Identification and Determination of Nonprotein Nitrogenous Substances in Corn Steep Liquor

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The identity and quantities of nonprotein nitrogenous substances, including amino acids, quaternary nitrogen compounds, and heterocyclic nitrogen compounds were determined in corn steep liquor because of their nutritional importance in animal feeds and in supplements for fermentation media. After extraction from the liquor with 2% trichloroacetic acid or 80% aqueous ethanol, these substances were separated on columns of cation exchange resins with buffers of increasing pH and quantitatively determined by specific spectrophotometric procedures. Of the total nitrogen in steep liquor 90% was extractable as nonprotein nitrogen: one half in free amino acids and ammonia. The four major free amino acids in the liquor are alanine, leucine, proline, and γ-aminobutyric acid. Choline and trigonelline are the primary quaternary nitrogen compounds. Major purine and pyrimidine derivatives are adenine, xanthine, cytidine, and guanosine. Steep liquor contains a much higher level of free nitrogenous constituents than the corn from which it was derived. Amino acid content varied among three batches of steep liquors from a single manufacturer.

Corn steep liquor, a by-product of the wet milling of corn for starch, is extensively used as a component of animal feeds and of media for the culture of microorganisms in industrial fermentations. It provides a rich source of nutrients, vitamins, and minerals, is especially high in nitrogenous constituents, and serves as a source of unidentified growth factors for poultry (*16*) and microorganisms (*12*). Steep liquors not only contain low-molecular-weight substances which are leached out of the corn grain, but also large amounts of substances derived from the degradation and fermentative conversion of proteins, carbohydrates, and nucleic acids during steeping. The identification and quantitative determination of the major nitrogenous compounds of corn steep liquors should provide more information concerning the nutritional value of the liquor and the nature of the processes giving rise to the liquor components during steeping.

Steeping is essentially a process of soaking grain in very dilute sulfurous acid to soften it for subsequent grinding and to facilitate starch liberation. It is accompanied by a bacterial fermentation. Compositional differences in the products available commercially result from variations in the process among different manufacturers and even among different batches from the same plant. Reviews by Bartling (*3*) and Liggett and Koffler (*13*) give a more detailed description of the commercial production and properties of steep liquors.

By means of amino nitrogen analyses Cardinal and Hedrick (*7*) demonstrated that free amino acids and peptides account for a large proportion of corn steep liquor nitrogen. Cardinal and Hedrick (*7*) and Aurich (*2*) used microbiological assays to determine several of the major amino acids and vitamins in the steep liquors. Zelinca and Hudek (*22*) identified γ-aminobutyric acid in steep liquor.

Since no systematic studies seeking to characterize and determine the major nitrogenous substances in this material have been reported, these materials were determined by ion exchange chromatography. The amounts of nitrogenous substances in steep liquors, as determined in the present study, and in whole corn extracts, reported previously (*9*), are compared in order to demonstrate the extent of polymer degradation and amino acid transformation that occurs during steeping. Three steep liquor samples from the same manufacturer were analyzed for total amino acids content to assess variation among batches.

Materials and Methods

Preparation of Samples for Analysis. Concentrated steep liquor sample 1 from a batch prepared August 10, 1961, and samples 2 and 3 from different batches prepared February 19, 1963, having 51, 51, and 54% dry solids, respectively, were obtained from a major industrial corn wet miller and stored at −10° C.

Extracts of steep liquor sample 1 prepared with two different protein precipitants, 2% aqueous trichloroacetic acid (TCA) and 80% ethanol in water, were analyzed to compare the effectiveness of these solvents in extracting nonprotein nitrogen (NPN). Sample 1 containing 20.4 grams of dry steep liquor solids was mixed with 30 ml. of 3.44% trichloroacetic acid so as to obtain a 2% trichloroacetic acid slurry. The mixture was stirred for 10 minutes and centrifuged at 4° C. No further precipitation was obtained by increasing the concentration of the trichloroacetic acid in the slurry. TCA was removed from the liquor solution by extracting twice with two volumes of diethyl ether. The 80% ethanol extraction using 3 grams of dry steep solids per liter of solvent was accomplished by the methods used in preparing whole corn extracts (*9*).

