THE NONPROTEIN NITROGEN OF THE ALASKA PEA, WITH SPECIAL REFERENCE TO THE CHEMICAL NATURE OF HUMIN NITROGEN[1]

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INTRODUCTION

The pea (*Pisum sativum*) and its seeds, in various stages of growth, have been studied by a number of investigators. Thus, Osborne and his associates (*19, 20, 21, 23*)[2] have isolated from pea seeds four proteins, namely, the globulins legumin and vicilin, the albumen legumelin, and a proteose. They have also reported the hydrolytic products of vicilin (*24*), legumelin (*25*), and legumin (*22*). From the work of Schulze and his collaborators it is known that the compounds choline (*34*) and trigonelline (*35*) occur in pea seeds. Sure and Tottingham (*37*), who studied the metabolic changes taking place in pea seeds during germination, concluded that amides accumulate in the shoot during all stages of germination, especially in the later stages when amino acids are diminishing. On the other hand, α-amino acids accumulated rapidly during the earlier stages of growth but decreased in later stages. From this they concluded that α-amino acids serve for amide production in the nitrogen metabolism of the etiolated pea plant. According to Boswell (*2*), the growth and ripening processes in pea seeds are characterized by a rapid decrease of sucrose, total soluble nitrogen, amides, basic nitrogenous substances, amino acids, and materials which produce humins upon hydrolysis; by an increase in starch, hydrolyzable polysaccharides, and insoluble nitrogen; and finally by a less rapid decrease in total nitrogen.

Humin nitrogen appears to be intimately interrelated with the more general humus question, so much so that there is some confusion in the literature regarding the occurrence and nature of the black substances. Thus, the terms "humin" and "humin nitrogen" are not infrequently employed to designate soil humus and its nitrogen as well as the humin and its nitrogen formed by hydrolysis of proteins. This is due to the fact that the humin nitrogen of the soil is derived principally from proteins and their degradation products through the action of acids or enzymes, just as the humin nitrogen discussed in the present paper is formed by hydrolysis of proteins and their products with mineral acids or through the activities of enzymes. Both types of humin are black or brown in color. In both cases, moreover, the proteins are primarily decomposed to amino dicarboxylic acids, such as aspartic and glutaminic acid; to diamino acids, such as histidine, arginine, and lysine; to monoamino acids, such as leucine, isoleucine, alanine, tyrosine, phenylalanine, tryptophane, etc. However, while

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[2] Reference is made by number (italic) to Literature Cited, p. 823.

these degradation products when formed by hydrolysis of proteins remain unchanged, when formed in the soil they undergo further disintegration. For instance, the amino dicarboxylic acid, aspartic acid, in the process of putrefaction through deamination, is first converted in the soil into succinic acid, which, on splitting off carbon dioxide (decarboxylization), is further changed to propionic acid; while the amino acid, glutaminic acid, is converted into butyric acid through simultaneous deamination and decarboxylization, according to the following schemes (*1*):

$$\underset{\text{Aspartic acid}}{COOH.CH_2.CH.NH_2.COOH} \xrightarrow{(+H_2)} \underset{\text{Succinic acid}}{COOH.CH_2.CH_2.COOH}$$

$$(+NH_3) \rightarrow \underset{\text{(Propionic acid)}}{CH_3.CH_2.COOH} \ (+CO_2);$$

$$\underset{\text{Glutaminic acid}}{COOH.CH_2.CH_2.CH.NH_2.COOH} \xrightarrow{(+H_2)} \underset{\text{Butyric acid}}{CH_3.CH_2.CH_2.COOH}$$

$$(+CO_2+NH_3).$$

The monoamino acid, leucine, which was shown to occur in Michigan peat soils (*26*), is through denitrification changed in the soil to leucinic acid (*43, p. 14*) according to the equation:

$$\begin{matrix}CH_3\\CH_3\end{matrix}\!>\!\underset{\text{Leucine}}{CH.CH_2.CH.NH_2.COOH} + \underset{\text{Nitrous acid}}{NOOH} =$$

$$\begin{matrix}CH_3\\CH_3\end{matrix}\!>\!\underset{\text{Leucinic acid}}{CH.CH_2.CH.OH.COOH} + N_2 + H_2O.$$

The diamino acid, lysine, also found in soils (*36*), is in the process of putrefaction through decarboxylization converted into the diamine cadaverine, according to the equation:

$$\underset{\text{Lysine}}{NH_2.CH_2.CH_2.CH_2.CH_2.CH.NH_2.COOH} =$$

$$\underset{\text{Cadaverine}}{NH._2CH._2CH._2CH_2.CH_2.CH_2.NH_2} + CO_2 (18).$$

The foregoing examples may suffice to illustrate what secondary changes the primary cleavage products of proteins may undergo in the soil. In addition to the humin substances formed in the soil or on hydrolysis of proteins with mineral acids, pigments are known that occur naturally in animal and plant tissues (hair, skin, eyes, etc.). These pigments, referred to as melanins by Schmiedeberg (*28*) and others, will not be considered here.

