[Contribution from the Chemistry Department of the Oregon Experiment Station.]

DISTRIBUTION OF NITROGEN IN THE ALFALFA SEED.

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The absence or small quantity of certain amino acids revealed by our methods of protein analysis and the beneficial results obtained in nutrition and maintaining a nitrogen balance when these proteins deficient in certain amino acids are supplemented by the missing amino acids or proteins containing these acids, gives proof to a correlation between chemical analysis and biological analysis of proteins. Little is known of the character of the nitrogen compounds in our legume hays and so far as the writer can ascertain no proteins have been isolated from either the hay or seed of alfalfa and red clover.

Not only is a study of the character of the nitrogenous compounds important from the standpoint of animal nutrition, but it is necessary if we are to know what effect a plentiful supply of plant nutrients has upon the quality of plant compounds compared to a limited quantity of fertilizer. For example, experiments at this Station[1,2] have shown that a liberal supply of sulfates caused an increase in the nitrogen content of alfalfa and red clover compared to the same legumes grown on soils receiving no sulfur fertilizer. Whether this nitrogen increase results from greater protein formation or from the elaboration of other nitrogenous compounds of less value is of great importance.

In beginning this study, the alfalfa seed was chosen as it contains some of the nitrogen compounds in a concentrated form synthesized by the alfalfa plant. The nitrogen constant of the seed is about 6%, equivalent to 37.5% of crude protein. The quality and quantity of the nitrogen compounds in the seed are of no immediate or practical value in feeding experiments, but, when it is considered that the seed contains the storage compounds which according to plant physiologists are synthesized in the leaves, one would expect the quality of the seed protein to be similar to that of the protein in the hay when cut at maturity or in the blossoming stage. Apparently then, any information obtained in determining the nitrogen compounds of the seed could be applied to a similar study of the hay. The first part of the investigation includes the preparation of a protein from the alfalfa seed and its analysis.

Experimental.

The seed was ground in a mill[3] until it was sufficiently broken up so that most of the fat could be extracted with petroleum ether. It was

[1] H. G. Miller, *J. Agr. Res.*, **17**, 87 (1919).

[2] F. C. Reimer and H. V. Tartar, *Oregon Exp. Sta. Bull.*, **1919**, No. 163.

[3] E. Abderhalden, "Handbuch der Biochemischen Arbeitsmethoden," **3**, 269, (1910).

then reground until it would all pass through a 60-mesh sieve. A fine powder could not be obtained until most of the fat was removed.

Working with a legume, one would expect that the proteins would consist largely of globulins and according to the amount of nitrogen extracted by sodium chloride solution, this was found to be the case. Five g. of the material was mixed with 200 cc. of 5% sodium chloride solution and shaken occasionally. After an hour the mixture was centrifuged and the turbid liquid poured off. This was repeated 4 times, using 100 cc. of 5% sodium chloride solution each time. In all 3.88% nitrogen was extracted, or 65.8% of the total. A 10% sodium chloride solution removed 3.96% nitrogen. A further extraction of this residue with three 100 cc. portions of 0.2% potassium hydroxide solution removed 1.45% of nitrogen.

Attempts to prepare a globulin by salt extraction were not very successful. Extracting the finely ground seed with 15 cc. of 5% sodium chloride solution to 1 g. of material gave a very viscous mixture, and great difficulty was experienced in preparing a clear liquid even by using paper pulp and a powerful press. One hundred g. of seed yielded about 1.2 g. of protein (*N* 15.6%) after dialyzing 1200 cc. of the filtered solution. Water and dilute alkali extracts were also difficult to work with. It was almost impossible to get a quantity of clear extract sufficient for protein preparation. The substance causing these solid particles to remain in suspension could be removed by adding 80% alcohol to twice the volume. This caused the separation of a large slimy mass of material composed chiefly of carbohydrates. But here there was danger of precipitating the protein in solution; however, it was found that the alkali solutions after precipitation with alcohol gave a large yield of protein when treated with dil. acetic acid.

