Changes in Dye Absorption Capacity of Wheat Embryos Undergoing a Browning Reaction

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The rate of changes occurring in wheat embryos conditioned to moisture levels ranging between 9 and 23% and stored for up to 12 days at 37° to 38° C. was followed by recording the dye absorption capacity of the germs. The germs show, during progressive storage and concomitant damage, decreased binding capacity for basic dyes. The basic groups of the protein molecule become, under these conditions, more highly ionized and capable of greater retention of acid dyes. However, the stronger retention is almost outweighed by the decrease in the number of available basic amino groups. The highly consistent and pronounced changes in absorption of basic dyes point to the possibility of using the method to detect incipient damage and to follow the rate and extent of damage.

ISOLATED WHEAT GERM, which is high in sugar and proteins, browns readily when stored at elevated temperatures and at high moisture levels. This darkening may be due to a browning reaction of the Maillard type, involving condensation of reducing sugars and amino groups.

The kinetics and equilibria of the browning reaction have been studied extensively by physical-chemical techniques. The principal tools have been polarimetry, cryoscopy, colorimetry, and potentiometry. The chemical methods employed include the formol titration and the Van Slyke determination of primary amino groups, and various methods for determining free reducing sugars. There is conflicting evidence regarding the possibility of using the mentioned chemical methods for the determination of amino acids or reducing sugars in sugar amino reactions. However, there is ample experimental evidence indicating a stoichiometric condensation between reducing sugars and amino groups in browning reactions (*3*).

Cole and Milner (*2*) found differences in absorbance (in the ultraviolet region) of extracts of germs dissected from damaged and normal grains. Similar results were obtained in the testing of the whole commercial wheat germ which had browned in laboratory storage at moderately elevated temperature and moisture content. McDonald and Milner (*6*) have indicated the major dependence of the browning reaction in wheat germ on the protein constituents. They have shown that the chromogens and fluorogens, which are developed in, and are characteristic of, the browning reaction, are formed at a stage following the initial reaction with proteins.

According to Hodge (*3*), the initial stage of browning cannot be detected by spectrophotometric measurements in the ultraviolet; however, before visible browning has begun, a strong absorption appears to announce the beginning of the intermediate stage. In the final stage of browning, the intermediates formed polymerize, and unsaturated, fluorescent, colored polymers are formed. Studies on the relationships between color production and fluorescence show that the fluorogens are precursors of the brown pigments but are not identical with them. Recently, Linko *et al.* (*5*) have shown that fluorescence of wheat embryos, stored at elevated temperature and high moisture content, increased only after discoloration was visually apparent, and particularly at moisture values beyond 15%. A decrease in nonreducing sugars and interaction with available free amino acids preceded marked increases in fluorescence.

A number of workers have found a drop in pH of solutions of proteins and glucose (*7*) or in pH of water extracts (*6*) of biological material during the course of nonenzymatic browning.

This work has been undertaken to follow the changes in dye absorption of wheat germ stored at various moisture levels, as a way to follow and correlate the differences with biochemical changes occurring in the germ. With acid dyes, the intensity of staining decreases as the pH increases; with basic dyes the intensity increases with increasing pH. As the browning reaction is concomitant with lowering of pH, this should be reflected in variations in dye absorption from dye solutions at various pH levels.

Materials and Methods

Germ. The fresh unprocessed wheat embryo tissue was obtained from a commercial source and was stored at +4° C. in a moisture-proof container until used. The granular germ was essentially free of bran and starchy endosperm. Its moisture content was 9%. Moisture content was determined by the 130° C. air-oven method (*1*).

Dyes. Methyl green basic dye; Color Index No. 684 from Coleman & Bell Co., Norwood, Ohio.

Acridine Orange basic dye; Color Index No. 788 from Coleman & Bell Co., Norwood, Ohio.

Orange G acidic dye; Color Index No. 27 from National Aniline Division, Allied Chemical & Dye Corp., N. Y.

