HISTOCHEMICAL CHARACTERIZATION OF WHEAT AND WHEAT PRODUCTS

IV. Mapping the Free Fatty Acids in Germinating Wheat[1]

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ABSTRACT

Changes in free fatty acid content of various tissues of wheat germinated for 8 days were followed by a histochemical method using the base of acridine orange in xylol. Using various substrates showed the staining method specific for fatty acids. Halved kernels stained and viewed under ultraviolet radiation indicated the scutellum as the main site of lipase activity. As the germination period increased, zones farther from the scutellum tissue gradually increased in fatty acids until, after about 8 days, measurable quantities of free fatty acids were present throughout the kernel.

Compared with oil-bearing seeds, particularly castor beans, sound wheat is a poor source of lipolytic enzymes. However, when moisture content is raised, especially on germination, wheat seedlings mobilize reserve storage fat for the developing embryo, and lipase activity increases (18). The lipase activity is located in the outer layers of wheat kernels. Pett (17), using dissected wheat kernels at various stages during germination for 48 hours, studied changes that occur in esterase activity in various tissues. Highest esterase content was associated with the scutellum, and germ lipase activity was considerably less than the scutellum lipase activity. Esterase content of the endosperm was fairly high, but much lower than in the scutellum. Bran contained a significant amount of esterase. Sullivan and Howe (21) found that both clear and low-grade flours had greater lipase activity than patent flours, germ, or bran. Extensively investigated lipids of wheat (14) are concentrated primarily in the embryo and aleurone layer and consist primarily of mixed monosaturated-di-unsaturated glycerides and tri-unsaturated glycerides.

Numerous investigators who have studied changes in fats and fatty acids during germination conclude that the oils disappear fairly rapidly during the course of germination (13,15). Several have reported an increase in fatty acids as germination of seeds progresses (1,2,5,12,16). However, a recent review (20) cites evidence that free fatty acids do not accumulate during the rapid consumption of fat in citrullus cotyledons grown in both light and darkness (3,9).

Methods for measuring lipase activity are based mainly on titration of fatty acids, hydrolyzed from a specified oil substrate under

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given conditions of time, temperature, and pH. Luchsinger *et al.* (11) developed a sensitive assay procedure based on the glycerol released from a mono-olein emulsion. Ferrigan and Geddes (4) used the procedure to study lipase activity of mill streams from hard red spring wheat. Cereal lipase normally is studied using an artificial substrate sometimes completely unrelated to the oil in the plant tested. The ideal substrate for lipolytic enzyme studies of wheat would be wheat fat (21). With regard to histological staining methods, proof of lipase activity generally is based on an adaptation of Gomori's technique to demonstrate and localize phosphatases in tissue section (8). Glick and Fisher (7) attempted to localize lipase in wheat grain using Gomori's histochemical method and Tween 40 as a substrate. Their failure to find enzyme action in any wheat sections was attributed to low lipase activity of wheat and possible low attack on the ester employed.

The present authors used a saturated solution of the base of acridine orange in xylol to map lipase activity in germinating wheat while it acts on the normally present oil substrate of the wheat kernel. Though no substitute for quantitative analysis, this method shows sites of lipase activity loci and permits tracing transformation of liquids within germinating seed tissues.

Experimental Work

Materials. Selected, sound kernels of hard red winter wheat were treated 2 minutes with a 1:1000 mercuric chloride solution to retard mold growth, rinsed with distilled water, and placed (on consecutive days) between two layers of moist filter paper in a covered Petri dish. The samples were germinated at room temperature for 8 days and then removed for testing.

Acridine orange (AO) is a basic dye (C.I. No. 788)[2]. The staining dye base was obtained by precipitating a 0.2% water solution of the filtered dye, by dropwise addition of *N* sodium hydroxide solution and an excess of 1 to 2 drops of the alkali. The precipitate was washed with water (pH 8.0) and, after vacuum-drying in a desiccator, 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 for use daily by shaking 15 minutes and filtering. The lipase used was soluble wheat germ lipase from Mann Research Laboratories.

Methods. Pictures were taken using UV radiation with a predomi-

[2] Coleman and Bell Company, Norwood, Ohio.

nant wave length of 3,660 A, and high-speed Ektachrome film. As it was desirable to obtain a background of the unstained wheat tissues, no effort was made to eliminate the small amount of visible light from the radiating UV source.

Results and Discussion

Use of the base of AO in staining lipid materials is based on findings by Knaysi (10). The free base of a number of dyes was reported to have colors different from their respective salts or soaps. The dye base is soluble in xylol but practically insoluble in water. Dyes recommended by Knaysi showed poor contrast, so a dye more suited to histological work was sought. AO dye shows, in addition to the above properties, a very pronounced polychromatic fluorescence, which gives a multiple color effect that depends on the concentration of dye bound by various tissues. The color changes from green through yellow-orange to red with increasing concentration.

Specificity. Several triglycerides, fatty acids, pyruvic acid, and lactic acid, were used to test the specificity of the staining reaction. A small drop or small piece of solid material was placed in the cavity of a spot plate and covered by adding 0.5 ml. of a saturated dye solution in xylol; the color was observed after 3 minutes both under

TABLE I

COLORATION OF VARIOUS SUBSTRATES WITH ACRIDINE ORANGE BASE, USING ULTRAVIOLET RADIATION

	SUBSTRATE	COLOR IN UV
0	Control	Green
1	Tristearin	Green
2	Tripalmitin	Green
3	Refined commercial corn oil	Green
4	Tween 80	Green
5	Crude wheat germ oil[a]	Yellow-orange
6	Crude corn oil[a]	Yellow-orange
7	Commercial soybean lecithin	Orange-reddish
8	Glycerine	Green
9	Tween 80 incubated for 2 hours with lipase	Small red droplets
10	Butyric acid	Red-orange
11	Lauric acid	Red-orange
12	Linoleic acid	Red-orange
13	Myristic acid	Red-orange
14	Octanoic acid	Red-orange
15	Oleic acid	Red-orange
16	Palmitic acid	Red-orange
17	Stearic acid	Red-orange
18	Undecylic acid	Red-orange
19	Lactic acid	Yellow-orange
20	Pyruvic acid	Yellow-orange

[a] Diethyl ether-soluble material, freshly prepared in the laboratory from wheat germ and ground corn, respectively.

daylight and under UV radiation. Results obtained with both sources were identical except that the UV gave better contrast; those reported in Table I were obtained using this source.