In the following table, the data show that the alcoholic-alkali solution contains more nitrogen than the alcoholic-salt solution, salt solution alone, water extract and the alcohol extract. The alcohol solutions contained water and 95% alcohol in the ratio of 1:1. The concentrations of the salt and potassium hydroxide in the solutions used were 5% and 0.5% respectively. Two g. of the seed and 100 cc. of the different solutions were shaken in centrifuge bottles and allowed to stand for 14 hours. The mixture was then centrifuged and a clear liquid was obtained in all cases except where water and sodium chloride solution were used. For total nitrogen, 25 cc. aliquots were taken. Amino acid nitrogen was determined on 10 cc. portions by the Van Slyke method to determine whether the alkali extraction method attacked the protein molecule exposing more amino groups than the other extractives. From the amount of gas liberated, there appears to be no alteration of the protein

molecule in this respect by the alkali. The larger volumes of gas liberated from the water and salt solutions which contained less total nitrogen may have been due to enzyme hydrolysis during the extraction period.

TABLE I.—NITROGEN EXTRACTED FROM ALFALFA SEED BY DIFFERENT REAGENTS.

Solvent.	Extractable N. %.	Gas liberated[a] from 10 cc. of solution by nitrous acid. Cc.	Temperature. ° C.	Pressure. Mm.
Alkali	4.65	2.0	27	757
Alkali alcohol	3.78	1.9	25	756
Salt	3.33	2.4	27	756
Salt alcohol	1.34	1.8	25	758
Alcohol	0.71	1.3	27	756
Water	2.01	2.6	24	757

[a] Results checked by duplicate determinations. No corrections for reagents.

Apparently there is no reason why the protein of the alfalfa cannot be extracted with dilute alkali. Using 0.2% potassium hydroxide solution it was possible to extract more nitrogen from the ground seed than with the other reagents and, furthermore, after adding acetic acid sufficient for coagulating the protein, there was little, if any, protein nitrogen remaining in solution. The protein preparation most likely contains different proteins when the latter are classified according to their solubility in different reagents and methods of isolation from solution. However, in this preparation, we have a product representative of the seed protein and in a concentrated form adapted to our methods of protein analysis.

For the preparation of protein, 50-g. samples of seed were used. Small quantities were ground with 0.5% potassium hydroxide solution in a mortar and then transferred to a glass jar. About 2000 cc. of the solution was used for the extraction. After standing for 2 or 3 hours, the volume of this mixture was then doubled by pouring in 80% alcohol. The slimy mass which precipitated out was allowed to settle. This supernatant liquid now filtered easily, yielding a clear filtrate. The precipitate in the jar and on the filter was washed 2 or 3 times with alcoholic solution. To the filtrate, dil. acetic acid was added drop by drop until there was distinct coagulation. After the precipitated protein settled the clear liquid was poured off and saved for further investigation. The protein was washed 3 or 4 times by decantation and then poured into centrifuge bottles where the protein was centrifuged out. It was then washed 3 times with dilute alcohol, gradually increasing the strength of the alcohol until absolute alcohol was used for dehydration. After each washing the precipitate was thrown out by centrifuging. The absolute alcohol was allowed to stand in contact with the precipitate for 4 or 5 hours after which it was thrown on the filter and washed with absolute alcohol followed by ether. The protein precipitate was then dried in a vacuum desiccator. If all lumps were broken up in the washing, the protein could be easily ground in the mortar. After drying at 110°, it weighed 10.32 g.

Analyses of 3 different preparations are given on page 909 in Table II. The carbon, hydrogen, nitrogen and sulfur are calculated on a moisture and ash-free basis, the ash on a moisture-free basis.

TABLE II.—ANALYSIS OF PROTEIN FROM ALFALFA SEED.

	Preparation I.		Preparation II.		Preparation III.	
C	52.94	52.82	53.18	52.92	53.12	53.74
H	7.01	6.97	6.89	7.00	6.88	6.97
N	15.66	15.36	15.67	15.65	15.58	15.32
S	...	...	0.69	...	0.70	...
Ash	0.94	...	0.89	...	0.90	...

