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NUTRIENT DISTRIBUTION IN GRAIN

Location of Nonprotein Nitrogenous Substances in Corn Grain

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Nonprotein nitrogenous substances in corn contribute to the flavor and nutritional quality of the processed grain in food products and feeds. Because their presence in dry-milled products is determined by their location in the kernel, the amounts of amino acids, quaternary nitrogen compounds, nucleosides, purines, and pyrimidines in germ, endosperm, and bran fractions were measured. These compounds were extracted with aqueous ethanol from the corn fractions, separated by ion-exchange chromatography, and quantitatively determined by specific spectrophotometric procedures. Amino acids, which contribute over 50% of the nonprotein nitrogen in whole corn or its fractions, are distributed almost equally between the endosperm and the smaller germ fraction, with minor amounts in the bran. The concentration of free amino acids in the germ is several times that in the endosperm. Only small differences exist between the types of amino acids found in germ and endosperm. Quaternary nitrogen and heterocyclic nitrogen compounds were primarily in the germ.

A SMALL but significant fraction of the nitrogen in whole corn grain has been shown to be present in low-molecular-weight substances such as free amino acids, amines, amides, quaternary nitrogen compounds, purines, and pyrimidines (*7*). These nonprotein nitrogenous substances contribute to the nutritional value, flavor, and other factors important to products derived from corn. For the preparation of certain foods and feed products from corn, the grain is dry-milled to yield endosperm, bran, and germ fractions which may be processed separately. Therefore, the disposition of the nonprotein nitrogen and its effect upon product quality will depend upon the initial concentration of these substances in the grain parts. This report describes a quantitative study of the distribution of the major nonprotein nitrogenous compounds in the parts of corn grain. The information obtained is also of interest relative to the roles of these substances in seed metabolism.

Determinations of these substances were facilitated by improved methods for the ion exchange chromatography of amino acids (*15, 18*), quaternary nitrogen compounds (*6*), and purines and pyrimidines (*8*). These procedures employed columns of finely pulverized classified cation exchange resins and sodium citrate buffer eluent. Improved resolution, decreased degradation of labile compounds, and increased rate of analysis were advantages of these methods.

Materials and Methods

Hand-Dissected Corn Samples. Whole grain yellow dent hybrid seed corn (Doubet Variety 25) was picked at maturity, air-dried, and immediately transferred to cold storage. This corn was grown under average farm conditions in central Illinois in 1960 on soil balanced in phosphorus, potash, and nitrogen but without added minor elements. Fertility level of the soil was medium high with a yield of 100 bushels per acre. The kernels were manually dissected into germ, endosperm, and combined tip cap and pericarp fraction according to the procedure of Hopkins and coworkers (*13*) as modified by Earle, Curtis, and Hubbard (*12*). Completeness of separation was determined by microscopic examination. Samples of whole corn, endosperm, and bran (combined tip cap and pericarp) were air-dried and ground in a hammer mill to pass a 0.027-inch screen. The germ was first flaked in a roller mill and its oil removed with petroleum ether before grinding. The germ contained 37.5% oil. The germ sample was analyzed as lipid-free material, but values in tables are corrected to whole germ. Moisture content of the ground samples was determined by drying under vacuum at 100° C. over phosphorus pentoxide (Table I). Data obtained with respect to yields of fractions, nitrogen content, and amounts of nonprotein nitrogenous constituents were converted to a moisture-free basis in all of the tables.