The purpose of this paper is to report the results of experimental work showing the nature of the humin nitrogen in the Alaska pea. These results were obtained in connection with research carried on for several years with the object of ascertaining the influence of various fertilizers on the yield and chemical composition of the pea.

REVIEW OF LITERATURE

There is a great deal of information regarding humus, the black organic matter which occurs in soils. Although the existence of Mulder's (*17*) hypothetical acids, humic, ulmic, geic, crenic, and apocrenic, has never been corroborated by succeeding investigators, much definite information has accumulated within the last 25 years concerning the chemical compounds of which humus is made up. Thus, the writer has shown that Michigan peat soils and Iowa loess soils contain considerable quantities of organic matter which on hydrolysis yields acid amides and monoamino and diamino acids (*6, 7, 8, 10, 11*), and that these compounds serve as sources of ammonia in soils (*9, 13*) since on their decomposition the liberated ammonia is largely incorporated in the soil.

The writer also predicted in 1914 (*12*) that peptones and proteoses would be found to occur in the soil. Their presence was actually demonstrated by Walters (*42*) in 1915. Schreiner, Shorey, and their associates have isolated from various soils a considerable number of nitrogenous and nonnitrogenous compounds (*16, 29, 30, 31, 32, 33, 36*) the identification of which has greatly added to the knowledge of soil humus and its nitrogen. While much literature is available on the nature of humus and especially of organic soil nitrogen, adequate information is lacking in regard to humin substances and humin nitrogen as obtained on hydrolysis of materials containing protein. Thus, in 1900, Kossel and Kutscher (*15, p. 168, 169*) wrote:

> Mulder was the first to report that humin substances are formed by the action of mineral acids on proteins. The mixture of these substances, which are chemically but little understood, is now referred to as melanoidinic acid. * * * We designate the nitrogen of the substances which remain in the barium sulfate and magnesium oxide precipitates as humin nitrogen. By this designation we do not mean to state anything.

In 1901 Kossel (*14*) stated: "Finally, on cleavage of proteins, there appear products whose relations to the aforementioned groups are not known. To these belong * * * humin substances."

It appears that up to the end of his researches (1927) Kossel never took up the question of humin nitrogen with the object of finding out its chemical nature, though in subsequent work he not infrequently had to do with humin substances. Grindley and Slater (*5*) in their quantitative determination of the amino acid content of feeding stuffs by the Van Slyke method obtained results which ranged in percentage of the total nitrogen from 3.95 per cent of humin nitrogen in blood meal to 15.79 per cent in alfalfa hay. In this connection they state that "on the average, from 8 to 10 per cent of the total nitrogen of the feeding stuffs is separated in the humin, which is an unknown mixture of secondary products * * * " (*5, p. 2768*). Roxas (*27*), who heated pure amino acids (in the presence of sugars) with water or with hydrochloric acid of different strengths in order to ascertain their rôle in humin formation, came to the conclusion that tyrosine, cystine, arginine, lysine, histidine, and tryptophane are responsible for humin formation, and that proline may be involved in that reaction under certain conditions. Gortner and Holm (*4*), who also worked with pure amino acids, have maintained that the black insoluble humin of protein hydrolysis is derived from tryptophane, while the soluble humin formed in the presence of

formaldehyde is derived from tyrosine. In order to find out the chemical nature of the humin nitrogen, the writer experimented directly with the humin substances as obtained on hydrolysis of the aqueous pea extracts. The results secured are recorded in the experimental part of this paper.

