Nutritionally Unavailable Niacin in Corn. Isolation and Biological Activity

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A substance containing bound niacin was extracted with 50% ethanol-water from commercial corn gluten, a source high in the vitamin. Rat feeding tests established that the niacin in this substance, like that of whole corn, does not contribute to the animal's vitamin requirement unless it is hydrolyzed with dilute alkali. The substance was separated from extracted proteins through its insolubility in water and its nonadsorption on a cation exchange resin. The isolate contains carbohydrate and nitrogenous compounds, as well as niacin. Gel filtration chromatography or countercurrent distribution did not alter its composition significantly. Its composition indicates a complex structure.

Niacin deficiency symptoms in animals and man have been associated for a long time with diets containing a high proportion of corn, although analysis of hydrolyzed corn samples established the presence of moderate amounts of the vitamin in the grain. Woolley (1946) suggested that corn contains a niacin antagonist. Kodicek (1940) attributed the inability of corn niacin to support animal growth to its being bound in a chemical linkage to substances not disrupted during assimilation. Kodicek *et al.* (1956) reported that animals could use this bound niacin after treatment of cornmeal with dilute alkali. Pearson *et al.* (1957) and Harper *et al.* (1958) have confirmed this observation.

To elucidate the nature of the nutritionally unavailable niacin-containing substance in corn, appreciable quantities had to be isolated. This bound niacin-containing material is highly concentrated in commercially prepared corn gluten, the protein-rich, water-insoluble fraction separated from endosperm during wet milling. Corn gluten is used extensively as a component of mixed feeds. The isolation, purification, properties, and nutritional behavior of the niacin-containing substance from gluten are reported.

MATERIALS AND METHODS

Corn and Milled Corn Fractions. The corn grain examined was a composite of commercial varieties of No. 1 grade yellow dent that had been stored under refrigeration. Dry-milled products from corn were either milled in this laboratory or commercially by the J. R. Short Milling Co., Mount Vernon, Ind. Wet-milled fractions were obtained from the Corn Products Co., Pekin, Ill. The corn gluten was taken directly from the commercial separators and dried in small quantities in a forced air oven at 40° C.

The dry- and wet-milled samples were ground in a hammer mill to pass a 0.027-sq. inch mesh screen after an initial coarser grind to minimize heating. Oil was extracted from germ samples before grinding.

Analytical Methods. Total niacin was determined after hydrolysis with either acid (Sweeney and Parrish, 1950) or base (Pelletier and Campbell, 1959) by measuring color produced by reaction with cyanogen bromide and sulfanilic acid. Alkaline hydrolysis was preferred when analyzing gluten and other protein-rich materials. Free niacin was determined in unhydrolyzed samples after ammonium sulfate precipitation of the bound niacin, as bound niacin also produces some color with cyanogen bromide (Sarkar *et al.*, 1962). Total niacin and free niacin were also determined microbiologically (Snell and Wright, 1941) with *Lactobacillus arabinosis* NRRL 531 as a check on the chemical method. Countercurrent samples were also assayed microbiologically for total niacin content. For total niacin, samples were hydrolyzed by autoclaving 15 minutes in 1*N* HCl. Acid was removed in a heated vacuum desiccator. All samples were adjusted to pH 7 before assay. For free niacin analysis, a nonsterile technique was used because sterilization of the material to be assayed causes some breakdown of the bound niacin to give free niacin. Microbiological assays and chemical analyses were in good agreement.

Nitrogen content was determined by the semimicro-Kjeldahl method. Microgram levels of nitrogen were analyzed colorimetrically after nesslerization of samples digested in 2*N* H_2SO_4 with $CuSeO_3$ as the catalyst. Amino nitrogen was measured by the colorimetric ninhydrin method of Yemm and Cocking (1955). Total carbohydrate was determined as glucose by the phenolsulfuric method of Dubois *et al.* (1956).