Isolation of Nonprotein Nitrogen Constituents. The previously described method for extracting nonprotein nitrogen from whole corn (*7*) was modified considerably to facilitate analysis of corn fractions. One hundred grams of the finely ground sample were stirred in 1 liter of 80% aqueous ethyl alcohol at room temperature for 10 minutes. The suspension was cooled to 4° C. and centrifuged to obtain a solution of material soluble in 80% ethanol. The residue was re-extracted with 1 liter of 80% ethanol, centrifuged at 4° C., and then washed with an additional volume of 200 ml. of cold 80% ethanol. Alcohol was removed from the combined washes and extracts by vacuum distillation. Zein and other water-insolubles were removed at various stages in the reduction of volume to prevent occlusion of minor constituents with the precipitate. Precipitates were washed before being discarded, and all washings were combined with the extract. The combined extract containing water-soluble nonprotein nitrogenous substances was concentrated to dryness under vacuum at a bath temperature of 40° C., then made up to volume with water. The amount of nitrogen extracted was determined by semimicro-Kjeldahl and compared with total nitrogen (Table I). All extracts

Table I. Nitrogen Distribution in Whole Corn and Its Fractions

Sample	% Moisture	Percentage[a] Weight	Total Nitrogen[a] Mg./100 g. in tissue	Total Nitrogen[a] Mg./100 g. in corn grain	Total Nitrogen[a] % in grain	Nonprotein Nitrogen[a] Mg./100 g. in tissue	Nonprotein Nitrogen[a] Mg./100 g. in corn grain	Nonprotein Nitrogen[a] % in grain
Whole grain	10.48	100.0	1390	1390	100	27.7	27.7	100
Germ	10.23	11.0	2970	330	24	102.4[b]	11.3	41
Endosperm	12.13	79.5	1270	1010	73	15.2	12.1	44
Bran	9.22	6.6	750	50	4	57.8	3.8	14

[a] Moisture-free basis.
[b] Analyzed as lipid-free material but corrected to whole germ.

were frozen during storage. Separate suitable portions of the whole corn, germ, bran, and endosperm extracts were diluted with pH 2.2 sodium citrate buffer to a total of 2 ml. for chromatographic analysis of each of the groups of nonprotein nitrogenous substances.

Separation of Constituents by Ion-Exchange Chromatography. Determination of Amino Acids, Amides, and Amines. Ninhydrin-positive substances were separated by chromatography on columns of Amberlite IR-120 resin and automatically determined in a Phoenix Model K 8000 amino acid analyzer by the procedures of Spackman, Stein, and Moore (*18*). In addition to the 150-cm. column used for the determination of the acidic and neutral components, a 50-cm. column was required to separate the numerous basic ninhydrin-positive substances. Extracts equivalent to 2.7 grams of corn, 2.5 grams of bran, 1.5 grams of germ, and 7.1 grams of endosperm were placed on separate columns.

The manual system (*15*) was employed to isolate the unresolved glutamine, asparagine, and serine peaks. Two-milliliter fractions were collected with a fraction collector and 0.5-ml. aliquots taken for analysis with ninhydrin (*16*). A sample from the combined peak tubes was hydrolyzed in 2.0*N* HCl at 100° C. for 2 hours. The resultant glutamic acid, aspartic acid, and serine were rechromatographed and determined automatically. Under these conditions amide hydrolysis was complete and determination of asparagine as aspartic acid was quantitative. Glutamine values are low because of partial degradation during ion-exchange chromatography.

To investigate the possible peptidal nature of certain of the ninhydrin-positive unknowns (Figure 1), corn extract containing 190 mg. of solids (2.7 mg. of nitrogen) was hydrolyzed with 200 ml. of 6*N* HCl under reflux for 24 hours (*11*). Humin was removed by filtration and the acid eliminated from the hydrolyzate by repeated concentration to dryness, followed by addition of water. The final dried residue was dissolved in an appropriate volume of buffer for amino acid analysis.

Several of the unknown ninhydrin-positive substances separated on the analytical columns were isolated in larger quantities for identification. Ten times the normal sample was applied to a larger diameter chromatographic column (1.8 cm.). By means of a stream splitter system (*20*), one fortieth of the effluent was directed into the automatic analyzer, where it was diluted appropriately with buffer, and amino acids were determined. The remaining effluent was distributed into tubes by means of a fraction collector.

