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# HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

## I. Histochemical Demonstration of Germ and Aleurone Using Acridine Orange[1]

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### ABSTRACT

Polychromatic fluorescent properties of acridine orange solutions were used to characterize wheat tissues and milled wheat products. A water solution of the dye can be used for multicolor staining of wheat tissues. Coloration varies with protein content.

A saturated xylol (xylene) solution of the base forms a brilliant fluorescence with the lipid material of either the aleurone or the germ so it can be used to distinguish these two wheat tissues in milled products.

Methods available to characterize wheat tissues in flour are limited, although this subject is one of considerable practical concern to the milling industry. Methods used depend on gross differences in the chemical composition of the various tissues of the wheat kernel. Such methods are useful only when a certain constituent is present in relatively large amount in one tissue and essentially absent in another — and only when differences are detectable by sensitive and reproducible chemical tests. Therefore, a dye adsorption test that characterizes wheat tissue in milled products should prove useful.

Fat concentration of the wheat kernel is highest in the embryo and the aleurone layer (10). According to Meyer (9), 2% of the wheat kernel is oil, while the content of lipids in the bran and the germ is 5–6% and 12–18%, respectively. Combined germ and aleurone content of a milled wheat product can be estimated from its oil content. However, the technique of determining the amount of lipid-rich tissue particles in ground flour depends on a number of factors such as the fol-

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lowing, which cause variations in the oil content of flour:

a) Tempering and milling procedures influence the behavior of the endosperm, pericarp, aleurone, and germ from various wheats (5,6).

b) Oil may be pressed from the germ during milling and so oil in the germ may decrease while it increases in the flour (12).

c) The analytical procedure to determine fat is not precise enough to detect minute differences of lipid-rich material in flour (10).

The difficulty of estimating, on the basis of fat content, the particles in flour originating from germ and aleurone tissues led to experiments with histochemical methods.

Two methods using acridine orange (AO) were investigated. First, a slightly acid water solution of the dye was used according to staining schedules recommended in the literature for animal tissues. A second procedure employed a saturated xylol (mixture of xylenes) solution of the base of the dye.

## Materials

Materials used were:

1. Longitudinal and transectional slices prepared from kernels of soft and hard winter wheat by the procedure of Grosh and Milner (4).

2. Various grades of commercially milled flour and samples of highly refined white flour with various amounts of purified and ground germ added. (Germ used, a commercial product, had extraneous material removed by hand under low magnification.) A whole-wheat meal ground to pass a 40-mesh sieve was also prepared.

## Methods

Experiments were carried out with AO, a xanthene dye (C.I. No. 788; Synonyms: Basic Orange 3 RN, Euchrysine 3 RXA). The fluorochrome (2) dye, whose purity and percentage of active compound were unknown, was obtained from Coleman & Bell Co., Norwood, Ohio.

The dye base was prepared by the following procedure outlined by Knaysi for preparation of the base of neutral red (7): 0.2 g. of dye were dissolved in 100 ml. water and filtered; the dye base was precipitated with *N* sodium hydroxide solution, and an excess of 1 to 2 drops of the alkali was added. The base precipitates imediately and can be filtered. The precipitate was washed with slightly alkaline water (pH 8.0) and, after air-drying, was kept in a stoppered glass bottle. The precipitated dye base remained stable for months.

A saturated solution of the dye base in xylol was prepared by shaking a 0.1% suspension for 15 minutes and then filtering. A shift

in color may take place after the dye base has stood for a time, so a fresh solution was prepared daily.

*Staining with an Aqueous Solution of Acridine Orange (AO).* This procedure was introduced in fluorescent microscopy by Bukatsch and Haitinger (1) and independently by Strugger (13) and has found wide use. The physico-chemical properties of the fluorescence were investigated by Zanker (16,17). Metcalf and Patton (8) discussed the use of ultraviolet illumination and fluorescent dyes, especially AO, and studied details of structure and physiological differences in biological materials. Emig (3) recommended AO to stain plant tissues because it stains them a fast clear brown or dark orange.

Many investigators have used AO in staining schedules in recent years. A partial list of references regarding acridine orange in staining animal tissues was given by Von Bertalanffy and Bickis (15).

AO dissolved in water showed a gradual change of the fluorescent color from green to red with intermediate orange when the concentration of the dye increased from 0.001 to 0.10%. Schümelfelder (14) found the red fluorescence to be due to the polymer of AO in stronger solutions. The intermediate colors of yellow and orange were due to different mixtures of a green fluorescent monomer and red fluorescent polymer cations. Scheibe and Eder (11) have reported similar results with AO bound in stained tissues.