Ponceau Xylidine acidic dye; Color Index No. 79 from Hartman-Ledon Co., Philadelphia, Pa.

All staining solutions were prepared in the same way. Dye and buffer solutions of twice the concentration were made up and equal quantities of each were mixed to obtain the final staining solution. The concentration of the acidic dye in the final staining solution was 0.02%; of the basic dye, 0.01%.

Buffer. McIlvaine's citric acid–secondary sodium phosphate buffer diluted 10 times, at pH values 2.0, 3.0, and 4.0.

Storage of Germ. Portions of the germ were conditioned in stoppered Erlenmeyer flasks at 9, 11, 13, 15, 17, 19, 21, and 23% moisture content, respectively, and stored at 37° to 38° C. for 12 days.

Samples were removed after 1, 2, 3, 4, 6, and 12 days and kept in a refrigerator (at +4° C.) until used.

Based on color and visual appearance during progressive deterioration, the wheat germ could be divided into the following groups:

A-1. Normal, sound, yellow germs
A-2. Slight browning
A-3. Browning, appearance dull and lifeless
A-4. Advanced browning
A-5. Extremely dark brown to black

These designations are used, along with subsequent data, for comparative purposes.

Staining. A weighed amount, 0.25 gram, of the tested germ was shaken in

a 15-ml. centrifuge tube with 3 ml. of the buffered dye solution. The tubes were shaken also after 15 and 30 minutes, after which time 12 ml. of distilled water were added and the contents of the tube mixed thoroughly. After the solution stood for 30 minutes, the contents were centrifuged till clear (10 minutes at 2000 r.p.m. in a Universal centrifuge generally obtained a clear centrifugate). The clear supernatant was decanted and the absorbance measured with a DU Beckman spectrophotometer.

The wave lengths employed, in millimicrons, were: 634 for methyl green, 497 for Acridine Orange, 538 for Ponceau Xylidine, and 470 for Orange G.

The results are given as absorbance of the supernatant. A high reading, therefore, indicates low dye absorption.

Results and Discussion

Only representative data are reported here, as the results are highly consistent.

To test the possible effect of the color of the water extract on dye absorption readings for each of the dyes employed, the absorbance of the buffered extracts (containing no added dye) was determined. This control test was carried out at the respective wave lengths at which the dye absorption tests were made. Generally, the absorbance of the solution was higher in the browner samples. However, the range of analytical data was very small for the test conditions described, and the absorbance of the blank extracts constituted a very small per cent compared with the proportion in the tests in which the dye had been added.

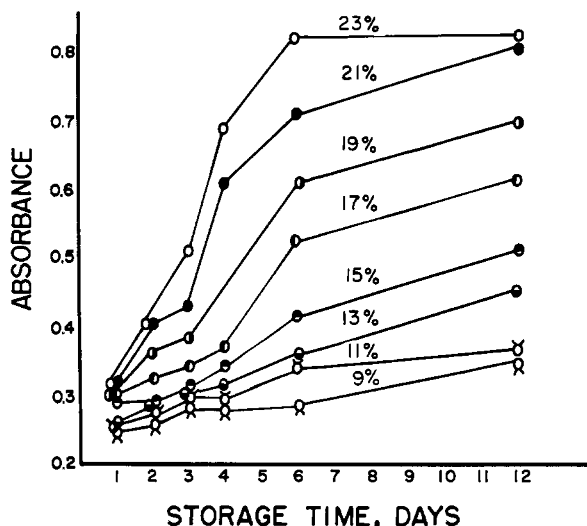

Figure 1. Dye absorption of wheat embryos from an Acridine Orange solution buffered at pH 2.0

Percentage figures refer to moisture content of conditioned embryos

Figure 1 presents the absorbance of the supernatant solution from wheat embryo shaken with Acridine Orange buffered at pH 2.0. The dye absorption showed a definite decrease in the samples stored at higher moisture levels and this decrease assumed progressively higher levels with storage. Although there was a slow but definite decrease in dye absorption even in samples containing 11% moisture, a marked decrease was observed in samples containing 13% moisture and above.

