[CONTRIBUTION FROM THE OFFICE OF PLANT PHYSIOLOGICAL AND FERMENTATION INVESTIGATIONS, WITH THE COÖPERATION OF THE OFFICE OF CEREAL INVESTIGATIONS, BUREAU OF PLANT INDUSTRY, UNITED STATES DEPARTMENT OF AGRICULTURE]

THE OCCURRENCE OF POLYPEPTIDES AND FREE AMINO ACIDS IN THE UNGERMINATED WHEAT KERNEL

BY S. L. JODIDI AND K. S. MARKLEY

Received April 23, 1923

Introduction

While we know, from the work of Osborne[1] and his associates, that the wheat kernel contains 5 proteins, we are altogether inadequately informed, to say the least, in regard to the nitrogenous constituents other than the proteins. According to Schulze[2] the non-protein nitrogenous compounds present in the seed of wheat make up 0.240% calculated on the basis of the dry seed, or 11.2% calculated on the basis of its total nitrogen.

Frankfurt[3] is doubtful with regard to the occurrence of amino acids in the wheat embryo, since his attempts to show their presence in alcoholic extracts of the embryo have led to negative results.

Richardson and Crampton[4] reported the presence of allantoin—a purine derivative—in the wheat embryo, while Frankfurt[3] showed the occurrence of the bases betaine and choline.

These results were confirmed by the work of Schulze,[5] who also reported the presence of asparagine in the wheat embryo, stating, however, that the quantity of those nitrogenous compounds is quite insignificant when related to the weight of the seed. Later Schulze[6] and Castoro reported the occurrence of arginine also, in the embryo, adding that its quantity is very small. In more recent work by Grindley[7] and his associates as well as by Nollau[8] very complete estimations of the amino acids in wheat, wheat bran and gluten were reported. Their data, however, refer to *hydrolyzed* wheat and its by-products.

No mention is made in the literature of the occurrence of polypeptides in the wheat kernel, as far as we are aware, despite the fact that they were discovered and described by Fischer[9] some 20 years ago. However, from the results reported in the experimental part of this paper it will be seen that the 4 wheat varieties investigated showed the presence of considerable quantities of free amino acids and polypeptides in the ungerminated wheat kernel.

[1] Osborne, "The Proteins of the Wheat Kernel," *Carnegie Inst. Pub.*, **1907**.

[2] Schulze, *Z. physiol. Chem.*, **41**, 455 (1904).

[3] Frankfurt, *Landw. Vers.-Sta.*, **47**, 456 (1896).

[4] Richardson and Crampton, *Ber.*, **19**, 1181 (1886).

[5] Schulze, *Chem.-Ztg.*, **18**, 799 (1894).

[6] Ref. 2, p. 467.

[7] Grindley, THIS JOURNAL, **37**, 2762 (1915); **45**, 815 (1923).

[8] Nollau, *J. Biol. Chem.*, **21**, 611 (1915).

[9] Fischer, "Untersuch. über Aminosäuren, Polypeptide und Proteine," J. Springer, Berlin, **1906**.

Experimental Part

For our work we have selected the wheat varieties Fultz, Marquis, Kubanka, and Kanred, because of their commercial importance. Fultz[10] represents a soft red winter wheat which is widely raised in the eastern half of the United States. Marquis,[10] introduced into this country from Canada in 1913, has become the leading variety of hard red spring wheat. Kubanka[10] is a widely grown commercial variety of durum wheat, usually outyielding the hard red spring wheats in the northern Great Plains because of its greater resistance to drought and rust. Kanred,[11] a pure-line selection from a wheat introduced into the United States from Russia in 1900, is a hard red winter variety extensively grown in the central Great Plains area.

For the experimental work the wheat samples were ordinarily ground in an electric buhr-mill to pass a 40-mesh sieve. Inasmuch as the results in this paper are largely based on the total and the protein nitrogen of the different wheat varieties a number of estimations, including ammoniacal nitrogen, were made. The average of these was taken as a basis for the calculations. It may be mentioned here that the *protein nitrogen* was estimated according to Stutzer's method outlined elsewhere,[12] while the ammoniacal nitrogen was determined in a vacuum according to Grafe's[13] method.