Chromatographic Determination of Quaternary Nitrogen Compounds. These compounds were separated on 50-cm. columns of IR-120 resin and eluted with sodium citrate buffers according to the procedures of Christianson and coworkers (*6*). Extracts equivalent to 60.0 grams of corn, 7.5 grams of germ, and 55.0 grams of endosperm were placed on separate columns. The substances were detected and quantitatively analyzed by spectrophotometric determination of their periodide derivatives (*6*, *19*).

Chromatographic Separation of Heterocyclic Nitrogen Compounds. Crampton and his coworkers (*8*) separated purines and pyrimidines on 0.9- × 60-cm. columns of Amberlite IR-120 using a buffer concentration gradient at constant pH. Because of the complexity of the mixture in corn extracts, this method did not give satisfactory resolution. By extending the column lengths to 150 cm. and by using sodium citrate buffer with stepwise pH increases, nucleosides, purines, and pyridines in the extracts were successfully resolved. The initial buffer schedule is similar to that used to separate amino acids (*15*). Additional eluent, consisting of pH 5.28 sodium citrate buffer, was used to elute the more basic compounds. Buffer compositions, temperatures, flow rates, and elution positions of known compounds are given in Figure 2. Extracts equivalent to 30.0 grams of corn, 3.4 grams of germ, and 40.0 grams of endosperm were placed on separate columns.

The eluted substances were detected by continuous scanning of the effluent at 260 mμ with a Vanguard Model 1056 automatic ultraviolet analyzer. More precise determinations were made by directly measuring the absorption of the 2-ml. effluent fractions at 260 mμ in a Beckman DU spectrophotometer equipped with 0.9-ml. capacity quartz absorption cells having a 1-cm. light path. Absorption values at 260 mμ of