Table I shows the variations in dye absorption (expressed as absorbance of the supernatant) of germ samples stained with Acridine Orange and methyl green solutions, buffered at various pH levels.

Table I. Effect of Storage for 12 Days at Various Moisture Levels on Absorption of Basic Dye at pH 2, 3, and 4

		pH				
		2.0	3.0	4.0	2.0	3.0
		Absorbance				
Moisture, %	*Appearance*	*Acridine Orange*			*Methyl Green*	
9	A_1	0.350	0.285	...	0.545	0.421
11	A_1	0.375	0.307	...	0.540	0.540
13	A_2	0.400	0.325	...	0.635	0.575
15	A_3	0.502	0.375	0.315	0.830	0.730
17	A_3	0.605	0.458	0.405	0.850	0.720
19	A_4	0.695	0.557	0.490	0.880	0.840
21	A_5	0.795	0.625	0.542	0.980	0.800
23	A_5	0.820	0.742	0.672	1.050	1.000

Table II. Effect of Storage for 12 Days at Various Moisture Levels on Absorption of Acidic Dye at pH 2 and 3

		pH			
		2.0	3.0	2.0	3.0
		Absorbance			
Moisture, %	*Appearance*	*Xylidine Ponceau*		*Orange G*	
9	A_1	0.245	0.402	1.33	1.48
11	A_1	0.300	0.418	1.37	1.45
13	A_2	0.270	0.365	1.27	1.38
15	A_3	0.243	0.330	1.17	1.32
17	A_3	0.255	0.317	1.17	1.34
19	A_4	0.285	0.382	1.20	1.35
21	A_5	0.265	0.365	1.22	1.42
23	A_5	0.332	0.430	1.32	1.38

These results seem to indicate a shift in the isoelectric point of the wheat germ during the storage conditions employed. The theory underlying this method of determining the isoelectric point has been outlined by Levine (*4*) during study on the apparent isoelectric point of cytoplasm. This has been established from the fact that positively charged ions of basic dyes combine with the negatively charged protein ions at pH values above the isoelectric point, and negatively charged ions of acidic dyes react with the positively charged protein ions at pH values below the isoelectric point. If a series of solutions is set up at different pH values, the intensity of staining will depend on the amount of ionization of the protein. During the staining with a basic and an acidic dye, two opposite sigmoid curves are obtained, and the isoelectric point is theoretically the pH value at which the curves cross. In practice, no true isoelectric point can be obtained, as the point of intersection of the two sigmoid curves depends on a great number of factors—i.e., the buffer system and its concentration, the dyes employed and their concentrations, and the effect of constituents other than protein.

Spectorov (*9*), in his studies on changes during the vernalization process in wheat, has recently pointed to the value of the dye absorption method to indicate a shift in the isoelectric point of the wheat embryo.

No attempt has been made to determine the exact value of a shift in the isoelectric point. This could probably be

done by electrophoretic studies. The difficulty in determining this shift by the dye absorption method is that at higher pH levels, the solubility of proteins becomes prohibitive, and it is practically impossible to obtain clear extracts under conditions normally employed. Additionally, at higher pH levels, there is a danger of precipitating the added dyes as well as extracting flavone pigments from the tested materials. Therefore, these studies had been confined to pH values of 2, 3, and 4, respectively. There is a consistent decrease in dye absorption of both basic dyes (the values are given as absorbance of the supernatant) with decreasing pH.

In observing the data for various germ samples stained with a solution at a specified pH level, the higher the damage, the lower the dye absorption. This trend is uniform in any of the pH levels employed and is consistent with findings of lowering of pH due to binding of basic amino groups.