The results obtained are presented in Table I.

TABLE I

PROPORTION OF TOTAL, PROTEIN AND AMMONIACAL NITROGEN IN THE UNGERMINATED WHEAT KERNEL

Variety of wheat	Total nitrogen Oven-dried wheat %	Protein nitrogen Oven-dried wheat %	Protein nitrogen Total nitrogen %	Ammoniacal nitrogen Oven-dried wheat %	Ammoniacal nitrogen Total nitrogen %	
Fultz[a] (C. I. 3598)	1.80	1.64	91.76	0.0026	0.142	
	±0.03	±0.003	±0.22	±.0004	±.023	Av. dev.
Kanred[b] (C. I. 5146)	2.83	2.47	87.01	.....	....	
	±0.01	±0.04	±1.31	.....	....	Av. dev.
Kubanka[c] (C. I.)	3.03	2.73	86.73	.0033	.109	
	±0.03	±0.02	±0.76	±.0003	±.01	Av. dev.
Marquis[d] (C. I. 3641)	3.04	2.65	87.26	.0033	.109	
	±0.03	±0.04	±1.29	±.0004	±.012	Av. dev.

[a] From the Arlington Farm, Roslyn, Va.; 1920.
[b] From the Hays Branch Expt. Sta., Hays, Kansas; 1922.
[c] 1920.
[d] From the Dickinson Sub-Station, Dickinson, N. D.; 1920.

[10] Clark, Martin and Ball, *U. S. Dept. Agr. Bull.*, **1074** (1922); *U. S. Dept. Agr. Farmers' Bull.*, **1280, 1281, 1301** (1922); **1304** (1923).

[11] *U. S. Dept. Agr. Circ.*, **194** (1921).

[12] Jodidi, Moulton and Markley, THIS JOURNAL, **42**, 1063 (1920).

[13] Grafe, *Z. physiol. Chem.*, **48**, 300 (1906).

In looking over Table I, in which the average is based in each case on at least three determinations, it will be noticed that the differences in the total nitrogen of the several varieties are rather marked and characteristic, the nitrogen percentage of Fultz being lowest (1.80), that of Kanred considerably higher (2.83), and that of Kubanka and Marquis higher than either of the others (3.03 and 3.04, respectively).

As to the protein nitrogen, the differences between the varieties when calculated to the oven-dried wheat follow about the order indicated in the case of the total nitrogen. However, when the protein nitrogen is referred to the total nitrogen we find that the differences between Kanred (87.01), Kubanka (86.73), and Marquis (87.26), are comparatively small, only Fultz showing a higher percentage (91.76).

With regard to the proportion of the ammoniacal nitrogen, which was estimated because it is formol-titrable along with the amino acids, it will be seen that the differences between the varieties are small, the total proportion of ammoniacal nitrogen for the three varieties examined being generally quite insignificant.

After a few preliminary experiments the methods finally adopted, in order to demonstrate the presence of amino acids in the wheat seed, were as follows.

Several flasks each containing 25 g. of "whole wheat" flour and 400 cc. of distilled water were shaken at room temperature for 2 hours and the contents filtered at once. The residue on the filter contained the undissolved substance, the bran, starch, etc. The clear filtrate, which was apparently free from starch, was concentrated on the water-bath. During evaporation, precipitates formed that displayed the xanthoprotein and biuret reactions and otherwise appeared to consist chiefly of proteins. These precipitates were separated by centrifugation. The supernatant liquid was evaporated on the water-bath to dryness and the residue extracted with 70% alcohol in order to remove the rest of protein[14] matter, inorganic salts, etc. The extract was filtered until clear, the alcohol distilled and the residue—a yellow sirup—dissolved readily[15] in hot water. The solution was made up to 100 cc., of which 2 portions of 20 cc. each were oxidized according to Kjeldahl's method to ascertain the quantity of nitrogen present. Of the remaining solution, 50 cc. was freed from carbon dioxide, phosphoric acid and coloring matter, and titrated with formol according to Sörensen's[16] method as applied by one[17] of us elsewhere (Method A).