The final aqueous extracts were made to volumes equivalent to 0.5 gram of original liquor solids per ml. Aliquots of the aqueous extracts were hydrolyzed in 6*N* hydrochloric acid at a total volume of 200 ml. under reflux for 24 hours to permit determination of amino acids contained in peptides. For use in determining amino acids in steep liquors equivalent amounts of the intact liquor were hydrolyzed also under the same conditions. The hydrolyzates were evaporated to dryness to remove acid and made to volume with water, so that the solutions were equivalent to 0.5 gram of original steep liquor solids per ml. Total nitrogen was determined on these extracts and hydrolyzates by the semi-micro-Kjeldahl method.

Since the concentration and analytical sensitivity varied for each group of constituents in the liquor, different solutions were prepared to yield samples suitable for application to separate columns for ion exchange chromatographic separation and analysis of the different classes of nitrogenous constituents. These solutions were obtained by diluting the extracts of hydrolyzates to appropriate volume based on their nitrogen content with 0.2*M*, pH 2.2 sodium citrate buffer. Two-milliliter aliquots of these buffered solutions which were applied to the columns contained 0.2 to 0.3 mg. of

Table I. Distribution of Nitrogenous Substances in Corn Steep Liquor Sample 1

	% of Total Nitrogen	
	80% ethanol	2% trichloroacetic acid
Total NPN	87.4	90.7
Free amino acids	37.0	36.7
Free ammonia	5.3	6.9
Peptides	36.2	40.3
Quaternary nitrogen	1.0	1.0
Heterocyclics	2.1	2.1
Unaccounted	5.8	3.7
Insolubles		
Protein	13.1	7.8
Unaccounted	0.5	1.5

Table II. Amino Acids in Steep Liquor Sample 1

	Mg. Nitrogen per 100 Grams Solids				
	Free in Extract		Total in Extract		After hydrolysis, in liquor
Amino Acid	TCA	Ethanol	TCA	Ethanol	
Alanine	345	338	556	576	579
Leucine	290	312	362	352	384
Proline	247	256	465	478	506
Ornithine	221	143	227	136	224
Lysine	205	203	368	253	363
Arginine	185	176	426	446	489
γ-Aminobutyric acid	174	174	177	198	174
Asparagine	167	195	...	...	...
Valine	153	165	298	276	290
Serine	119	132	263	245	263
Glycine	100	115	433	415	433
Phenylalanine	95	86	122	117	129
Isoleucine	78	82	141	149	151
Threonine	76	89	191	196	197
Methionine	68	80	103	110	94
Glutamic acid	45	53	472	486	515
Histidine	75	65	318	207	348
Aspartic acid	12	14	244	226	245
Ethanolamine	6	8	8	7	8
Tyrosine	Trace	Trace	27	38	47
Cystine	Trace	Trace	88	70	125

nitrogen for determination of free, peptidal, or total amino acids; 35 to 40 mg. nitrogen for quaternary nitrogen compounds; and 10 to 15 mg. nitrogen for heterocyclic nitrogen compounds.

Separation and Determination of Extracted or Hydrolyzed Constituents. Generally, amino acids were determined automatically on a Phoenix amino acid analyzer (Model K-8000) following the procedure of Spackman, Stein, and Moore (*18*). The 30° to 50° C. system was used with a 150-cm. column to facilitate separation of the amides, glutamine and asparagine, from serine. The 50-cm. column was also used in separating the basic amino acids in order to resolve the large number of these substances in the steep liquor.

Columns of classified Amberlite IR-120 cation exchange resin were also employed for separation and manual determination of amides and amino acids (*9,15*), quaternary nitrogen compounds (*10*), and aromatic heterocyclic compounds (*9*). The column size, temperature, buffer schedules, and flow rates used were those indicated in the references as giving the most effective resolution of these groups of nitrogen compounds. Quaternary nitrogen compounds were quantitatively analyzed by a modification (*10*) of the procedure of Wall and coworkers (*20*). Purines, pyrimidines, and related substances were detected automatically in the effluent by a Vanguard ultraviolet analyzer scanning at 260 mμ. The fractions containing ultraviolet-absorbing materials were reanalyzed with a Beckman DU at 260 and 280 mμ.