EXPERIMENTAL WORK

Peas of grade No. 4 [3] grown at the Arlington Experiment Farm, Rosslyn, Va., on a plot treated with muriate of potash at the rate of 300 pounds per acre, and peas of the same grade grown on an untreated plot, were employed in these experiments. [4] The untreated plot was naturally fairly fertile, but it received no fertilizer during the entire growing season. After drying in an electric oven at 50° C. for about two days, peas from the potash-treated plot were ground in a coffee mill and passed through a 40-mesh sieve. Six 1-liter flasks, each containing 25 gm. of the ground material, received 500 c. c. each of boiling water from which the ammonia had been removed by long boiling. The flasks were then kept on a steam bath for 30 minutes, after which their contents were centrifuged, filtration being almost impossible because of the large quantity of starch present. The solid residues in the centrifuge tubes were treated for the second time with hot water in the manner described. All the extraction liquids were concentrated on the steam bath and centrifuged again. In order to free the extract from proteins, the supernatant liquid of the centrifuge tubes (about 300 to 400 c. c.) was boiled for about five minutes, then acidified with 0.5 c. c. of glacial acetic acid previously diluted with water. The extract was then centrifuged, after which the supernatant liquid of the centrifuge tubes was treated with zinc carbonate in excess and with some lead acetate and boiled for about eight minutes. The whole was then centrifuged, the supernatant liquid of the centrifuge tubes filtered through a Büchner funnel provided with two soft filters, and this filtrate refiltered through a double-folded filter, when a clear, light-brown filtrate was obtained. This was made up with water to 500 c. c., of which two portions of 10 c. c. each were oxidized according to the Kjeldahl method to determine the quantity of nitrogen present in the extract.

An equal quantity (500 c. c.) of purified extract was similarly prepared from peas grown on the untreated plot, and the total nonprotein nitrogen was determined in the same manner as before.

NITROGEN OF ACID AMIDES

Four portions, a, b, c, and d of 120 c. c. each, of the purified extract of peas grown on the potash-treated plot, and another four portions, a′, b′, c′, and d′, from peas raised on the untreated plot, were transferred, respectively, to eight 1-liter Kjeldahl flasks. To each of these was carefully added, with constant stirring, 3.6 c. c. of concentrated sulphuric acid, making about a 5 per cent solution (by weight) of sulphuric acid. The flasks with their contents were then placed in glycerin baths and boiled under reflux condensers for two hours at

[3] Peas of No. 4 grade will not pass through a screen having round holes eleven thirty-seconds of an inch in diameter but will pass through holes twelve thirty seconds of an inch.

[4] The writer wishes to acknowledge his indebtedness to V. R. Boswell, senior horticulturist, Division of Horticultural Crops and Diseases, for supplying the pea samples used in this investigation.

about 135° C. On cooling, the contents of each flask was almost neutralized with sodium hydroxide solution and the ammonia distilled with cream of magnesia into receiving flasks containing tenth-normal sulphuric acid. The results obtained are recorded in Table 1.

TABLE 1.—*Acid amide nitrogen in the Alaska pea, expressed as percentage of the total nonprotein nitrogen in the purified extract from peas grown in potash-treated and in untreated soil*

GROWN IN POTASH-TREATED SOIL

Portion	Extract used for distillation	Total nonprotein nitrogen	Acid amide nitrogen	
	C. c.	*Mgm.*	*Mgm.*	*Per cent*
a	120	417.528	47.2277	11.31
b	120	417.528	48.2084	11.55
c	120	417.528	47.7881	11.45
d	120	417.528	47.7181	11.43
Average			47.7356	11.43

GROWN IN UNTREATED SOIL

a′	120	259.752	20.1604	7.76
b′	120	259.752	19.7401	7.60
c′	120	259.752	19.2077	7.39
d′	120	259.752	19.6280	7.56
Average			19.6841	7.58

NITROGEN OF AMINO ACIDS

The residues from acid amide nitrogen determinations which remained in the Kjeldahl flasks and which, in addition to magnesium oxide, contained also humin substances whose chemical nature will be demonstrated later, were repeatedly extracted with boiling ammonia-free water. The extracted materials were filtered and thoroughly washed. All filtrates and washings from the residues a and b, representing the peas from the potash-treated plot, were together concentrated on the water bath to a small volume. The resulting dark-brown sticky sirup was easily soluble in water, even at room temperature, but practically insoluble in strong alcohol. When treated with 96 per cent alcohol the alcohol gradually became permeated with a considerable quantity of a salt. About 2 to 3 volumes of 96 per cent alcohol were added, stirred well, and left standing overnight. The following morning the whole was filtered on a Büchner funnel containing two soft filters, the filtration proceeding rapidly. The salt on the filter was washed with 85 per cent alcohol and this was followed by extraction of the salt with alcohol in the Soxhlet apparatus until the siphoning alcohol appeared almost colorless. The alcohol-moist salt, which weighed 41 gm. and was completely soluble in cold water, was identified as sodium sulphate mixed with an insignificant quantity of magnesium sulphate. All filtrates, washings, and extracts were united and further concentrated on the water bath to small volume. This was filtered through a double folded filter and made up with water to 100 c. c., one-half of which (m), 50 c. c., was used directly for the determination of the amino nitrogen according to the method

of Van Slyke, and the other half (n), 50 c. c., was employed for the estimation of the peptide nitrogen.