The flour extracts ordinarily showed a neutral or a very slightly acid reaction. The probability that the slight acidity could have a hydrolyzing effect upon the nitrogen compounds in the flour extracts seemed to be quite remote. However, in order to remove any doubt the experiment was repeated with the difference that all evaporations took place under reduced pressure. We refer to this modification as Method B.

While operations incidental to the two foregoing methods remove the proteins, they do not accomplish it quantitatively. Inasmuch, however, as proteins containing ly-

[14] Except gliadin, which is soluble in the alcohol.

[15] The very small residue on the filter displayed protein reactions due undoubtedly to some gliadin present.

[16] Sörensen, *Biochem. Z.*, **7**, 48 (1907).

[17] Jodidi, THIS JOURNAL, **33**, 1236 (1911); **34**, 98 (1912); **40**, 1031 (1918).

sine, as is the case with glutenin and leucosin occurring in the wheat kernel, have been shown to contain free amino groups which are formol-titrable,[18] the complete removal of the proteins from the aqueous solutions prior to their titration with formol seemed necessary. Method A, therefore, was modified as follows. The dried alcoholic extract of the flour was dissolved in hot water, the solution brought to boiling, carefully acidified with acetic acid, boiled for a few minutes and filtered. To the filtrate freshly prepared lead hydroxide[19] and some lead acetate were added. The mixture was boiled for a few minutes and filtered. The clear filtrate was then concentrated on the water-bath to 100 cc. and treated as outlined in Method A. We refer to the results secured in this way as those of Method C. To do away with any possible changes due to evaporation under ordinary pressure, Method C was further modified so as to concentrate the extracts in a vacuum. This modification is referred to as Method D. For lack of material however, not all four methods could be applied to each variety. Since the reaction of amino acids with formaldehyde is reversible according to the equation, $NH_2RCOOH + CH_2O \rightleftarrows CH_2 = NRCOOH + H_2O$, excess of formaldehyde was applied to make sure that the forward reaction takes place quantitatively. The results obtained are recorded in Table II.

TABLE II

PROPORTION OF AMINO ACID NITROGEN IN THE UNGERMINATED WHEAT KERNEL

Variety of wheat	Method applied	Oven-dried %	Air-dried %	Total nitrogen %	Water-soluble nitrogen %
Fultz	A	0.033	0.029	1.85	11.13
	B	.029	.026	1.61	9.72
	C	.033	.029	1.85	11.12
Marquis	A	.048	.043	1.56	10.09
	B	.060	.054	1.95	12.63
	D	.055	.050	1.81	11.67
Kubanka	A	0.047	0.043	1.55	11.41
	B	.037	.034	1.21	8.92
	D	.039	.036	1.28	9.41
Kanred	B	.066	.059	2.32	16.04
	D	.067	.060	2.38	16.46

The most outstanding feature of the results in Table II, in which the figures represent the average of at least two estimations, seems to us to be the fact that each of the four methods applied, while differing from the others to a certain extent, indicates appreciable quantities of amino acids, the proportions (in round figures) for Kubanka, Fultz, Marquis, and Kanred being respectively 10, 11, 11, and 16% of amino acid nitrogen calculated to the water-soluble nitrogen, and 1.4, 1.8, 1.8, and 2.3%, calculated to the total nitrogen.

Concerning acid amides, it is true that asparagine was reported by Schulze[6] and Castoro as well as by Frankfurt[20] to occur in the wheat embryo. However, these authors failed to give information as to the *varieties* in which it occurs, nor did they give the *percentage* in which it is present. It was, therefore, deemed of sufficient interest to obtain data on those subjects. The way we proceeded was as follows.

The alcoholic extract of a definite quantity of flour prepared as described in Method A was freed from alcohol by distillation. The residue was taken up with water and made

[18] *Z. physiol. Chem.*, **81**, 274 (1912).

[19] Hoppe-Seyler, "Handbuch der Physiologisch-Pathologisch Chemischen Analyse," A. Hirschwald, Berlin, **1909**, p. 393.