Characterization of Separated Compounds. The amino acids, amides, and amines were identified by position of elution and by the ratio of absorbances of their ninhydrin reaction products at 440 and 570 mμ (*18,21*). Ornithine was also determined by analysis of effluent fractions from manual columns (*14*) by the method of Chinard (*8*). Quaternary nitrogen compounds and heterocyclic nitrogen compounds were characterized by comparing elution positions with known standards. Identities of isolated compounds were further verified by comparing R_f's with those of known substances by paper chromatography, using two or more solvent systems after the pooled, concentrated effluent fractions containing nitrogenous substances were desalted by appropriate techniques (*9,10*). The procedures for paper chromatography have been summarized by Block (*6*). Aromatic nitrogen compounds were also characterized by their ultraviolet spectra. Pentose (*11*) and phosphorus determinations (*1*) were made upon some of the substances.

Results

Nitrogen Distribution. Steep liquor sample 1 was studied in greater detail than the others. It contained 7.3 grams of total nitrogen per 100 grams of solids, distributed into various classes of nitrogenous material as shown in Table I.

The slightly greater yield of extractable nitrogen with 2% TCA than with the 80% ethanol reflects a higher solubility of peptidal and other material in the TCA solution. This is in agreement with Bell (*5*), who has shown that various agents commonly used to precipitate protein give different yields of NPN. Also, a higher content of ammonia in the TCA extract may result from degradation of amide groups of asparagine, proteins, or other substances. Almost half of the extracted nitrogen was present in free amino acids or ammonia, and most of the remaining NPN was in the peptides which could be converted to amino acids by acid hydrolysis (Table I).

Slightly less than 1% of the extracted nitrogen is in quaternary nitrogen compounds and an additional 2% in nucleosides, purines, and pyrimidines. A small fraction of the extracted nitrogen was found in ultraviolet-absorbing compounds not retained on the cation exchange resin. These may be acidic nucleotides or other nucleic acid derivatives, now under study.

Amino acid analysis of the hydrolyzate of the original whole steep liquor indicates that 93% of its nitrogen is accounted for by amino acids or ammonia either free or bound in proteins or peptides. After correcting for the TCA-soluble peptidal material and free amino acids, 8% of the total steep liquor is accounted for as TCA-insoluble protein. This amount accounts for about 85% of the TCA-insoluble nitrogen. Similarly, most of the alcohol-insoluble material is contained in peptide or protein-bound amino acid (Table I).

Amino Acids, Amides, and Amines. Major amino acids occurring free in the extracts or in hydrolyzates of the extracts and in the intact steep liquor sample 1 are listed in Table II. The four major free amino acids in the liquor are alanine, leucine, proline, and γ-aminobutyric acids; these are related to the major amino acids of corn proteins, if γ-aminobutyric acid is considered an enzymatic degradation product of glutamic acid. Concentration of glutamic acid, which is a major component of corn proteins, is low in steep liquor.

Some differences were observed in the relative effectiveness of 80% ethanol and 2% TCA to extract different categories of free amino acids. The results summarized in Table II indicate that 80% ethanol gives slightly higher yields of nonpolar amino acids, such as leucine and valine, and of acidic amino acids, such as glutamic, whereas 2% TCA was more efficient for extracting the basic amino acids, especially ornithine. Use of smaller volumes of these extractants than those designated exaggerated these differences in effectiveness of extraction.

For most of the free amino acids, a proportional increase in amount occurred upon hydrolysis of the extract or whole steep liquor. Exceptions in which

Table III. Free Amino Acids in Whole Corn and in Steep Liquors

Amino Acid	Mg. Nitrogen per 100 Grams Corn: Whole corn (9)	Liquor extract (TCA)
Total free amino acids	15.24	218.00
Ammonia	1.55	34.43
Asparagine	4.28	11.52
Proline	2.90	17.04
Alanine	1.92	23.81
Arginine	0.92	12.77
γ-Aminobutyric acid	0.69	12.01
Glutamic acid	0.45	3.11
Glycine	0.35	6.90
Serine	0.34	8.21
Histidine	0.34	5.18
Aspartic acid	0.29	0.83
Glutamine	0.25	...
Lysine	0.21	14.15
Tyrosine	0.21	...
Valine	0.17	10.56
Ethanolamine	0.11	0.35
Threonine	0.10	5.24
Phenylalanine	0.07	6.56
Isoleucine	0.06	5.38
Leucine	0.06	20.01
Ornithine	0.02	15.25
Methionine	0.02	4.69