Two portions of 10 c. c. each of m were oxidized by the Kjeldahl method to ascertain the total nitrogen present. In two other portions of 10 c. c. each the amino nitrogen was estimated by Van Slyke's method.

The extracts, filtrates, and washings from the residues a′ and b′, representing the peas from the untreated plot, were separately concentrated on a water bath and made up to 100 c. c. While the first 100 c. c., a′, was used directly for the determination of the amino nitrogen, the other 100 c. c., b′, was reserved for the estimation of peptide nitrogen. Two portions of 25 c. c. each of the solution a′ were Kjeldahlized in order to ascertain the total nitrogen present. In two other portions of 10 c. c. each the amino nitrogen was estimated by the nitrous acid method of Van Slyke. The results are presented in Table 2.

TABLE 2.—*Amino nitrogen in the Alaska pea, expressed as percentage of total nitrogen in extract of humin substances from peas grown in potash-treated and in untreated soil* [a]

GROWN IN POTASH-TREATED SOIL

Portion and analysis No.	Total nitrogen in 50 c. c. of solution	Amino nitrogen in 50 c. c. of solution	
	Mgm.	*Mgm.*	*Per cent*
m (1)	234. 18	14. 1050	6. 01
m (2)	235. 16	14. 2450	6. 07
Average	234. 67	14. 1750	6. 04

GROWN IN UNTREATED SOIL

Portion and analysis No.	Total nitrogen in 100 c. c. of solution	Amino nitrogen in 100 c. c. of solution	
	Mgm.	*Mgm.*	*Per cent*
a′ (3)	206. 00	17. 660	8. 56
a′ (4)	206. 56	17. 101	8. 29
Average	206. 28	17. 381	8. 43

[a] The figures in this table represent the average of at least two determinations.

HUMIN NITROGEN FORMED ON HYDROLYSIS OF POLYPEPTIDES

According to Fischer (*3*), the hydrolysis of polypeptides proceeds in a manner quite similar to that of proteins, as far as strength of acids and time necessary for hydrolysis are concerned. Accordingly the 50 c. c. of solution n, representing the potash-treated plot, and two 50 c. c. portions of solution b′[5], representing the untreated plot, all of which were reserved for the peptide nitrogen estimations, were transferred to 800 c. c. Kjeldahl flasks.[6] To these enough concentrated hydrochloric acid was added to make a concentration of 20 per cent, and the whole boiled in glycerin baths at a temperature

[5] The 100 c. c. of solution b′ was divided into two 50 c. c. portions, m′ and n′.

[6] Large flasks are necessary because at the beginning of hydrolysis the substance foams badly.

of about 130° C. for 12 hours, reflux condensers being used. Each hydrolysate which contained a considerable quantity of black substance was then diluted somewhat with water and filtered separately through a Büchner funnel containing one hardened filter. The humin substance was washed thoroughly with hot, ammonia-free water. Oxidized by the Kjeldahl method the humins of portions n and n′ gave 50.38 and 12.0906 mgm., corresponding respectively to 21.47 and 11.72 per cent of the total nitrogen present as humin nitrogen.

NITROGEN OF POLYPEPTIDES

The filtrates and washings from the humin substances were evaporated in the water bath to complete dryness in order to get rid of the hydrochloric acid. The dark-brown residues were moistened with a few drops of acetic acid, taken up with hot water, filtered, and made up to 50 c. c. In two 10 c. c. portions of this solution the total nitrogen was estimated according to the Kjeldahl method. In two other 10 c. c. portions the amino nitrogen was determined by Van Slyke's nitrous acid method. The data secured are reported in Table 3.