[20] Ref. 3, p. 453.

up to 100 cc. Of this solution 2 portions of 20 cc. each were oxidized according to Kjeldahl's method to find out the quantity of nitrogen present. To 50 cc. of the remaining solution hydrochloric acid was added to a concentration of 20% and the whole boiled under a reflux condenser for 30 minutes, since it was ascertained that under these conditions asparagine[21] splits off its amide nitrogen as ammonia quantitatively. Inasmuch, however, as it was noticed that the quantity of ammoniacal nitrogen increases with the duration of the hydrolysis, another extract was treated as described above, except that the hydrolysis was allowed to continue for 12 hours. The evaporated hydrolysate was distilled with magnesium oxide and the ammonia thus obtained titrated with standard acid. The results secured are summarized in Table III.

TABLE III

PROPORTION OF ACID AMIDE NITROGEN IN THE UNGERMINATED WHEAT KERNEL

	Ammoniacal nitrogen split off by hydrolysis with acid forms							
	Acid amides (Hydrolyzed for 30 minutes)				Compounds other than acid amides (Hydrolyzed for 12 hours)			
Variety of wheat	Oven-dried %	Air-dried %	Total nitrogen %	Water-soluble nitrogen %	Oven-dried %	Air-dried %	Total nitrogen %	Water-soluble nitrogen %
Fultz	0.026	0.023	1.46	8.76	0.006	0.005	0.34	2.08
Marquis	.058	.052	1.91	12.33	.036	.033	1.18	7.63
Kanred	.053	.047	1.88	12.99	.017	.015	0.59	4.12
Kubanka	.052	.048	1.72	12.61	.032	.029	1.05	7.75

The numbers in Table III, each of which ordinarily represents the average of two or three individual estimations, need some explanation. When hydrolyzed for but 30 minutes the flour extract yields the total nitrogen of acid amides as ammonia, which is recorded in Cols. 2 to 5. However, when hydrolyzed for 12 hours it yields as ammonia both the nitrogen of acid amides and that of compounds other than acid amides, such as allantoin, which was shown to be present in the wheat embryo[22] and is known to split off ammonia[23] when boiled with acids. Hence, it is necessary to subtract the ammoniacal nitrogen obtained on hydrolysis of the extract for 30 minutes from that secured by hydrolysis for 12 hours in order to obtain the ammoniacal nitrogen of compounds other than acid amides. It is this *difference* that is recorded in the last 4 columns. It will be noticed that the percentage of acid amide nitrogen in Fultz is much lower than in Marquis, Kanred and Kubanka, which show practically no difference among themselves in this respect. Similarly, the nitrogen from compounds other than acid amides (allantoin and similar substances) is lowest in Fultz, higher in Kanred and still higher in Marquis and Kubanka.

The occurrence of polypeptides or substances of a peptide character in vegetable and, generally speaking, in biological materials appears to have been variously reported by a number of investigators.

[21] Jodidi, Kellogg and True, *J. Agr. Research*, **15**, 398 (1918).

[22] *J. Landw.*, **52**, 322 (1904). Ref. 4.

[23] Abderhalden, "Handbuch der Biochemischen Arbeitsmethoden, Urban and Schwarzenberger, Berlin, **1910**, vol. 2, p. 514.

Thus, by hydrolyzing gliadin Osborne and Clapp[24] succeeded in isolating a dipeptide of proline and phenylalanine. Levene and Wallace[25] by the action of trypsin on gelatin, obtained a polypeptide which on hydrolysis yielded proline and glycocoll while Levene and Beatty[26] by the action of 25% sulfuric acid on gelatin, managed to obtain peptide-like substances. By the action of pancreatin on different proteins Fischer and Abderhalden[27] obtained a polypeptide that was made up of all the proline and phenylalanine, and in part of other amino acids that could be obtained by hydrolysis of the proteins in question. Hart[28] reported that the water-soluble nitrogen of feeding stuffs is largely composed of free amino acids and peptide linkings. A few years later the occurrence of peptides in healthy and mosaic-diseased spinach[29] and cabbage[30] was reported.