Table IV. Variations in Amino Acid Content between Batches of Steep Liquor

Steep Liquor Sample	Mg. Nitrogen per 100 Grams Solids: 1[a]	2[a]	3[a]	Dev. from Av., %
Total nitrogen	7270	7140	7650	±2.7
After hydrolysis	7210	7160	7570	2.3
Alanine	578	481	531	±6.1
Glutamic acid	515	510	486	2.3
Proline	506	459	496	9.9
Arginine	488	865	767	20.5
Glycine	433	375	415	3.3
Leucine	384	366	378	1.7
Lysine	363	355	352	1.2
Histidine	348	418	393	6.6
Valine	290	265	354	11.3
Serine	262	219	231	7.0
Aspartic acid	245	253	251	1.3
Ornithine	224	43	126	47.2
Threonine	197	173	190	5.0
γ-Aminobutyric acid	174	104	128	19.2
Isoleucine	151	135	145	3.9
Phenylalanine	129	125	132	1.3
Cystine	125	128	Trace	66.6
Methionine	94	87	98	4.2
Tyrosine	47	87	55	25.4
Ethanolamine	8	7	10	15.2

[a] Average of two separate determinations.

no increase was noted are asparagine (which is converted to aspartic acid) and ornithine and γ-aminobutyric acid (which are not components of the proteins or peptides). Since ethanolamine content did not increase significantly after hydrolysis, little phospholipid containing this substance is in the extract. There are more glutamic acid, glycine, histidine, and aspartic acid bound in the protein and peptides of corn steep liquor than occur free in the liquor. The detection of high levels of amino acids not present in the proteins and differences in the proportions of free and protein-bound individual amino acids indicate that the free amino acids are not merely products of proteolysis but that some have also been derived from metabolic transformations of other amino acids during steeping.

In Table III the amount of free amino acids in corn grain (*9*) is compared to that in steep liquor extracted from the same weight of corn. A yield of 6.9 grams of steep liquor solids per 100 grams of corn (*13*) is used for the computation. The total free amino acids in liquors is fifteenfold greater than that in an equivalent amount of corn. The greatest increases in amino acids in the steep water relative to that free in corn are in ornithine, leucine, methionine, isoleucine, valine, threonine, and lysine. The first four are in low concentration as free components of the corn extracts. Of the amino acids free in the grain, only asparagine is in sufficient quantity to account for a significant portion of that in the liquor. Glutamine, free in appreciable amounts in whole corn, is absent from steep liquor. The amide peak represents only asparagine. Glutamine is either metabolized by the microorganisms or hydrolyzed by the acid.

Variation of Total Amino Acid Content in Different Steep Liquor Samples. The total amino acid and nitrogen analyses of the three different samples of steep liquor are given in Table IV. Although the total nitrogen content is similar in the three samples, major differences are noted in individual amino acids such as arginine, orithine, γ-aminobutyric acid, cystine, and tyrosine.

Quaternary Nitrogen Compounds. The relative amounts of trigonelline and choline found in the steep liquor and in whole corn are compared in Table V. Trigonelline is present, free, in steep liquor and in corn in similar amounts. Therefore, its presence in steep liquor is primarily due to extraction of the free substance during steeping. Some liquid or other materials containing choline are also present in the liquor, as evidenced by the higher choline content in hydrolyzed liquor.

Heterocyclic Aromatic Nitrogen Compounds. Most of the ultraviolet-absorbing compounds contained in an extract of corn steep liquor have been characterized as nucleosides, purines, pyrimidines, and pyridine derivatives. The amounts of these substances in corn steep liquor as compared to whole corn are tabulated in Table V. The nucleosides (uridine, cytidine, and guanosine) and the purine (adenine) in steep liquor show considerable increases over those in corn. But some compounds in the steep liquor, including trigonelline, uracil, and hypoxanthine, seem to be primarily derived from free substances from the grain.

Table V. Quaternary Nitrogen and Heterocyclic Nitrogen Compounds in Whole Corn and in Steep Liquor Sample 1

Compound	Liquor: Mg. nitrogen/100 grams solids	Liquor: Mg. nitrogen/100 grams corn	Corn, Mg. Nitrogen/100 Grams Corn (9)
Trigonelline	4.1	0.29	0.24
Choline	44.2	3.09	1.91
Choline[a]	65.1	...	...
Uridine	11.8	0.83	0.11
Uracil	3.0	0.21	0.10
Xanthine	33.3	2.30	...
Hypoxanthine	10.5	0.77	0.43
Guanosine	20.4	1.41	0.07
Adenosine	4.8	0.33	0.26
Cytidine	27.4	1.89	...
Adenine	36.6	2.53	0.28
Cytosine	4.8	0.33	...