Feeding Tests. Groups of five weanling male rats of the Wistar strain were fed test diets for about 5 weeks. Growth was measured by daily weighings. The basal diet was based on one described by Pearson *et al.* (1957).

Growth of rats on control diets depended upon the casein content of the diet, since tryptophan can be converted to niacin by the rat. Carpenter *et al.* (1960) and Harper *et al.* (1958) demonstrated that diets containing only 3.5% casein retarded growth more in the absence of niacin and gave a better response to that vitamin. However, with the 9% casein diet used in these experiments, niacin deficiency could be demonstrated effectively.

EXPERIMENTAL

Distribution of Niacin in Corn-Milled Fractions. To test their suitability as sources of bound vitamin, dry- and

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wet-milled corn fractions were assayed chemically for niacin after alkaline hydrolysis. Their total niacin content is summarized in Table I. Corn gluten had the highest concentration of total niacin in the fractions tested, 111 μg. per gram. The major portion of this niacin was bound in a water-insoluble form. The properties of this bound niacin were similar to those of the major niacin-containing substance in corn (Christianson *et al.*, 1960). Most of the steep-liquor niacin was soluble in water in contrast to the niacin in corn gluten or whole corn. Pearson *et al.* (1960) reported on the extraction of bound niacin from corn steep liquors.

Extraction of Niacin-Containing Substances from Corn Gluten. Ethanol-water solutions were the most effective for extracting niacin-containing material from ground corn gluten. The most satisfactory extractant was 50% ethyl alcohol. Extraction was performed by stirring 10 grams of gluten in 100 ml. of ethanol solution for 20 minutes at 4° C. The lower temperature was selected because it minimized the solution of nitrogenous impurities, primarily zein, without reducing niacin yields (Table II). Extractions of 1-kg. quantities of gluten were conducted in 10 liters of 50% ethanol at 4° C. for 20 minutes with 60% yields.

Recovery of Niacin from Alcoholic Extracts. One-liter portions of the alcoholic extract were placed in 6-foot lengths of Visking tubing (75/32) and then dialyzed against 10 liters of water at 4° C. for 24 hours. The water was changed at 4-hour intervals. The isolate was recovered by lyophilizing the total contents of the dialysis bags. One kilogram of gluten yielded 12.9 grams of amorphous yellow powder containing 0.2% niacin and 6.9% nitrogen.

Diet Tests on Corn, Gluten, and Isolate from Gluten. To demonstrate that the niacin of corn, corn gluten, and the water-insoluble fraction of the alcohol extract of corn gluten was unavailable because of its chemical linkage, rat feeding experiments were conducted upon both the original and hydrolyzed materials.

Alkali-treated corn was prepared by stirring 1 kg. of cornmeal in 1 liter of 0.1*N* NaOH for 90 minutes. Lesser quantities of corn gluten and corn guten isolate were similarly treated in proportional volumes of this alkaline solution. Additional alkali was added when necessary to ensure that the pH of solution was not below 12. After this treatment, the slurry was adjusted to pH 7 with hydrochloric acid, and the total product was lyophilized to dryness. Care was taken to retain all solubles which contained the bulk of free niacin shown by McDaniel and Hundley (1958).

To ensure that the response in the diet tests was not an artifact due solely to alkaline treatment, the bound niacin was also hydrolyzed by autoclaving 1 kg. of corn in 1 liter of 1*N* sulfuric acid at 120° C. for 30 minutes. The pH was adjusted to 1 and the niacin recovered by extracting with ether for 48 hours in a continuous liquid-liquid extractor as described by Frankenburg *et al.* (1953).

The nutritional availability of the niacin in these various preparations was determined by substituting them for all or part of the corn in the rat diets. Table III summarizes the composition of the test diets and their chemical analysis for total and free niacin. Bound niacin is the difference between total and free niacin. In other diets, synthetic niacin or niacin extracted from hydrolyzed corn was added to ascertain the growth response to known amounts of niacin.