The wheat sections were covered with a 1% acetic acid solution mixed with an equal amount of 0.01% solution of AO and stained for 5 minutes. The procedure was established after a series of tests in which the concentration of dye, time of staining, and concentration of acid were varied. Minor changes in the techniques do not affect the test, except that solution of dye concentrated to 0.1% diminished the effectiveness of clear tissue differentiation, and solutions diluted to 0.001% were rather ineffective. The stained wheat sections were observed with either ordinary light or ultraviolet radiation (having a predominant wave length of 3,660 Å) and with either low-power magnification or without visual aids.

*Staining with a Saturated Xylol Solution of the Base of Acridine Orange.* Free bases of several common basic dyes may have different colors from their salts or soaps. The dye bases are soluble in neutral fat, xylol, and alcohol but practically insoluble in water. The extent the color of a dye base solution shifts, in a fat solvent or fat, toward the color formed on interaction with free fatty acids was found to be stoichiometric. The color shift is due to intramolecular arrangement of the base upon soap formation, and the color is independent of the nature of the fatty acids.

Despite recommendations (7), experiments in the authors' laboratory, using the base of neutral red in staining wheat sections or wheat flour to detect free fatty acid-rich particles or layers, gave unsatisfactory results.

Excellent contrast was obtained when the AO base was employed. In addition to the differences in solubility and shift in color shown by many dye bases, AO exhibited pronounced polychromatic fluorescence, with variations of dye concentration in a stained tissue preparation.

Wheat sections were stained 5 minutes or longer (depending on the thickness of sections) by covering the material in the cavity of a spot plate with a xylol solution of the dye base. The stained material was blotted on filter paper and observed after about 5 minutes.

With sufficiently thin (about 20 microns) sections, the following procedure was used: Sections were placed on filter paper (Whatman No. 5) and at 1-minute intervals spotted three times with a drop of the dye base solution. The excess dye was adsorbed by the paper and the sections were transferred to a microscope slide for examination. No differentiation or counterstaining was needed.

Differences in color were seen immediately under ultraviolet radiation in the dark, but exposure to the light source radiation for about 10 minutes gave more brilliant colors.

To test wheat flour or ground wheat, a 0.25-g. sample was shaken for 15 minutes with 2 ml. of the dye base in xylol in a small test tube, then poured over filter paper (9-cm. Whatman No. 4) on a Büchner funnel and allowed to drain. The material could be tested on the filter paper after it was transferred to a Petri dish, allowed to dry (about 10 minutes), and observed under low magnification (24 or 48×, depending on particle size), employing a UV lamp as the source of radiation. The background of the filter paper, which shows a faint fluorescence, is not objectionable, but in case better contrast is desired the particles can be transferred, after drying, to a new sheet of paper.

Pictures were made with UV radiation on Ektachrome high-speed films.

## Results and Discussion

*Aqueous Solution of Acridine Orange.* Viewed under ordinary light, both the pericarp layers and the germs, when stained with a water solution of AO, showed a rather intense dye uptake of yellow-brown. On examination of tissues exposed in the dark to a source of ultraviolet radiation, germ and pericarp showed (under 96×

magnification) the following multicolored fluorescent effect:

Scutellum (cotyledon) — bright yellow-green
Plumule and coleoptile — yellow-green
Aleurone — slight but distinct blue-green
Inner pericarp — orange-red
Outer pericarp — green-yellow
Starchy endosperm — unstained, light blue-violet background

The differences in color of the various tissues in the sectioned wheat kernel seemed to be due to both the selectivity and intensity of dye adsorption by the layers and the effect of polychromacy of the dye. Staining with AO gives contrasting colors that help distinguish the structural elements of tissues without applying the rather complicated techniques of multiple stains. The method eliminates difficulties due to fixation, dehydration, counterstaining, etc.; the low concentrations applied reduce the danger of artifacts (8).

To identify wheat tissues in a milled product, flour or wheat meal was tested also by the procedure given for the dye base method, employing the aqueous staining solution instead of the xylol base solution.

When the dry paper was observed under low magnification (24×), germ and aleurone particles showed strong yellow-green fluorescence, though the fluorescence was not as brilliant as in the tests using the xylol solution of the base.

Figures 1 to 4 appear at the end of Part IV, page 129.

*Saturated Xylol Solution of the Base of Acridine Orange.* Figures 1 and 2 are pictures obtained on cross and longitudinal sections under low-power magnification, using a predominant radiation of 3,660 Å. No effort was made to cut out the small amount of visible light, as it was desirable to see the background of the nonfluorescing wheat layers.