Results of analysis of the protein by the Van Slyke method[1] are given in Table III. 2.0106 and 2.0000 g. quantities of moisture-free protein equivalent to 0.3150 and 0.3108 g. of nitrogen respectively were used in the hydrolysis of Sample 2. For Sample 3, 2.1576 and 3.3800 g. of protein equivalent to 0.3360 and 0.5124 g. of nitrogen were taken for analysis. Each was hydrolyzed with 100 cc. of 20% hydrochloric acid for 24 hours. The phosphotungstates of the bases were decomposed by the amyl-alcohol—ether method.[2] The amide nitrogen was determined by using magnesium oxide in place of lime. The precipitation of the bases was carried on in a 250 cc. centrifuge bottle and the precipitate was separated from the filtrate by centrifuging. The clear liquid was poured through a filter to retain any particles of precipitate not adhering to the compact mass. In washing the precipitate, 25-cc. quantities of wash solution were used. The large particles in the precipitate could be easily broken up with a stirring rod and a very thorough washing accomplished. The phosphotungstates of the basic amino acids were again centrifuged out, the nature of the precipitate causing an easy separation. This was repeated thrice, using the same quantity

TABLE III.—ANALYSIS OF PROTEIN BY THE VAN SLYKE METHOD.

	Sample 2.				Sample 3.				Average.
	G.	%.	G.	%.	G.	%.	G.	%.	%.
Amide N	0.0270	8.58	0.0264	8.49	0.0449	8.76	0.0297	8.83	8.67
Humin N absorbed by magnesia	0.0152	4.83	0.0129	4.14	0.0211	4.12	0.0128	3.81	4.22
Humin N insoluble in amyl alcohol	0.0004	0.13	0.0002	0.06	0.0015	0.30	0.0009	0.27	0.19
Humin N soluble in amyl alcohol	....	....	....	....	0.0028	0.52	....	....	....
Arginine N	0.0654	20.75	0.0703	22.61	0.1088	21.23	0.0704	20.95	21.38
Histidine N	0.0214	6.78	0.0124	4.00	0.0299	5.83	0.0170	5.06	5.42
Lysine N	0.0162	5.14	0.0206	6.63	0.0277	5.40	0.0244	7.26	6.11
Cystine N	0.0035	1.11	0.0031	0.99	0.0038	0.75	0.0040	1.19	1.01
Amino N of filtrate	0.1492	47.36	0.1470	47.26	0.2488	48.55	0.1626	48.30	47.87
Non amino N of filtrate	0.0184	5.84	0.0169	5.43	0.0208	4.08	0.0199	5.92	5.32
Total N recovered[a]	0.3167	100.52	0.3098	99.61	0.5101	99.54	0.3417	101.59	100.19

[a] Total nitrogen corrected for solubility of bases.[3]

[1] D. D. Van Slyke, *J. Biol. Chem.*, **10**, 15 (1912).

[2] D. D. Van Slyke, *ibid.*, **22**, 281 (1915).

[3] Abderhalden, *loc. cit.*

of wash solution. While this method of washing the precipitate has only been compared to the one recommended by Van Slyke[1] with this protein, it was found more satisfactory because it was easy to bring all particles of the precipitate in contact with the wash solution and the operation of transferring the precipitate from the filter paper to the flask was avoided. The decomposition of the bases can be done in the bottle after washing in the few particles of precipitate on the filter. The arginine was determined by the Koehler modification[2] of the Van Slyke method.

The above analysis indicates that the protein contains the basic amino acids; arginine, histidine, lysine, and cystine. Hopkins-Cole reagent gave the tryptophane test with the protein.

Determination of the free amino nitrogen was made upon the protein. A solution was prepared by grinding a quantity of protein equivalent to 79.92 mg. of nitrogen with dil. acetic acid and the volume made up to 50 cc. Free amino nitrogen was determined on 10 cc. portions of this colloidal solution. Before removing each portion, the solution was thoroughly shaken and each aliquot was washed completely into the reaction chamber with distilled water. Amyl alcohol was used to prevent foaming and corrections were made for the reagents used. The temperature was 22° and pressure 752 mm.

TABLE IV.—FREE AMINO NITROGEN OF THE PROTEIN COMPARED WITH THE LYSINE NITROGEN.

Total N in 10 cc. Mg.	N gas from 10 Cc.	Average. Cc.	Amino N in 10 cc. Mg.	Ratio of amino N to total N. %.	One-half lysine N Van Slyke method. %.
15.98	0.8	0.9	0.501	3.13	3.06[a]
15.98	1.0	...	...	...	..
15.98	0.9	...	...	...	..

[a] From Table III.