Table I. Total Niacin Content of Dry- and Wet-Milled Products of Corn Grain

Material	Total Niacin Content, μg./G. Sample
Whole corn	22
Dry-milled fractions	
Germ	43
Hominy meal	29
Hull	55
Corn bran	30
Wet-milled fractions	
Dry germ	23
Short germ	54
Gluten feed	33
Gluten meal	47
Gluten (direct from separators)	111
Corn steep liquors	105

Table II. Effect of Variables on Extraction of Niacin and Nitrogen from Corn Gluten

G. Gluten per 100 Ml. Extractant	Ethanol in Extractant, %	Temp., °C.	μg. Niacin per G. Gluten Extracted	Mg. Nitrogen per G. Gluten Extracted	μg. Niacin per Mg. Nitrogen
4	70	4	61	5.4	11.6
4	50	4	66	3.5	18.7
4	20	4	44	1.7	26.2
10	50	4	62	2.7	23.0
10	50	25	68	17.7	3.8

Experiment 1, Table III, confirms observations that the alkaline treatment of whole cornmeal increases available niacin in the test diet to levels that increase growth. The increased growth is a function of the liberated free niacin as indicated by comparison with the growth of animals fed diets supplemented with synthetic or corn-derived niacin. In Experiment 2, the addition of 7% corn gluten to cornmeal did not improve growth rate of the rats. Alkaline treatment of the added corn gluten accelerated growth to almost that attained upon addition of a comparable amount of niacin. Incomplete chemical hydrolysis of the bound niacin during preparation of this diet accounts for the slightly lower weight gain. Similarly, growth was not enhanced by addition of the niacin-containing material isolated from gluten by extraction with ethanol (Experiment 3). The addition of a small amount of the alkaline-treated isolate gave a growth response proportional to the niacin released by the alkali.

These results establish that the bound niacin of corn gluten cannot be used by the rat and that extraction and isolation procedures do not alter its nutritional unavailability.

Purification of Bound Niacin Isolates. The water-insoluble niacin-containing material from alcoholic extracts of gluten had a high nitrogen content (6.9%).

Table III. Growth Responses of Rats on Diets Containing Alkali-Treated and Niacin-Supplemented Corn, Corn Gluten, and Isolate from Corn Gluten[a]

Expt.[b]	Test Material	Niacin Content, μg./G. Bound	Niacin Content, μg./G. Free	Weight Increase, G. Initial	Weight Increase, G. Final	Weight Increase, G. Gain
1	Cornmeal 40%	18.3	3.0	40.8	97.0	56.2
	Alkali-treated cornmeal	5.3	16.3	40.4	141.2	100.8
	Cornmeal + 11.3 μg. niacin per gram of meal	18.3	14.3	40.8	123.4	82.6
	Cornmeal + 11.3 μg. niacin extracted from acid-treated cornmeal	18.3	14.3	40.4	120.6	80.2
2	Cornmeal 40%	18.3	3.0	45.8	130.8	85.0
	33% cornmeal + 7% corn gluten	36.5	2.5	45.8	134.8	89.0
	33% cornmeal + 7% alkali-treated corn gluten	27.5	11.5	45.8	171.0	125.2
	33% cornmeal + 7% corn gluten + 12.3 μg. niacin per gram of meal	36.5	15.3	46.2	187.2	141.0
3	Cornmeal 40%	18.3	3.0	40.2	103.2	63.0
	Cornmeal + gluten isolates, 3.6 mg./kg.	36.8	3.0	40.2	96.6	56.4
	Cornmeal + gluten isolates alkali hydrolyzed, 3.9 mg./kg.	18.7	21.5	40.2	145.0	104.8
	Cornmeal + 18.3 μg. niacin per gram of meal	18.3	21.3	40.2	149.3	100.1