TABLE 3.—*Peptide nitrogen in the Alaska pea, expressed as perceniage of total nitrogen in extract of humin substances from peas grown in potash-treated and in untreated soil* [a]

GROWN IN POTASH-TREATED SOIL

Portion and analysis No.	Total nitrogen in 10 c. c. of solution	Peptide nitrogen plus amino nitrogen (originally present in solution) in 10 c. c.		Peptide nitrogen [b]
	Mgm.	*Mgm.*	*Per cent*	*Per cent*
n (1)	32.657	14.340	43.91	37.87
n (2)	33.400	14.512	43.45	37.41
Average	33.029	14.426	43.68	37.64

GROWN IN UNTREATED SOIL

Portion and analysis No.	Total nitrogen in 50 c. c. of solution	Peptide nitrogen plus amino nitrogen (originally present in solution) in 50 c. c.		Peptide nitrogen [b]
	Mgm.	*Mgm.*	*Per cent*	*Per cent*
m′ (3)	81.6085	40.6610	49.59	41.16
m′ (4)	82.3790	41.2180	50.27	41.84
Average	81.9938	40.9395	49.93	41.50
n′ (5)	80.5575	40.689	50.58	42.15
n′ (6)	80.3475			
Average	80.4525	40.689	50.58	42.15
Average of m′ and n′			50.26	41.83

[a] The figures in this table represent the average of duplicate determinations.
[b] On the basis of the nonprotein nitrogen.

NITROGEN OF DIAMINO AND OF MONOAMINO ACIDS

The residues c and d, which remained in the Kjeldahl flasks after the ammonia had been distilled off (see p. 815) were thoroughly

extracted with boiling ammonia-free water and freed from humin by filtration. The filtrates on concentration were separated from the sulphates of sodium and magnesium as already described and the final concentrated solutions made up with water to 100 c. c. In two portions of each solution of 10 c. c. each the nitrogen was estimated by the Kjeldahl method. The remaining 80 c. c. portions were made up with water to 100 c. c. each. To each of these were added 5 gm. of sulphuric acid and 30 c. c. of a solution containing 20 gm. of phosphotungstic acid and 5 gm. of sulphuric acid per 100 c. c. After 24 hours the phosphotungstates were filtered out and washed in the usual manner. Portion c was used for the qualitative tests. On being freed from phosphotungstic acid it showed the following reactions:

Acidified with sulphuric or hydrochloric acid, it gave with phosphotungstic acid a heavy, grayish-white precipitate; with phosphomolybdic acid, a yellow precipitate; with mercuric chloride, in the presence of barium hydroxide, a gray flocculent precipitate; with silver nitrate, a grayish-white precipitate soluble in excess of ammonia. These reactions are characteristic of diamino acids.

Portion d was Kjeldahlized to ascertain the quantity of basic nitrogen present. Unfortunately this estimation was lost.

The filtrate from the phosphotungstate of both flasks was diluted with three volumes of ammonia-free water, almost neutralized with calcium hydroxide and then made distinctly alkaline with barium hydroxide. Next the whole was saturated with carbon dioxide, brought to a boil, then filtered on a Büchner funnel containing two soft filters and washed well with hot ammonia-free water. The residue on the Büchner funnel was again extracted with hot water. All filtrates and washings were concentrated on the water bath to small volume and filtered. The filtrate from an insoluble grayish-white residue, which was identified as a mixture of gypsum and calcium carbonate, was made up to 100 c. c. In three 10 c. c. portions of this solution the total nitrogen was determined by the Kjeldahl method, while in three other 10 c. c. portions the amino nitrogen was estimated according to the Van Slyke method. The analyses showed 4.07, 4.19, and 3.95 per cent, averaging 4.07 per cent, of monoamino nitrogen.

CHEMICAL NATURE OF HUMIN NITROGEN FORMED ON HYDROLYSIS OF ACID AMIDES

It seems best to present this subject in three parts, dealing with (1) the proportion of humin nitrogen found in the peas, (2) the cleavage of the humin nitrogen into a soluble and an insoluble fraction, and (3) the percentage of amino nitrogen found in the soluble fraction.

Proportion of Humin Nitrogen in Peas

Each of the residues a and b,[7], as well as the residues a′, b′, c′, and d′, remaining in the Kjeldahl flasks after the ammonia had been driven off (p. 865), received 25 c. c. of hot ammonia-free water, and was then brought to a boil and allowed to settle, after which the supernatant liquid was decanted on a Büchner funnel containing a hardened filter.

[7] The residues c and d were used for estimation of the diamino and monoamino nitrogen.

The treatment was repeated with each of the solid residues until the last washings ceased to give a precipitate with barium chloride. Each residue was now transferred to a Büchner funnel, washed with hot water, and the suction allowed to continue until the substance became dry. It was then mechanically removed from the filter to the Kjeldahl flask as completely as possible, traces being washed down by means of the wash bottle. The last traces were dissolved in dilute sulphuric acid and added to the same flask. The nitrogen determinations by the Kjeldahl method of the residues a and b yielded 27.0113 and 29.1548 mgm. of nitrogen, corresponding, respectively, to 6.47 and 6.98 per cent of the total nonprotein nitrogen in the form of humin nitrogen, the average being 6.73 per cent for the peas grown on the potash-treated plot.