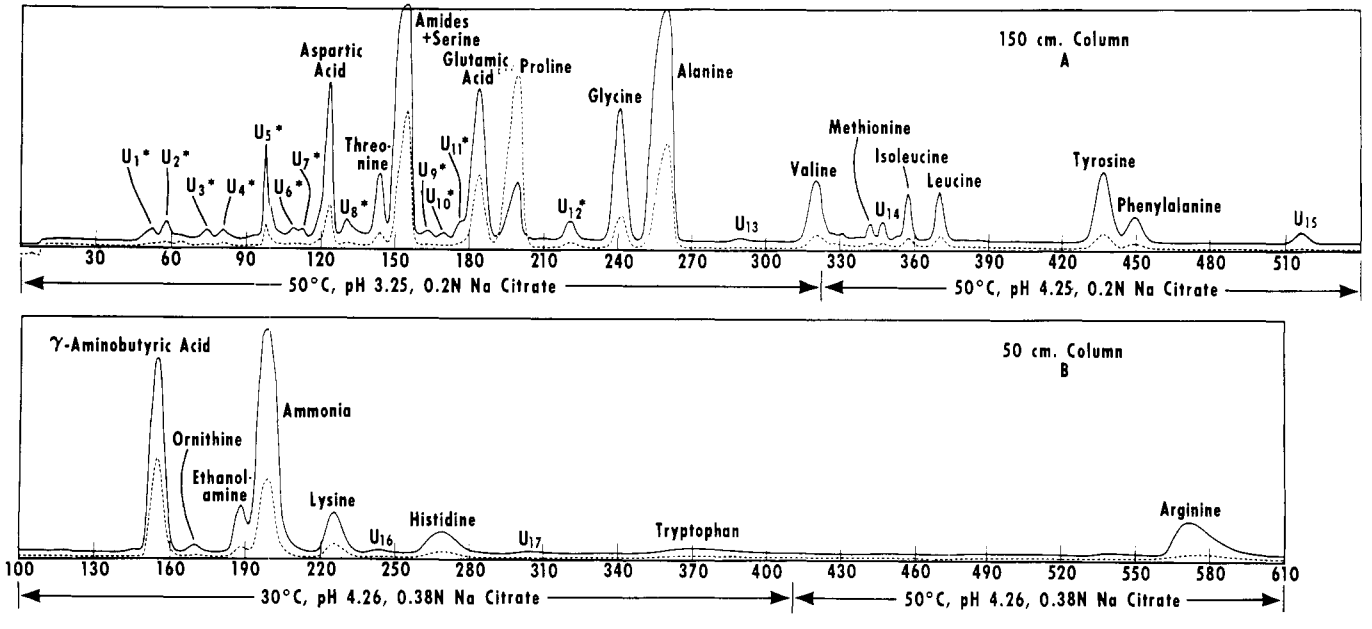

Figure 1. Automatic amino acid analysis of extracts of whole corn

Substances hydrolyzable by acid

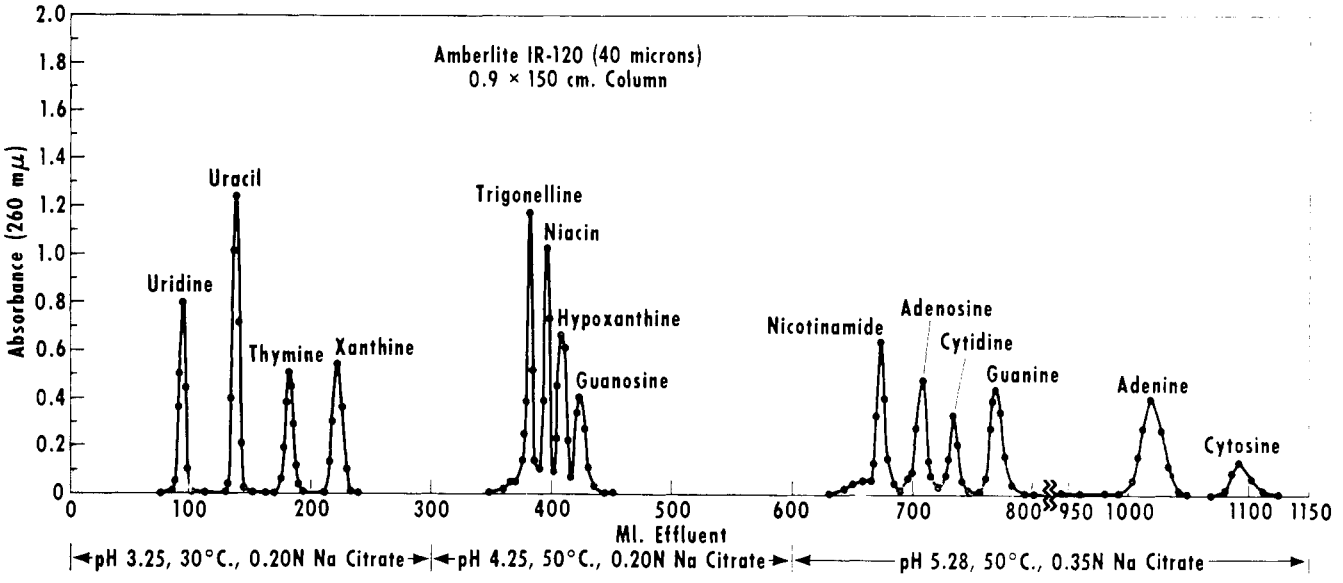

Figure 2. Separation of synthetic mixture of known purines, pyrimidines, nucleosides, and other ultraviolet-absorbing aromatic nitrogen compounds by ion-exchange chromatography

From 50 to 150 mg. of each substance applied

standard solutions of the compounds in buffers corresponding to the eluent were used to calculate concentration.

Characterization of Individual Constituents. The identity of the compounds eluted from the columns was tentatively established by comparison of their elution positions with that of knowns. Paper chromatography, specific chemical procedures, and absorption spectra were used to verify the identifications. The ratios of absorbances at 440 and 570 mμ of ninhydrin reaction products were useful in characterizing amino acids and other ninhydrin-positive compounds (*22*). Pentose estimation (*10*) and phosphorus determination (*1*) were conducted on selected isolated peaks to establish the presence of nucleosides or nucleotides.