[a] Basal diet, in per cent: sucrose 40.8, corn oil 5.0, vitamin-free casein 9.0, niacin-free vitamin mix 0.8, choline chloride 0.1, DL-cystine 0.4, HMW salts 4.0. Sodium phosphate, NaH_2PO_4, was added at 1% level to reduce urolithiasis (Knoebel, 1959). The niacin-free vitamin mixture (in sucrose) contributed the following vitamins in mg. per 100 g. of diet: thiamine·HCl, 0.2; riboflavin, 0.3; pyridoxine, 0.25; calcium pantothenate, 2.0; inositol, 10.0; biotin, 0.01; and folic acid, 0.02. Vitamins A, D, E, and K were given in oil weekly by dropper. The salt mixture was purchased from a commercial source and contained the formulation as described by Hubbell *et al.*, 1937.
[b] Experiments 1 and 3, 35 days; Experiment 2, 42 days.

Kodicek (1960) has stated that bound niacin is precipitated upon removal of alcohol from the extracts of gluten due to occlusion with the water-insoluble zein. Removal of this protein was therefore desirable. Zein has been adsorbed on columns of fine mesh Amerlite IRC-50 resin, a weak cation exchanger, from aqueous alcohol solutions by Craine *et al.* (1961). The ion exchanger, IRC-50 in the carboxyl form, was equilibrated with 50% ethanol. Alcoholic extracts of gluten containing bound niacin isolate were reduced to one sixth the original volume by vacuum distillation (bath temperature less than 40° C.). The concentrated extracts were reconstituted to 50% with ethanol and applied to a column, 3.2 × 30 cm. The column was washed with three bed volumes of 50% ethanol at a flow rate of 24 ml. per hour. The wash solution contained 96% of the originally extracted niacin and 45% of the extractable nitrogenous materials (Figure 1). Under these conditions, maximum nitrogenous material was removed from the extract.

Upon removal of the alcohol from the IRC-50 wash by vacuum distillation, 65% of the niacin isolate could be collected by centrifugation. Since most of the nitrogen in the extract was water-soluble, it could be eliminated by decanting the supernate (Figure 1). The bound niacin was collected in a Beckman Spinco ultracentrifuge, Model L, Rotor No. 21, run at 2000 r.p.m. for 1 hour at 4° C. The final precipitate was lyophilized and stored at 0° C. This product contained 0.6% niacin, 2.7% nitrogen, and 35.9% hexose. The yield was 600 mg. per 100 grams of gluten.

Criteria for Homogeneity of Bound Niacin. Attempts were made to dissolve or distribute bound niacin isolate in various solvents to determine whether or not the isolate might be further purified or fractionated. Weighed quantities were mixed with selected volumes of a single-phase or two-phase solvent system. Concentrations of niacin and hexose were determined in each phase. Table IV summarizes these data. The bound niacin was essentially insoluble in absolute ethanol, chloroform, and hexane. In the Sevag system, the bound niacin precipitated at the interphase. The precipitate contained percentages of total niacin and hexose similar to that of the original isolate. Bound niacin was distributed between the

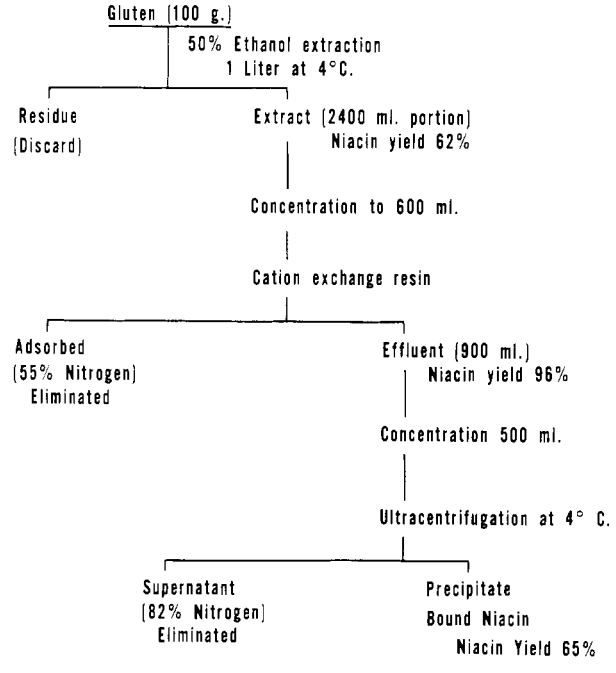

Figure 1. Scheme for the isolation of bound niacin from corn gluten and purification of the isolate with respect to nitrogen