The appearance of the extract obtained by using a large volume of alkali solution indicated that it might be used for direct precipitation of the protein. It was found, however, that the nitrogen content of the protein so obtained was only 13.05%. Furthermore, on concentrating the alcohol-free solution after removal of the protein, a transparent gelatinous mass separated out and it was necessary to add the alcohol here in order to obtain a solution satisfactory for further investigation.

The following experiment and table show in a general way the distribution of nitrogen in the seed. Six g. of the ground seed, equivalent to 0.3540 g. nitrogen, was extracted with 600 cc. of 0.5% potassium hydroxide in 300 cc. quantities and then washed with 200 cc. of water using 100 cc. portions. Separation of the liquid and solid was accomplished by centrifuging. The total nitrogen in the residue was determined. The protein was precipitated in the usual way. After con-

[1] *Loc. cit.*

[2] A. E. Koehler, *J. Biol. Chem.*, **42**, 267 (1920).

centrating the solution on the water-bath to about 250 cc., alcohol was added and total nitrogen was determined in the precipitate. The alcohol was then distilled off and 20% lead acetate solution added until no further precipitate formed; and the total nitrogen content of this precipitate was determined. The lead was removed from the solution with hydrogen sulfide and excess of the latter driven off by boiling. Sulfuric acid was then added to 5% concentration after concentrating to 100 cc. A 20% solution of phosphotungstic acid in 5% sulfuric acid was added until no further precipitate formed. After standing for 24 hours the precipitate was filtered off, washed and decomposed with barium hydroxide, and excess of barium was removed by carbon dioxide in a boiling solution. Total nitrogen and amino nitrogen were determined in this solution. Excess of sulfuric and phosphotungstic acids in the filtrate were removed with barium hydroxide and excess of barium with carbon dioxide. Total nitrogen and amino nitrogen determinations were made.

TABLE V.—QUANTITATIVE STUDY OF DISTRIBUTION OF NITROGEN IN ALFALFA SEED.

	N. G.	Total N. %.	Amino N. G.
Protein	0.2170	61.30	....
Alcohol precipitate	0.0029	0.82	....
Lead acetate precipitate	0.0238	6.72	....
Phosphotungstic acid precipitate	0.0319	9.01	0.0122
Filtrate from bases	0.0370	10.45	0.0033
Nitrogen in residue (unextractable)	0.0322	9.10	....
Nitrogen in barium precipitates[a]	0.0092	2.60	....

[a] By difference.

Complete extraction of the nitrogen was not obtained and of the several residues examined, the nitrogen content varied from 9 to 10%. It appears that when the seed is ground to sufficient fineness all of the nitrogen can be extracted. Extracting material that passed through a 100-mesh sieve resulted in a residue containing only 7.8% nitrogen and the ratio of the nitrogen precipitated by acetic acid to the nitrogen in the filtrate was 2.17: 1 while in the above table it was 2.07: 1. Apparently the greater part if not all of the nitrogen in the residue is protein nitrogen. The nitrogen in the alcohol precipitate is no doubt due to nitrogen compounds that were adsorbed by the precipitate. Alcoholic precipitation before protein removal adsorbs more nitrogen and causes a lower protein yield. The lead acetate precipitation probably contains proteose nitrogen. The exact nature of the phosphotungstic acid precipitate could not be ascertained from the small amounts of residue obtained. Solutions treated with nitrous acid by the Van Slyke method liberated gas to account for 38.3 to 43.5% of the nitrogen in three different residues obtained. The total amount of gas measured from the nitrous acid treatment in the filtrates was always the same. Three different analyses from 50-g. quantities of seed yielded 26.9, 27.5, and 26.7 mg. of nitrogen but the

total amount of nitrogen in the different filtrates varied greatly depending on how the barium precipitates were treated. Hydrolysis of the filtrate produced ammonia nitrogen, humin nitrogen, a black precipitate formed and the gas liberated by nitrous acid increased from 27 to 36 mg. The filtrate solution reduced Fehling's solution. The solution from the phosphotungstic acid precipitate did not give the Molisch test. On hydrolysis, ammonia was formed, soluble humin but no black precipitate. The gas liberated by nitrous acid increased from 96 to 110 mg. Adding a little glucose before hydrolysis gave a black precipitate. Attempts to detect definite compounds did not prove successful. Investigation will be continued later when larger amounts of protein are prepared for more complete quantitative data on all the amino acids present.