For paper chromatography of isolated constituents, the fractions constituting a detected peak were pooled, and the nitrogenous substance was isolated from the buffered effluent. The solutions of ninhydrin-positive substances were desalted for paper chromatography on 2.5- × 15-cm. columns of Dowex-2 (OH^- form) according to the procedure of Dreze, Moore, and Bigwood (*9*). The water-insoluble periodide complex of the quaternary nitrogen compounds was formed, separated from the supernatant, and then dissociated to yield a solution of the substance free of excessive salts (*6*). Combined effluent fractions containing aromatic heterocyclic nitrogen compounds were passed through 1- × 3-cm. columns of Darco G-60 acid-washed charcoal. After washing the columns with water to remove buffer, the adsorbed substances were eluted with ammonia–ethanol–water (10:25:65) as employed by Magasanik and Brooks (*14*). The salt-free solutions of nitrogen compounds were concentrated to dryness under vacuum and dissolved in 0.5 ml. of water. The R_f's of the compounds upon unidimensional descending paper chromatography were compared with those of knowns by using several solvent systems. Techniques for paper chromatographic separation and detection have been summarized by Block, Durrum, and Zweig (*5*).

Results

Three different extracts of whole grain did not differ by ±4% in nitrogen content; so the extraction procedure is reproducible. In general, two chromatographic analyses were performed on at least two different extracts. Replicate analysis for the major components agreed within ±5%.

There is 0.03 gram of nonprotein nitrogen (NPN) in 100 grams of whole corn. NPN constitutes 2% of the total corn nitrogen. The germ contains not only a higher concentration of NPN than the endosperm but also a higher ratio of NPN to total nitrogen (Table I). Despite the differences in concentration, the amounts of NPN are about the same in the germ and endosperm because the latter has about eight times as much tissue as the germ. Concentration of NPN in bran is intermediate between that of germ and endosperm, but the total amount is much less than that in the other two tissues.

Germ contains the greatest portion of free amino acids, although it is a small part of the kernel (Table II). Free

Table II. Free Amino Acids in Whole Corn and Its Fractions

(Mg. nitrogen/100 grams)

Amino Acid	Whole Corn	Germ		Endosperm		Bran	
		Sample[a]	Corn	Sample	Corn	Sample	Corn
Asparagine	4.28	21.14	2.33	1.80	1.43	2.08	0.14
Proline	2.89	15.94	1.75	1.09	0.86	1.04	0.07
Alanine	1.92	7.23	0.80	0.95	0.76	1.00	0.07
γ-Aminobutyric acid	0.69	2.12	0.23	0.36	0.29	0.45	0.03
Glutamic acid	0.45	2.97	0.33	0.19	0.15	0.33	0.02
Serine	0.34	1.21	0.13	0.17	0.14	0.08	0.01
Aspartic acid	0.29	0.49	0.06	0.18	0.14	0.28	0.02
Glycine	0.35	0.95	0.11	0.27	0.21	0.52	0.03
Arginine	0.92	2.18	0.24	0.47	0.37	0.18	0.01
Glutamine	0.25	1.88	0.21	0.06	0.05	0.35	0.02
Histidine	0.34	0.82	0.09	0.19	0.15	0.10	0.01
Lysine	0.21	0.44	0.05	0.12	0.09	0.12	0.01
Tyrosine	0.21	0.56	0.06	0.14	0.11	0.13	0.01
Threonine	0.10	0.52	0.06	0.07	0.06	0.16	0.01
Ornithine	0.02	Trace	...	0.02	0.02	0.09	0.01
Ethanolamine	0.11	0.34	0.04	0.06	0.05	0.12	0.01
Phenylalanine	0.07	0.15	0.02	0.06	0.05	0.09	0.01
Leucine	0.06	0.18	0.02	0.06	0.04	0.13	0.01
Valine	0.17	0.66	0.07	0.11	0.08	0.18	0.01
Isoleucine	0.06	0.17	0.02	0.04	0.03	0.09	0.01
Methionine	0.02	0.06	0.01	0.01	0.01	...	...
Total	13.75	60.01	6.63	6.42	5.09	7.52	0.51
Ammonia	0.99	1.19	0.13	1.19	0.95	9.48	0.63
Total	14.74	61.20	6.76	7.61	6.04	17.00	1.14

[a] Analyzed as lipid-free material but corrected to whole germ.