The corresponding figures for the residues a′ and c′, from peas raised on the untreated plot, were 28.5384 and 26.0586 mgm. of nitrogen, corresponding to 10.98 and 10.03, the average being 10.51 per cent of the total nonprotein nitrogen in the form of humin nitrogen.

Cleavage of Humin Nitrogen into a Soluble and an Insoluble Fraction

The residues b′ and d′ were hydrolyzed in 1-liter Kjeldahl flasks with 100 and 200 c. c. of 37 per cent hydrochloric acid, respectively, for 12 hours. The hydrolyzing mixtures were kept boiling quietly at about 135°–140° C. At the expiration of this time the hydrolysates were evaporated on the water bath to dryness, the residues moistened with a few drops of acetic acid, then taken up with hot ammonia-free water, filtered, and washed with hot ammonia-free water. The almost coal-black humin residues which remained on the filters were transferred quantitatively to the respective Kjeldahl flasks in which hydrolysis had taken place and oxidized by the Kjeldahl method. They yielded, respectively, 11.8525 and 10.7177 mgm. of nitrogen, corresponding to 4.56 and 4.13 per cent of the total nonprotein nitrogen in the form of residual humin nitrogen. Since the total humin nitrogen obtained was equal to 10.98 and 10.03 per cent, it is evident that 41.54 and 41.18 per cent of the residual humin nitrogen, respectively, was not affected by the hydrolysis with 37 per cent hydrochloric acid, remaining as humin nitrogen under the conditions described.

Amino Nitrogen in Soluble Fraction

The filtrate and washings from the residual humins were concentrated separately on the water bath and made up to 50 c. c. After a preliminary examination of the solutions had been made, two aliquots of 10 c. c. each were oxidized according to the Kjeldahl method, and in two other portions of 10 c. c. each the amino nitrogen was estimated according to Van Slyke's nitrous acid method. The results secured were as follows: On an average, 50 c. c. of the solutions corresponding to the humin residues b′ and d′ were found to be equal to 16.6350 and 16.6020 mgm. of nitrogen corresponding to 6.40 and 6.39 per cent of the total nonprotein nitrogen in the form of soluble nitrogen, as obtained by hydrolysis with 37 per cent hydrochloric acid. The estimations, according to the Van Slyke method, gave the following results: Two 10 c. c. portions of b′ and two 10 c. c. aliquots of d′ gave, on an average, 52.36 and 52.41 per cent, respectively, of amino nitrogen. The data on the humin nitrogen are presented in Table 4.

TABLE 4.—*Humin nitrogen of the Alaska pea as affected by hydrolysis with 37 per cent hydrochloric acid*

Humin residue	Total humin nitrogen (on hydrolysis of acid amides with 5 per cent sulphuric acid)		Residual humin nitrogen (on hydrolysis of the humin substances with 37 per cent hydrochloric acid)			Soluble nitrogen (on hydrolysis of the humin substances with 37 per cent hydrochloric acid)			Residual humin nitrogen plus soluble nitrogen			Amino nitrogen		
	Weight	On the basis of the nonprotein nitrogen	Weight	On the basis of the nonprotein nitrogen	On the basis of the total humin nitrogen	Weight	On the basis of the nonprotein nitrogen	On the basis of the total humin nitrogen	Weight	On the basis of the nonprotein nitrogen	On the basis of the total humin nitrogen	Weight	On the basis of the soluble nitrogen [a]	On the basis of the total humin nitrogen
	Mgm.	*P. ct.* [b]	*Mgm.*	*P. ct.*	*P. ct.*	*Mgm.*	*P. ct.*	*P. ct.*	*Mgm.*	*P. ct.*	*P. ct.*	*Mgm.*	*P. ct.*	*P. ct.*
b'----------	28. 5384	10. 98	11. 8525	4. 56	41. 54	16. 6350	6. 40	58. 29	28. 4875	10. 96	99. 83	8. 7010	52. 36	30. 52
d'----------	26. 0586	10. 03	10. 7177	4. 13	41. 18	16. 6020	6. 39	63. 71	27. 3197	10. 52	104. 89	8. 7093	52. 41	33. 39
Average	-------	10. 51	-------	4. 35	41. 36	-------	6. 40	61. 00	-------	10. 74	102. 36	------	52. 39	31. 96

[a] This soluble nitrogen was obtained on hydrolysis of the total humin nitrogen with 37 per cent hydrochloric acid for 12 hours.

