins of both cocksfoot and rye-grass, but the cystine and methionine were not altered to a significant extent. Slag had opposite effects on the two species. The effect of applying a dressing of ammonium sulphate at the rate of 1 cwt. per acre to rye-grass, 3 weeks before cutting, was to reduce the percentage of N as amino-N in the hydrolysed protein from 65 to 57 and to effect smaller reductions in the contents of cystine and methionine.

SUMMARY

Protein preparations have been made from sixty-three samples of *Trifolium repens*, *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*, by mincing and extraction with a buffer solution (pH 9.2), precipitation at pH 4.5, washing with alcohol and ether and finally drying in a vacuum.

The proteins contained 15–43% of the total N in the plants. The amount of N in the protein was on the average about 11%, being slightly higher in the preparations from the clover than in those from the grasses. The clover protein also contained a rather greater proportion of amino-N (67%) after hydrolysis than the grass proteins (60–63%).

The distribution of S as cystine, methionine and sulphate was estimated by hydrolysis with HI followed by determination of the cysteine and homocysteine in the digest and of methyl iodide, sulphide and sulphite in the absorption train. The values for methionine were much higher by the methyl iodide method than by the homocysteine method; expressed as percentage of N in the protein the averages were 1.35 and 1.03% respectively. The amounts of cystine (1.16%) and methionine were essentially the same for all four species but they were significantly greater in samples which had reached or passed flowering stage than in samples of young grass or aftermath or grass which had been kept short by grazing. The reverse was the case for sulphate-S which accounted for from about 8 to 29% of the total S measured. Neither site nor manuring, with the exception of a late dressing of N, seemed to influence the results.

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