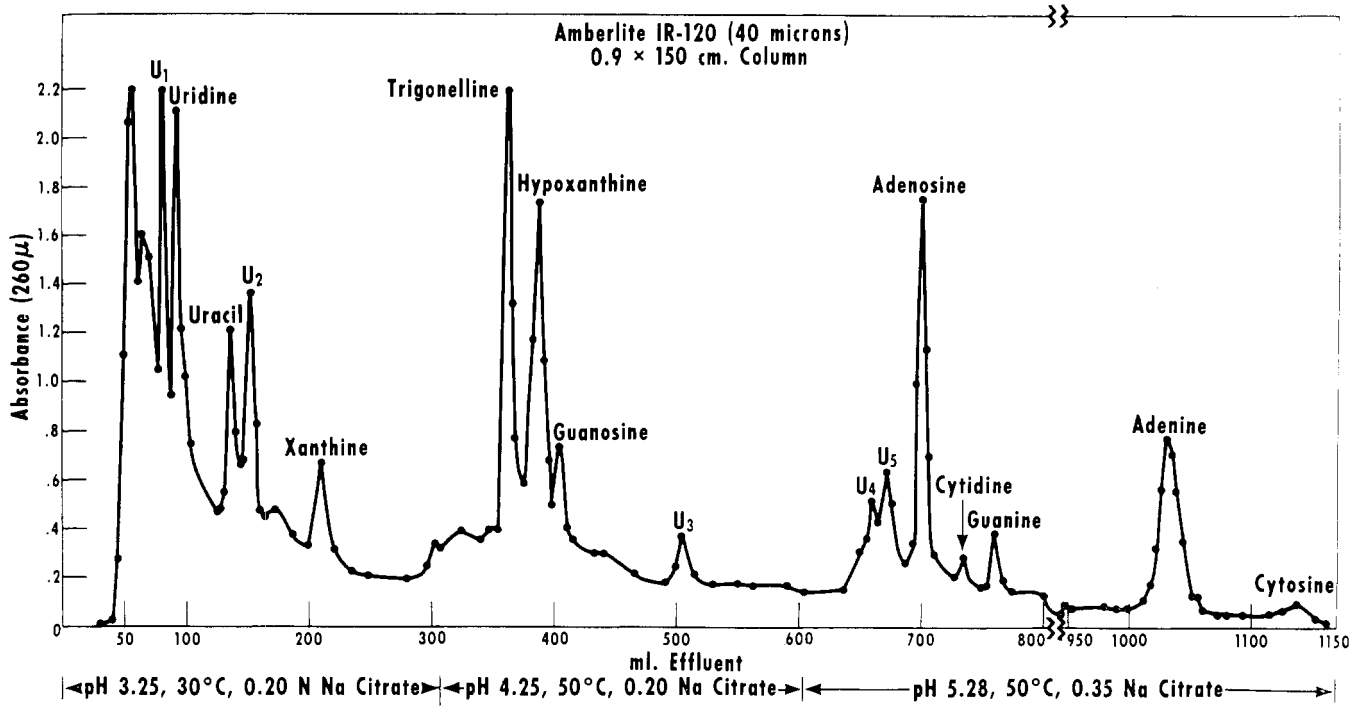

Figure 3. Separation of heterocyclic aromatic nitrogen compounds present in extract of whole corn