Niacin yields are based on amounts from preceding step. See text for experimental details

Table IV. Distribution of Corn Gluten Isolate Components

Conditions	Isolate Weight, Mg.	Solvent Volume, Ml.	Total in Solutions, % Niacin	Total in Solutions, % Carbohydrate
Solvent				
Absolute ethanol	108	20	9.6	5.0
Chloroform	107	40	5.0	4.0
Hexane	120	40	5.0	1.0
Sevag distribution				
Chloroform-butanol-ethanol-water (4:2:1:2)	207			
Upper phase		50	5.2	10.5
Middle phase		...	72.0	62.0
Lower phase		50	27.0	28.2
Two-phase systems				
Hexane-ethanol-water (1:2:2)	217			
Upper phase		50	0	0
Lower phase		40	98.3	98.6
Chloroform-ethanol-water (1:2:2)	160			
Upper phase		100	39.7	...
Lower phase		50	60.2	...
Chloroform-ethanol-water (1:2:2), pH 3	160			
Upper phase		100	60.6	60.0
Lower phase		50	39.4	37.6

two phases of the chloroform-ethanol-water system. Acidification of this system altered the distribution coefficient to favor the more polar phase. The acidified two-phase system was suitable for countercurrent distribution studies since it gave rapid, sharp, phase separations.

A 200-tube Craig, all-glass, countercurrent distribution apparatus was used. Thirty liters of chloroform, ethanol, and water mixture (1:2:2) acidified to 0.004*N* with HCl were equilibrated for 18 hours. To each tube were added 40 ml. of the lower phase. About 400 mg. of isolate, as obtained by the scheme in Figure 1, were dissolved in 40 ml. of the upper phase which was placed in the 0 tube. The isolate was distributed through 200 tubes with the addition of fresh upper phase to the 0 tube after each transfer. The run was completed in 23 hours. The two-phase system was rinsed from each tube and made completely miscible by addition of 15 ml. of absolute ethanol. Total niacin, carbohydrate, and nitrogen were run on aliquots of these fractions as given in Figure 2. The ratio of niacin to carbohydrate and nitrogen varied only slightly during the distribution. Ultraviolet absorption spectra of certain fractions were also compared. The concentration of a substance having a maximum absorption at 310 mμ decreased with progressive distribution. A bathochromic shift to 370 mμ at alkaline pH also characterized this component. Its distribution indicates that it was resolved from the major portion of the isolate and was retained in the first few tubes because of its greater solubility in the more nonpolar phase.

The bound niacin isolate was also fractionated by gel filtration to establish homogeneity in molecular size. Sephadex G-50 (medium) was equilibrated with 50% ethanol by stirring for 18 hours. A column, 1.8 × 35 cm., was packed under a flow rate of 18 ml. per hour. The purified isolate (30 mg.) was dissolved in 2 ml. of 50% ethanol at a flow rate of 36 ml. per hour. The absorbance of each 3-ml. fraction was read at 260 mμ. Two ultraviolet-absorbing compounds were resolved. Fractions were

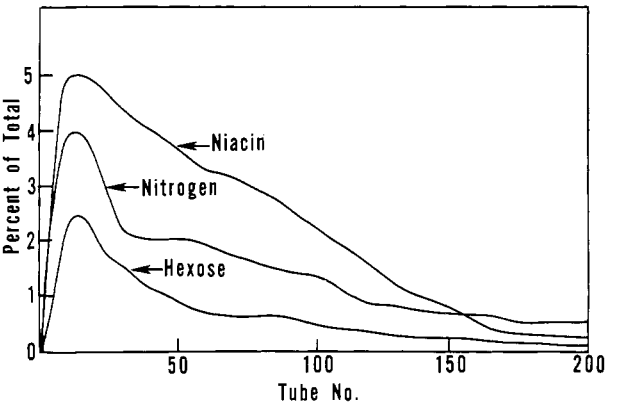

Figure 2. Countercurrent distribution of bound niacin in a 200-tube Craig apparatus