[a] Hydrolyzed corn steep liquor.

Discussion

The high content of free amino acids in steep liquor indicated by Cardinal and Hedrick (*7*) has been confirmed. The present studies have demonstrated for the first time the occurrence in corn steep liquor of significant quantities of nucleic acid degradation products. A number of compounds isolated by chromatography are still unidentified but may have biological significance. The compounds determined and characterized in the present studies are those responding to one of the three methods of effluent analysis employed and must be present in sufficient amounts to yield a reasonable peak. Important growth factors may go undetected by these procedures.

The greater concentration of low-molecular-weight nitrogenous com-

pounds in corn steep liquors compared with the corn kernel establishes that degradative processes reducing polymeric grain constituents, such as proteins and nucleic acids, to smaller components are active during steeping. Four types of such processes may occur: enzymatic hydrolysis due to activation of enzymes of the grain, hydrolysis due to the acid present, hydrolysis due to enzymes elaborated by the lactobacilli, and disulfide cleavage due to the sulfurous acid.

These studies and other investigations indicate that the sulfurous acid and bacterial action are the prime causes of protein degradation during corn steeping. Russo, Watson, and Heiman (*16*) observed that corn steeped at pH 3 with sulfurous acid but without accompanying lactic acid fermentation yielded almost as much water-extractable nitrogen as when fermentation occurred. This extracted nitrogen must be contained in polypeptides, since low ratios of amino nitrogen to total nitrogen were encountered in unfermented steep liquors by Liggett and Koffler (*13*). Turner and coworkers (*19*) have demonstrated that the sulfurous acid disrupts disulfide bonds in zein, thereby solubilizing the zein. The finding of asparagine accompanied by little aspartic acid indicates that the elevated temperature and acid are not highly effective in cleaving amide bonds; the amide bond of asparagine is more acid-labile than peptide bonds. Enzymic hydrolysis by the bacteria must be responsible for the extensive degradation of the solubilized proteins, as evidenced by the requirement of active fermentation to produce steep liquors of high amino nitrogen content (*13*).

The extraction of free low-molecular-weight materials from the entire grain during steeping is highly effective. For instance, trigonelline, an alkaloid, is contained in the steep liquor in amounts equivalent to that in direct extracts of the grain. Since this substance is probably not elaborated during steeping, it must be concluded that it is extracted quantitatively during steeping. Trigonelline is concentrated primarily in the germ rather than in the endosperm (*9*). Thus steeping is capable of removing substances from the germ as well as the endosperm, although the germ is not disrupted during wet milling as is the endosperm.

Metabolic transformation of low-molecular-weight compounds also occurs during steeping. A striking example is the formation of the amino acid, γ-aminobutyric, which is present in corn grain in low levels, whereas it is found free in steep water in high amounts. The γ-aminobutyric acid is present exclusively as the free material because its concentration is not increased in hydrolyzates of the steep liquor or its extract. In contrast, the concentration of free glutamic acid is negligible in the steep liquor, although this amino acid is in high concentration in endosperm proteins and in steep liquor proteins. It must be concluded that during steeping free glutamic acid is enzymatically decarboxylated to γ-aminobutyric acid. Since both corn (*4*) and certain microorganisms (*17*) have enzymes capable of catalyzing this process, it is not possible to ascribe the cause of this reaction definitively. Similarly, xanthine is not found in corn extracts but is present in steep liquor in high levels. Evidently guanine and adenine from nucleic acid are converted by enzymatic processes to xanthine during steeping.

Table IV shows that some variations in total amino acids occur in different preparations of steep liquor from the same manufacturer. Differences are most marked in the basic amino acids, especially ornithine and arginine. Analysis of the hydrolyzed samples of the three liquors reveals that the decreases in arginine content are accompanied by increases in ornithine concentration. Possibly ornithine is derived from chemical or biological alteration of the arginine, which varies in steep liquor batches.

The analytical methods developed in this report may serve to analyze selected batches of the product that result in marked differences in fermentation response. A correlation may be found between the concentration of certain constituents and the effectiveness of steep liquor in certain uses. The data presented emphasize that steep liquor is a rich source of building blocks for cellular growth derived from a complex extraction and degradation of corn components. The analysis should prove useful in explaining some of the biological activity of the material and should stimulate efforts at better control of the commercial steeping process.

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