Extract equivalent to 26.9 grams of corn

amino acid concentration is almost eightfold higher in the germ than in the endosperm. The major amino acids in all three fractions, in order of concentration, are asparagine, proline, alanine, and γ-aminobutyric acid. Together these constitute 60% of the characterized ninhydrin-positive substances in each seed part. The fraction of total amino acid, which each of these amino acids constitutes, is about the same in all three tissues. Most of the other amino acids were more highly concentrated in the germ, but a few were present in larger quantities in the endosperm; these include glycine, aspartic acid, and lysine. Although the distributions of the major free amino acids in the seed parts were similar, there were distinct differences in distribution among the others.

A number of ninhydrin-positive substances giving smaller peaks were detected in germ, bran, and endosperm extracts which were not identified in the previous study (*7*). Their separation in whole corn along with the more common amino acids is illustrated in Figure 1. Upon acid hydrolysis of the extracts and chromatography of the resulting solution, the disappearance of certain of the unknown peaks and the 8% increase in nitrogen in known amino acids indicated that these peaks were peptides. The most significant increases in amino acids after hydrolysis were those for aspartic, glutamic, and glycine. Several of the unidentified peaks, however, were acid-resistant (U_4, U_{10}, U_{13}, U_{14}, U_{15}, U_{16}, and U_{17} in Figure 1). On the basis of elution position as compared to those of known compounds, the unknowns may be homoserine (U_{10}), α-aminobutyric acid (U_{13}), alloisoleucine (U_{14}), β-alanine (U_{15}), 1-methylhistidine (U_{16}), and 3-methylhistine (U_{17}). Only β-alanine and homoserine were isolated in large enough quantities to permit confirmation of their identity by paper chromatography. Both of these amino acids have been previously established as constituents of the corn kernel (*2*, *3*).

Table III. Quaternary Nitrogen and Heterocyclic Nitrogen Distribution in Whole Corn and Its Fractions

	Mg. Nitrogen/100 Grams Sample			Mg. Nitrogen/100 Grams Corn	
	Corn grain	Germ[a]	Endosperm	Germ	Endosperm
Quaternary nitrogen compound					
Betaine	0.10	0.48	...	0.06	...
Trigonelline	0.24	0.90	0.08	0.10	0.07
Choline	1.71	6.12	0.66	0.67	0.52
Heterocyclic nitrogen compound					
Uridine	0.11	0.69	0.02	0.08	0.02
Uracil	0.07	0.37	0.02	0.04	0.02
Adenosine	0.26	1.63	0.07	0.19	0.05
Adenine	0.28	0.80	0.15	0.09	0.13

[a] Analyzed as lipid-free germ but corrected to whole germ.

Choline, trigonelline, and betaine, the three major quaternary nitrogen compounds in corn, are concentrated in the germ with lesser amounts in the endosperm (Table III). Of the three in this class, choline is present in the greatest amounts.

Heterocyclic nitrogen compounds contained in extracts of whole corn and its fractions were separated under conditions noted in Figure 3. The major ultraviolet-absorbing constituents are trigonelline and adenosine, with smaller amounts of adenine, uridine, and uracil. Over 70% of each substance is located in the germ, except adenine, which is in the endosperm in a slightly greater amount (Table III). Several of the peaks in Figure 3 have not been identified. Nucleotides and other acidic substances eluted very early and were not resolved on these columns.

The distribution of nonprotein nitrogen among the various classes in each of the fractions of corn is compared in Table IV. In each of the corn fractions the free amino acids are the predominant group of constituents determined. They constitute about 60% of the nonprotein nitrogen in the germ, 50% in the endosperm, and 30% in the bran. The quaternary nitrogen compounds contain 8% of the extracted NPN in germ but only 5% of the NPN in endosperm. The heterocyclic compounds constitute only about 5% of both endosperm and germ NPN. The amounts of quaternary and heterocyclic nitrogen compounds in bran were small, so that accurate analysis was not possible. Significant amounts of nonprotein nitrogen remain to be identi-