Although lipid material also is present to a certain extent in the endosperm and bran, it is only the germ and aleurone layer that show a distinct and brilliant red fluorescence due to the presence of fatty acids.

Figures 3 and 4 illustrate how easily the particles originating from the embryo or aleurone layer can be detected in ground whole wheat or milled flour. The figures were obtained under conditions similar to those outlined for wheat sections with the particles observed under higher magnification.

The aleurone and germ particles stand out clearly in the field, even though both the oil, mechanically exuded during milling, and other components, containing small concentrations of lipid materials, impart a faint pink shade.

Work to determine the extent of damage in wheat germs, as well

as lipase activity in cereal grains and microorganisms, is continuing, using the procedure for staining of wheat sections and lipid-containing material in milled wheat products.

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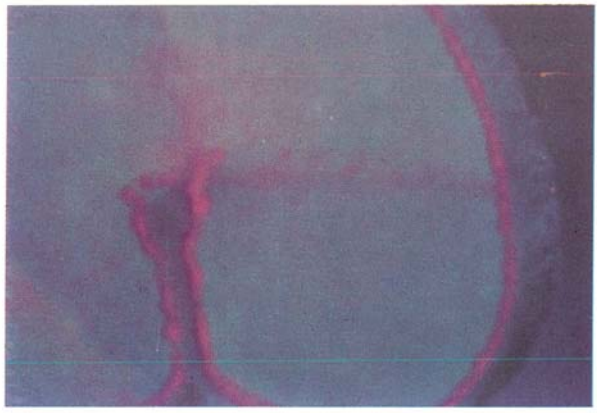

Fig. 1, page 107. Cross-section of kernel (50×), showing coloration of the aleurone viewed by reflected ultraviolet light.

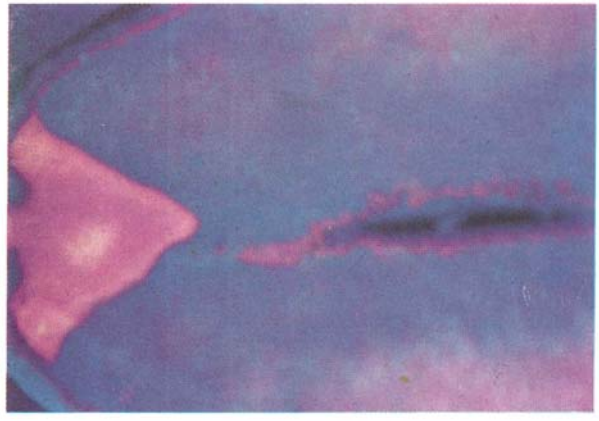

Fig. 2, page 107. Longitudinal section through both cheeks, crease, and part of germ (20×), showing coloration of aleurone and germ viewed by reflected ultraviolet light.

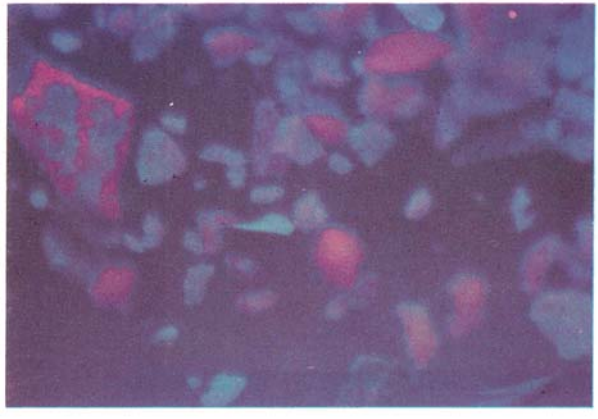

Fig. 3, page 107. Ground wheat particles (20×), showing coloration of particles originating from aleurone or germ tissues.

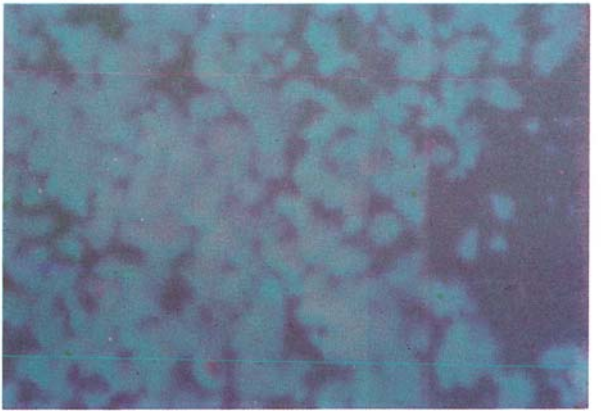

Fig. 4, page 107. Flour (50×), showing coloration of particles originating from aleurone or germ tissues.