For further inquiry into the distribution of nitrogen in the seed, 10 g. quantities were hydrolyzed with 20% hydrochloric acid for 24 hours and analyzed according to Van Slyke's method of protein analysis. This method applied to the hydrolytic products of seeds has many errors to contend with as reported by different workers. The hydrolysis of protein in presence of carbohydrates leads to the formation of humin nitrogen[1] and also the presence of nonprotein nitrogen compounds causes an error in the calculation of the different amino acids.[2] Brewster and Alsberg[3] have shown that when yeast nucleic acid is subjected to the Van Slyke procedure 15% of the total nitrogen of the acid appears in the arginine fraction, although nucleic acid contains no arginine. They state that the nitrogen calculated as arginine nitrogen probably comes from the purine and pyrimidine compounds.

In the following Table VI the percentage of arginine nitrogen is far less than that obtained from the hydrolysis of the protein. Ten g. of seed gave 0.0909 g. of arginine nitrogen. The protein in 10 g. of seed would give 0.0908 g. of arginine nitrogen. The latter result was obtained by multipying the nitrogen in the residue plus the nitrogen precipitated by acetic acid by the percentage of arginine nitrogen in the protein. These results make it appear that there is no arginine in the filtrate from acetic acid precipitate of protein or any compounds other than arginine in the basic precipitate from the hydrolytic products of seed which will give ammonia when boiled with strong alkali. The nature of that portion of the basic precipitate not decomposed by amyl alcohol and ether is not known except that the ratio of total nitrogen to nitrogen liberated by nitrous acid is about 4:1 suggesting that it may be basic nitrogen not belonging to the amino acids. The determinations were made by

[1] R. A. Gortner and G. E. Holm (other references are given), THIS JOURNAL, **39**, 2477 (1917).

[2] H. Steenbock, *J. Biol. Chem.*, **35**, 1 (1918).

[3] J. F. Brewster and C. L. Alsberg, *J. Biol. Chem.*, **37**, 367 (1919).

dissolving the precipitate in dilute alkali and taking aliquots for each determination. The percentage of humin nitrogen from the seed hydrolysis is larger, as would be expected. The differences in percentages of the different fractions found in the products of hydrolysis of the whole seed and seed protein, while not great in some cases show clearly a difference in the quantity if not quality of the nitrogen groups in the protein separated from that in the filtrate.

TABLE VI.—ANALYSIS OF THE HYDROLYTIC PRODUCTS OF ALFALFA SEED BY THE VAN SLYKE METHOD.

Sample	1. G.	2. G.	1. %.	2. %.	Average.
Amide N	0.0445	0.0448	7.44	7.42	7.43
Humin adsorbed by magnesia	0.0420	0.0365	7.02	6.05	6.53
Humin N insoluble in amyl alcohol	0.0164	0.0082	2.74	1.30	2.02
Humin N in amyl alcohol extract	....	0.0300	...	4.98	4.98
Arginine N	0.0908	0.0892	15.17	14.79	14.98
Histidine N	0.0405	0.0407	6.76	6.75	6.75
Lysine N	0.0517	0.0458	8.63	7.58	8.11
Cystine N	0.0035	0.0037	0.59	0.61	0.60
Amino N of filtrate	0.2606	0.2667	43.54	44.06	43.80
Non amino N of filtrate	0.0329	0.0339	5.50	5.62	5.56
Total N recovered[a]	0.5829	0.5995	97.39[b]	99.16	100.76

[a] Corrected for solubility of bases.[1]

[b] Humin N in amyl alcohol not determined.

Summary.

1. The alkali extract of alfalfa seed was easily filtered by first precipitating carbohydrate material with alcohol.

2. Protein precipitated by adding acetic acid to the alkali extract represented 60% of the total nitrogen.

3. Analysis of this protein by the Van Slyke method showed that it contained the basic amino acids histidine, arginine, lysine, and cystine. Tryptophane was also present.

4. Free amino nitrogen of the protein corresponded nearly to one-half of the lysine nitrogen found.

5. Distribution of the total nitrogen in the seed was made by the Van Slyke method and by precipitating different fractions in the alkali extract of the seed.

6. A more convenient way was chosen for handling the phosphotungstic acid precipitate of the bases than the procedure recommended by Van Slyke.

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[1] Van Slyke, *J. Biol. Chem.*, **10**, 15 (1912).