Table IV. Distribution of Nonprotein Nitrogenous Substances in Corn

Substance	Nitrogen, Mg./100 Grams Tissue				Nitrogen, Mg./100 Grams Corn		
	Corn	Germ[a]	Endosperm	Bran	Germ	Endosperm	Bran
Nonprotein	27.7	102.4	15.2	57.8	11.3	12.1	3.8
Amino acids and ammonia	14.7	61.2	7.6	17.0	6.8	6.1	1.1
Quaternary	2.1	8.9	0.7	...	1.0	0.6	...
Heterocyclic	0.7	3.5	0.3	...	0.5	0.2	...
Peptides	2.3	...	...	...	...	...	...
Not characterized	7.9	28.8	6.6	...	...	...	...

[a] Analyzed as lipid-free material but corrected to whole germ.

fied in whole corn, germ, endosperm, and especially bran fractions. This nitrogen may be contained in nucleotides, amines, or peptides that have not been characterized.

Discussion

The free nonprotein nitrogenous substances of whole corn grain as reported here are qualitatively similar to those found in earlier studies (*7*), but some quantitative differences are observed. The corn used in this study contained 27% more extractable nonprotein nitrogen than the composite commercial corn analyzed earlier; most of this difference results from larger amounts of asparagine and proline in this corn. The variations in quantities may be due to differences in varieties, storage conditions, and temperature of drying used in the two investigations. In the previous studies varietal differences were minimized by using a composite commercial sample; however, the effect of drying and storage was not controlled. In these experiments, a specific hybrid seed was picked at maturity, air-dried, and immediately transferred to cold storage Some quantitative differences in individual substances can also be explained by improved methodology in isolation and separation. Amide and nucleoside degradation was decreased under the present milder extraction. Automatic analysis with longer buffered columns resulted in better separation and greater sensitivity of detection with increased accuracy in calculation due to lower and more consistent blanks. Numerous substances not found previously were detected in the extracts by employing the procedures described above.

The quaternary nitrogen compounds were more highly concentrated in the germ, which on development becomes anabolically active. Trigonelline occurs almost entirely in the germ, which is consistent with its role of precursor for the pyridine moiety of nicotinamide adenine dinucleotide during germination (*4*). Betaine in the germ may serve, along with choline, as a methyl donor during biosynthetic processes.

Purine and pyrimidine bases, which are predominantly in the germ along with their nucleosides, may be related to nucleic acid biosynthesis. Presence of thymine, a component of DNA, is indicated in Figure 3. Although quantitation was not possible, the peak was identified by paper chromatography. The bases in largest amount are those also associated with enzymatic cofactors and phosphate transfer. Only adenine occurs in endosperm in appreciable amounts. This base is a component of a nucleotide involved in starch biosynthesis (*17*). Results of a systematic study of nucleotides in corn fractions and cellular particles will be reported later.

Since marked differences in the total amino acid composition of the germ and endosperm proteins of corn occur (*21*), it might be anticipated that these differences are reflected in the free amino acid compositions of the two tissues. The endosperm proteins are richer in glutamic acid and leucine and poorer in the basic amino acids than the germ proteins. However the proportions of the major free amino acids of both germ and endosperm are similar. Asparagine and alanine, amino acids of greater metabolic activity, predominate in both tissues. Proline and γ-aminobutyric acid (which is derived by enzymatic transformation from glutamic acid) are also present in large amounts in both tissues. But leucine, which is a major constituent of zein, is present in relatively low quantities in the germ. The basic amino acids are also slightly higher in content in the endosperm, although the germ proteins are richer in these components. It must be concluded that residual free amino acids in field-dried mature corn do not reflect the amino acid composition of proteins in the same cells.

These results and the analytical procedures employed should be of value for studies on the effect of drying, storage, and processing conditions upon the amounts and quantities of nonprotein nitrogenous constituents and their relationship to seed viability and nutritional and milling quality.

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