[b] The figures in columns 2 and 3 refer to humin residues a' and c', which are, however, equivalent to humin residues b' and d'.

It will be seen from Table 4 that there was found for b' 4.56 per cent of residual humin nitrogen and 6.4 per cent of soluble nitrogen, totaling 10.96 per cent of nitrogen, which accords well with the 10.98 per cent of total humin nitrogen found. The figures for d' were 4.13 per cent of residual humin nitrogen and 6.39 per cent of soluble nitrogen, totaling 10.52 per cent nitrogen, which is also in reasonable agreement with the 10.03 per cent of total humin nitrogen found. It will be noticed further that the amino nitrogen found (column 14) on the basis of the soluble nitrogen, averaged 52.39 per cent. The question naturally arises: What is the nature of the remaining 47.61 per cent? While a complete answer to this question can be given only by further investigation, it seems fairly safe to assume that a considerable part, if not all, of the 47.61 per cent is made up of amino acids also. In this connection the following facts must be borne in mind.

Roxas (*27*) has shown that tyrosine, cystine, and tryptophane, as well as the diamino acids are responsible for humin formation and that proline may also be involved in this reaction under certain conditions. These compounds, however, do not react with all of their groups on nitrous acid to evolve elementary nitrogen. Thus, Van Slyke has demonstrated (*38, 39, 40, 41*) that tryptophane reacts with one-half of its nitrogen, histidine with one-third, arginine with one-fourth, while proline and oxyproline react with none. It may be added that lysine practically reacts only with the α-amino group on nitrous acid within five minutes, the time which is ordinarily used in the Van Slyke method, while the Ω-amino group requires one-half hour for complete reaction. Consequently, the elementary nitrogen evolved by the Van Slyke method respresents only a part of the amino acid nitrogen. Whether all of the amino acids under consideration occur in the aqueous extract of the Alaska

pea has not yet been investigated. It is certain, however, that diamino acids are present, as already shown in this investigation. Furthermore, from the work of Osborne and his associates (*19*, *20*, *21*, *23*), it is known that the pea seed contains the proteins legumin, vicilin, and legumelin, all of which contain proline, tyrosine, and tryptophane, in addition to the diamino acids (*22*, *24*, *25*). These facts render it quite probable that the aqueous extract of the Alaska pea contains these amino acids. This will be readily comprehended when it is borne in mind that for the protein synthesis going on in leguminous and, generally speaking, in higher plants, not all of the amino acids are used up quantitatively. A small portion of the amino acids ordinarily remains as such in the seed either because the anabolic process has not been fully completed or because slight catabolism of the proteins takes place under the influence of proteolytic enzymes.

The percentage of amino nitrogen, calculated on the basis of the total humin nitrogen, is on an average but 31.96 per cent (last column). Undoubtedly this is due to the causes just outlined and also to the fact that the percentage of soluble nitrogen is only 61.00 per cent (column 9). It is possible that the proportion of soluble nitrogen would have been greater had methods been used different from the one employed in this work.

To facilitate a comparison of all the results obtained they are presented in summary form in Table 5.

TABLE 5.—*Nonprotein-nitrogen content of peas grown in potash-treated and in untreated soil*

	Peas grown in potash-treated soil	Peas grown in untreated soil
	Per cent [a]	*Per cent* [a]
Acid amide nitrogen	11.43	7.58
Humin nitrogen formed on hydrolysis of acid amides with 5 per cent sulphuric acid	6.73	10.51
Amino nitrogen	6.04	8.43
Humin nitrogen formed on hydrolysis of polypeptides with 20 per cent hydrochloric acid	21.47	11.72
Peptide nitrogen	37.64	41.83
Residual nitrogen	16.69	19.93
Total	100.00	100.00
Diamino nitrogen	([b])	([c])
Monoamino nitrogen	4.07	([c])

[a] Average per cent of total nonprotein nitrogen. [b] Lost. [c] Not determined.