A very diligent search of the literature revealed the fact that polypeptides have thus far never been reported to occur in the *ungerminated wheat kernel.*

If the aqueous flour extracts contain polypeptides, they must, when hydrolyzed long enough, yield free amino acids according to the equation,

$$\underset{\text{Polypeptide}}{NH_2RCONHR^1COOH} + H_2O = \underset{\text{Amino acids}}{NH_2RCOOH + NH_2R^1COOH}$$

Hence, the increase in amino acids after hydrolysis over that present before hydrolysis must correspond to the free amino groups of the peptides.

The following method was adopted in order to demonstrate the presence of polypeptides in the wheat kernel.

The preparation of the aqueous and alcoholic extracts up to the point where the alcohol is distilled was effected as outlined above. In order to remove the protein matter completely, the dry residue from the alcoholic extract was dissolved in hot water. The solution was brought to boiling, and treated successively with acetic acid, and then with freshly prepared lead hydroxide and a little lead acetate, the mixture being boiled and filtered after each treatment. The final clear filtrate was concentrated in a vacuum and made up to 100 cc. In two 20cc. portions of this the nitrogen was estimated according to the Kjeldahl method. To 50 cc. of the remaining solution hydrochloric acid was added to a concentration of 20%. This solution was boiled under a reflux condenser for 12 hours. The hydrolyzed substance was then evaporated to dryness, transferred quantitatively to a Kjeldahl flask and, in order to remove the ammonia, distilled with *one*[31] g. of magnesium oxide previously reduced to cream with water. The magnesium oxide residue in the Kjeldahl flask was thoroughly extracted with hot water and filtered. The filtrate and washings were concentrated and made up to 100 cc., of which 2 portions of 20 cc. were analyzed by the Kjeldahl method, while the remaining 50 cc. was used for formol-titration. The data secured are contained in Table IV.

Examination of Table IV, whose figures represent the average of three or four individual determinations, reveals the fact that the 4 wheat va-

[24] Osborne and Clapp, *Am. J. Physiol.*, **18**, 123 (1907).
[25] Levene and Wallace, *Z. physiol. Chem.*, **47**, 143 (1906).
[26] Levene and Beatty, *ibid.*, **49**, 247 (1906).
[27] Fischer and Abderhalden, *ibid.*, **39**, 81 (1903).
[28] Hart, *J. Biol. Chem.*, **22**, 477 (1915).
[29] Ref. 12, p. 1061.
[30] Jodidi, THIS JOURNAL, **42**, 1883 (1920).
[31] Jodidi and Moulton, *ibid.*, **41**, 1526 (1919).

rieties investigated contain considerable proportions of peptide nitrogen, the percentages for Kanred, Fultz, Marquis, and Kubanka being, respectively, 26.86, 28.09, 32.20 and 37.76 calculated to the water-soluble nitrogen, and 3.89, 4.67, 4.98 and 5.13 calculated to the total nitrogen.

TABLE IV

PROPORTION OF PEPTIDE NITROGEN IN THE UNGERMINATED WHEAT KERNEL[32]

Variety of wheat	Amino acid nitrogen found after hydrolysis				Amino acid nitrogen found before hydrolysis				Peptide nitrogen			
	Oven-dried %	Air-dried %	Total nitrogen %	Water-soluble nitrogen %	Oven-dried %	Air-dried %	Total nitrogen %	Water soluble nitrogen %	Oven-dried %	Air-dried %	Total nitrogen %	Water-soluble nitrogen %
Fultz	0.116	0.101	6.44	38.75	0.032	0.028	1.77	10.66	0.084	0.073	4.67	28.09
Marquis	.205	.185	6.75	43.66	.054	.049	1.77	11.46	151	.136	4.98	32.20
Kanred	.177	.157	6.23	43.11	.066	059	2.34	16.25	.111	.098	3.89	26.86
Kubanka	.196	.180	6.48	47.67	.041	.037	1.35	9.91	.155	.143	3.13	37.76

While by the method as outlined for the peptide nitrogen determination the proteins proper are quantitatively removed from the flour extracts, this may not be fully the case with the proteoses present in wheat and with the peptones whose formation during the flour extraction, in minute amounts, is very hard to prevent. It is evident that hydrolysis of these compounds, if present, just as the hydrolysis of peptides, is bound to raise the amino acid content of the flour extracts. It seemed imperative, therefore, to devise a method by which the proteoses and peptones are removed completely before hydrolysis takes place. We proceeded as follows.