Individual tubes were analyzed for total niacin, nitrogen, and hexose according to methods described in text

combined according to the eluted peaks, concentrated, and analyzed for total hydrolyzable niacin, nitrogen, and hexose. Ninhydrin equivalents of leucine were also determined on a 6*N* HCl-hydrolyzed aliquot of each combined fraction to locate the ninhydrin-positive moiety (Table V). The largest ultraviolet-absorbing component (Fraction 26-50) accounted for the major portion of the total niacin, nitrogen, leucine equivalents, and hexose. The second peak (53-80) contained 20% by weight of the isolate but no

Table V. Separation of Bound Niacin on Sephadex: Analysis of Effluent Fractions

Combined Fractions	Per Cent of Total Weight	Niacin	Nitrogen	Hexose	Leucine equivalents
26–50	80	98	94	95	99
53–80	20	0	6	5	0

niacin. Its properties were identical to those of the substance partially separated by countercurrent distribution.

Countercurrent distribution and gel filtration gave no evidence for the dissociation of niacin from the carbohydrate and the major portion of the nitrogenous substances, including the ninhydrin-positive constituent.

DISCUSSION

Data presented confirm earlier evidence that the major fraction of niacin in whole corn is nutritionally unavailable to the rat before alkaline hydrolysis. The total niacin in corn gluten also remains unavailable after wet milling of the grain. Bound niacin in corn gluten is probably identical to that in mature corn. However, industrial steeping at 60° C. at pH 4 in sulfurous acid solution for 48 hours alters certain properties of corn proteins (Turner *et al.*, 1965) and, possibly, the total physical structure of bound niacin. Under the mild tempering conditions of dry milling, the major quantity of niacin in these fractions would also remain unavailable. These products, as well as gluten, find an extensive outlet in animal feeds.

The alkaline treatment used in these diet studies possibly could destroy certain factors in whole corn or gluten that inhibit utilization of niacin. However, there is a close correlation between the chemically determined release of bound niacin to the free vitamin and the biological activity of the preparations. Furthermore, in studies in which the hydrolysis is limited to material isolated from corn or gluten, the possibility of changes in niacin antagonists or proteins is eliminated.

The bulk of niacin in corn grain is concentrated in the aleurone layer (Heathcote *et al.*, 1952). It would not be expected that zein, the major protein of gluten, and bound niacin are associated in the cell because of their differences in morphological distribution. Finding that alcohol-extracted bound niacin from gluten can be prepared fairly free of zein established that these materials are not chemically combined. Kodicek *et al.* (1956) also reported that certain purified preparations of commercial zein did not contain bound niacin.

The bound niacin in gluten appears to be a complex substance composed of 35% carbohydrate, primarily glucose; 2% peptide on the basis of leucine ninhydrin equivalents; 0.6% niacin; and the rest aromatic or heterocyclic nitrogen compounds. Several different approaches were investigated for fractionating bound niacin from contaminants in the isolate by physical means. Although a portion of the ultraviolet-absorbing material was dissociated from the bound niacin, the complex isolate was homogeneous with respect to niacin, ninhydrin-positive substances, and carbohydrate.

The composition of the isolate from corn gluten is similar to that reported for wheat bran by Das and Guha (1960) and by Kodicek and Wilson (1960). However, the larger amounts of nitrogenous materials in this corn gluten appear to influence the solubility of the product since isolates from wheat bran are water-soluble. Bound niacin can easily be isolated from corn gluten in good yields for further characterization. Linkage of niacin to this complex material is being studied.

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