DISCUSSION

Table 5 shows that of the nonprotein nitrogen, the percentage of acid amide nitrogen is higher in the peas grown in potash-fertilized soil (11.43) than in those grown in untreated soil (7.58), while the percentage of amino and peptide nitrogen is somewhat higher in the latter (8.43 and 41.83, respectively) than in the former (6.04 and 37.64, respectively). Of these compounds the amino acids are known to be nutritious, whereas opinions differ as to nutritive value of acid amides. The writer does not know of any experiments dealing directly with the value of polypeptides from the standpoint of nutri-

tion. However, judging from the fact that in the nitrogen metabolism of the higher plants the polypeptides stand between the amino acids on the one hand and the proteins on the other, and that both of these have high nutritive value, it seems fairly safe to assume that the polypeptides have nutritious qualities. Now the sum of amide, amino, and peptide nitrogen differs very little in the peas from the two plots, being 55.11 per cent in peas from potash-treated soil and 57.84 per cent in those from untreated soil. For the reasons given it may be said that so far as the nutritive value of their nonprotein compounds is concerned, the peas from the two plots are essentially equal. These compounds, however, constitute but a fraction of the total nitrogen.

The percentage of polypeptides in the Alaska pea is strikingly high, as it is in some other vegetables examined by the writer. It would seem, therefore, to be very desirable that the polypeptides receive further study. The percentage of humin nitrogen, too, is considerable; a fact which suggests the importance of research with a view to finding out more about the nature of humins and especially of humin nitrogen.

From the work of Roxas (*27*) as well as of Gortner and Holm (*4*) it is known that humin is formed from certain amino acids heated with mineral acids in the presence of sugars, aldehydes, or ketones. Since the aqueous extract of the Alaska pea contains appreciable quantities of amino acids and sugar, it is easy to understand why conditions are favorable for the formation of humin. Gortner and Holm (*4*), who experimented with pure amino acids and came to the conclusion that tyrosine and tryptophane are responsible for the soluble and insoluble humin, respectively, did not deal with the chemical nature of the humin substances. Roxas (*27*), who also experimented with pure amino acids, claimed to have demonstrated that in the case of cystine, tyrosine, arginine, and lysine the humin nitrogen is due to a reaction rather than to an adsorption. He suggested that the amino acids tryptophane, arginine, and histidine have labile hydrogen atoms that enable them to give condensation products with carbohydrates, with the formation of a ring. On the other hand, Grindley and Slater (*5*) believe that with the exception of tryptophane, which enters into chemical combination with carbohydrates, the amino acids are to a greater or less extent adsorbed by the humin substances formed by the action of mineral acids upon carbohydrates. The fact demonstrated in this paper, that the humin nitrogen can be split by simple hydrolysis into amino nitrogen to the extent of about 32 per cent, renders it highly probable that the humin substances obtained from the Alaska pea have peptide linkings.

SUMMARY

Seeds of the Alaska pea (grade No. 4) grown in potash-treated and in untreated soil are shown to contain, respectively, as percentages of the total nonprotein nitrogen: Acid amide nitrogen, 11.43 and 7.58 per cent; humin nitrogen formed on hydrolysis of the acid amides, 6.73 and 10.51 per cent; amino nitrogen, 6.04 and 8.43 per cent; peptide nitrogen, 37.64 and 41.83 per cent; and humin nitrogen formed on hydrolysis of the polypeptides, 21.47 and 11.72 per cent.

Since the sum of the more or less nutritive constituents, namely, the amide, amino, and peptide nitrogen, in peas grown in potash-treated and untreated soil, is 55.11 and 57.84 per cent, respectively, it is concluded that there is no essential difference in the nutritive value of such peas so far as the nonprotein nitrogen compounds are concerned. These, however, make up but a fraction of the total nitrogen.

The humin nitrogen resulting from hydrolysis of the acid amides was shown to be split by hydrolysis with 37 per cent hydrochloric acid into a soluble portion containing 61 per cent of the total humin nitrogen in soluble form, and an insoluble portion, with 41.36 per cent residual humin nitrogen. The soluble portion contained 52.39 per cent amino nitrogen, which means that 31.96 per cent of the total humin nitrogen was converted into amino nitrogen by the hydrolysis. It is believed, however, that the actual percentage of amino nitrogen is considerably higher, since by Van Slyke's nitrous acid method only a part of the nitrogen of certain amino acids (tryptophane, proline, oxyproline, and hexone bases) is split off in elementary form. Some of these amino acids (Kossel's so-called hexone bases) have been shown actually to occur in the aqueous extracts of the Alaska pea. On theoretical grounds, as outlined in this paper, it is probable that the other amino acids occur in the extracts. Inasmuch as the humins obtained from pea materials yielded amino nitrogen on simple hydrolysis, it seems safe to conclude that they had peptide linkings.

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