The dry alcoholic extract of a definite amount of flour, prepared as already outlined, was dissolved in water and made up to 200 cc. Two portions of 10 cc. each were used for nitrogen estimation. The remaining 180 cc. was made up to 200 cc. and divided into 2 equal parts. To each of those were added 5 g. of sulfuric acid mixed with 30 cc. of a solution containing 20 g. of phosphotungstic acid and 5 g. of sulfuric acid per 100 cc. After about 24 hours the precipitates were filtered off and washed with a solution containing 2.5 g. of phosphotungstic acid and 5 g. of sulfuric acid per 100 cc. Combined filtrates and washings were freed from phosphotungstic and sulfuric acids by treatment with baryta whose excess was precipitated with carbon dioxide. The mixture was then brought to boiling and filtered. The residue on the filter was extracted with ammonia-free water. The filtrate and washings were concentrated under reduced pressure, made up to a definite volume, and the peptide nitrogen was estimated after hydrolysis with hydrochloric acid as outlined above.

By this method in which proteoses and peptones, if present, are completely precipitated by phosphotungstic acid, it was found that Kanred, Fultz, and Marquis contain respectively 27.05, 29.35 and 35.14% of peptide nitrogen as against 26.86, 28.09 and 32.20% recorded in Table IV (last column). It is true that the results obtained by the last outlined method,

[32] While the results presented in this paper are based upon standard methods, the complexity of the substances contained in plant materials renders it extremely difficult, if at all possible, to effect a strictly quantitative separation of the various compounds. This should be borne in mind when examining the data in Tables II to IV.

the details of which will be given in a subsequent paper, are not strictly comparable with those of Table IV for the reason that phosphotungstic acid precipitates, in addition to proteoses and peptones, also diamino acids, ammonia, bases like choline, betaine, etc. Broadly speaking, however, the results obtained by the last method fully corroborate the outstanding fact presented in Table IV that the wheat varieties in question contain in their ungerminated kernel considerable proportions of peptide nitrogen. The presence of peptides in the wheat kernel may have considerable physiological significance, such as in protein synthesis and more generally in nitrogen metabolism, because of the fact that they form a necessary link between the amino acids on the one hand and the proteins on the other.

Summary

The results thus far obtained permit drawing the following conclusions.

1. The wheat varieties investigated contain peptides in their ungerminated kernels, the percentages of peptide nitrogen for Kanred, Fultz, Marquis, and Kubanka being, respectively, 26.86, 28.09, 32.20 and 37.76, on the basis of the water-soluble nitrogen, and 3.89, 4.67, 4.98 and 5.13, calculated to the total nitrogen.

2. The wheat varieties under consideration contain free amino acids in their ungerminated kernels, the proportions (in round figures) for Kubanka, Fultz, Marquis, and Kanred being, respectively, 10, 11, 11 and 16% of amino acid nitrogen, calculated to the water-soluble nitrogen, and 1.4, 1.8, 1.8 and 2.3%, calculated to the total nitrogen.

3. The proportions of acid amide nitrogen in the ungerminated kernel of the varieties Fultz, Marquis, Kubanka and Kanred are, respectively, 8.76, 12.33, 12.61 and 12.99%, calculated to the water-soluble nitrogen, and 1.46, 1.91, 1.72 and 1.88% calculated to the total nitrogen.

WASHINGTON, D. C.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

THE CATALYTIC REDUCTION OF NITRO COMPOUNDS. II. GAMMA-NITRO KETONES

BY E. P. KOHLER AND N. L. DRAKE

Received June 14, 1923

The first step in the reduction of nitro compounds, the transition from nitro to nitroso compounds or, in the case of primary aliphatic nitro compounds, to oximes is as yet a complete mystery. Since γ-nitro ketones on hydrogenation would form substances in which active hydrogen and a carbonyl group are in a relation that is favorable for intramolecular condensation, it seemed possible that they might serve better than simpler nitroparaffins for permitting an insight into this process. With this end in view we have reduced the following 3 nitro ketones.