With the acidic dyes, one would expect a higher dye absorption at the lower pH level. This is the case with either of the two acidic dyes employed at both the pH 2 and pH 3 levels, as indicated in Table II. Surprisingly, however, here is no one directional trend in the acid dye absorption of the samples of varying extent of damage tested at a specified pH level.

A shift toward lower pH values, as a result of binding of basic amino groups, should be reflected in higher dye absorption (or lower absorbance of supernatants). No such uniform trend has been recorded. The explanation seems to be that the extent of absorption of acidic dyes depends on two factors acting in opposite directions during storage, shift in pH toward higher acidity and increased acidic dye absorption, concomitant with binding of basic amino groups by reducing sugars and rendering them unavailable for the negative ions of the acidic dye. In Table II, note that though there was no one directional trend for both dyes employed at either pH level, there was a consistent increase in dye binding in the less damaged samples (moisture levels of 15% and below), and only in the highly damaged samples was the staining intensity decreased. This may indicate the relative availability of the basic amino groups in the lightly damaged samples, and decreasing availability of amino groups with progressive damage of the wheat with increasing moisture or length of storage.

Results reported in this investigation are based on studies made on a substrate in which the total protein content was constant. The absorption of acid dyes has been shown (*8*, *10*) to depend on the total number of basic amino groups, and subsequently on the protein level of the tested substrate. Thus, in assessing changes in samples of variable protein content, which undergo a browning reaction, both the protein level and availability of amino groups will affect the amount of bound dye. In testing commercial samples, it might therefore be desirable to express the results on a constant protein basis.

The possible application of the principle of dye binding, as a measure of soundness or storability of grain, and of heat treatment of soy flour to improve its biological value, is being investigated.

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Received for review November 21, 1960. Accepted February 16, 1961. Contribution No. 349, Kansas State University, Agricultural Experiment Station, Manhattan, Kan.

RADIATION STERILIZATION OF FOODS

Comparison of the Radiosensitivities of the Fat-Soluble Vitamins by Gamma Irradiation

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One of the problems in the use of ionizing radiation for sterilizing foods is the destruction of essential micronutrients. Knowledge of radiation lability of vitamins is also important in radiation biology. Although there have been numerous reports on the irradiation of the fat-soluble vitamins A, D, E, and K (*10*), no definitive study has been made of their comparative radiosensitivities under uniform conditions. By irradiating these compounds individually, in pure solution, the complicating factors of interaction with and protection by other solutes may be avoided. By the use of a relatively inert hydrocarbon, iso-octane, as irradiation medium, it was hoped to minimize solvent effects on the vitamins.

Vitamins and Vitamin Assays. Except for α- and β-carotene, all vitamins were obtained from Nutritional Biochemicals Corp. The original concentration of each was the same for both aerobic and anaerobic irradiation.

Vitamin E (*d l* - α - tocopherol), whether irradiated in iso-octane, tributyrin, or lard, was determined by the ferric chloride–1,1′-dipyridyl method (*11*), except that the reaction time was shortened to $1^1/_2$ minutes. To prevent phasic separation, 1 or 2 ml. of acetone were included in each tube.

Initial vitamin A acetate concentrations were determined by the ultraviolet absorption method (*1*) applied directly to the iso-octane solutions. Because irradiation increased the ratio of absorbance at 310 mμ to absorbance at 325 mμ, per cent retentions of vitamin A acetate in irradiated solutions were calculated on the basis of absorbance at 334 mμ.

Mixed α- and β-carotenes were prepared from commercially canned diced carrots (*5*) and assayed by absorbance at 440 mμ.

Vitamin D_3 in iso-octane was determined from its ultraviolet absorbance (*3*). Because the shape of the spectral maximum at 265 mμ was distorted in the more highly irradiated solutions, residual vitamin D was calculated on the basis of absorbance at 274 mμ. Concentrations of vitamin D in salmon oil were determined by the antimony trichloride method (*8*) without saponification.

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