Results summarized in Table I show that the staining is highly specific in differentiating between lipids and fatty acids derived from lipid material. However, any acidic substance soluble in xylol caused a shift in color of the AO base solution; this was observed on addition of lactic or pyruvic acid. These intermediary metabolites are likely to be found in germinating wheat but not necessarily a result of storage-fat breakdown. This should constitute only a minor limitation to the use of the method. The solubility of pyruvic and lactic acid in xylol is low, especially compared with their practically infinite solubility in solvents normally employed (i.e., 50% alcohol) in acidimetric determinations of lipase activity.

Distribution of Fatty Acids in the Wheat Kernel. Major sites of high lipid concentration, and generally free fatty acids in a dormant sound wheat kernel, are the germ and the aleurone. (See part I of this series, Figs. 1 and 2; p. 129.)

Wheat sections, prepared with a freezing microtome from totally untreated material, were stained by covering with the dye solution in the cavity of a spot plate. After 5 minutes, the sections were blotted on filter paper and allowed to air-dry. On observation under UV radiation, the germ and aleurone showed a brilliant red fluorescence in contrast to the small dye uptake resulting from the low concentrations of fatty acids of the endosperm. Compared with controls, stained diethyl ether or xylol-extracted wheat sections showed little coloration of the germ or aleurone layer. Such practically fat-free sections were subsequently covered with a thin layer of crude wheat germ oil, prior to staining. After 3 hours, to allow the wheat to adsorb oil, the oil-covered sections showed a brilliant fluorescence on staining.

Finally, wheat kernels, kept overnight between moist filter paper, were divided into three lots. The distal ends of the kernels of one lot were injected, by means of a syringe, with 0.01 ml. of distilled water. Kernels of a second lot received an equivalent volume of a 0.1% suspension of lipase; and the third lot was treated with a heat-inactivated suspension of lipase preparation. The kernels were kept between moistened filter paper at room temperature 18 hours, then halved and air-dried 1 hour. The air-dried halves were stained and observed. After staining with AO, the wheat kernels to which active lipase was added were more deeply colored than the controls. The difference in color intensity was far less than that observed between

sound kernels and kernels germinated for longer periods (about 8 days), indicating that the distribution of lipase is not the only factor governing the presence of fatty acids in various tissues.

Results of these tests indicate that the staining method is well suited for following the location of fatty acids produced by lipase activity in germinating wheat.

Figures 1 through 6 appear at the end of this article.

Before discussing the results of changes in the interior of the wheat kernel, it is pertinent to consider Figs. 1 and 2 which show the dorsal side of wheat halves, with emerging shoots. The staining technique permits one to observe the presence of the thin cutin lamellae which are known to be formed on the outer walls of the epidermal cells of leaves and stems. Cutin (19) is a complex mixture consisting mainly of unsaturated fatty acids that have undergone condensation and oxidation. The relatively high concentration of fatty acids in the thin varnishlike skin on the surface governs the dye uptake and brilliant coloration.

Changes during Germination. Figures 3 to 5 show clearly that the scutellum is the major site of lipase activity in the germinating wheat. Activity in the aleurone layer remained fairly constant. Even at advanced stages of germination (Fig. 4), parts of the wheat endosperm near the aleurone but distant from the scutellum were low in free fatty acids.

Figure 6 shows the pattern of fatty acid distribution in germinating maize.

The results show that during germination the enzymes elaborated by the scutellum eventually reach the more distant parts of the kernel until, at the final stages of germination, all lipid material may be converted to free fatty acids which become available to the developing seedling. Though the concentration of the endosperm fat is low, the quantity of fat present is about one-third of that in the whole kernel. It is possible that during the later stages of germination the reserve fat of the endosperm is used, along with the more concentrated sources of lipid material in the germ and aleurone.

Although the results obtained show that progressive germination is concomitant with the presence of free fatty acids in new "zones" more distant from the scutellar site of major lipase activity, it is impossible to account for the source of these fatty acids on the basis of the staining technique employed. The results of Pett (17) point to a redistribution of lipases on germination of wheat, resulting in an abrupt fall of the lipase activity in the scutellum along with a small increase in other tissues. Malhotra (13) has shown that in

germinating maize, fat decreased in the seed from 4.9 to 4.1% during 8 days of germination. When the endosperm and embryos of maize were separated at the different stages of germination before being analyzed, fat increased in the endosperm with a corresponding decrease in the embryo.

The amount of free fatty acids present in different parts of a germinating wheat kernel therefore may be governed, in addition to conditions of growth, by a number of additional factors such as: (a) breakdown of lipids due to the secretion of lipases from the scutellum or activation of lipases at new sites of the endosperm, which would bring about a net increase in fatty acids; (b) redistribution of lipids, but not changing total fatty material content in the whole kernel.

The answer to these questions cannot be obtained by the staining method employed, but the method can supplement information obtained by chemical methods. It is simple and shows high specificity. It is promising as it permits staining fatty acids specifically and not fats or oils as such.

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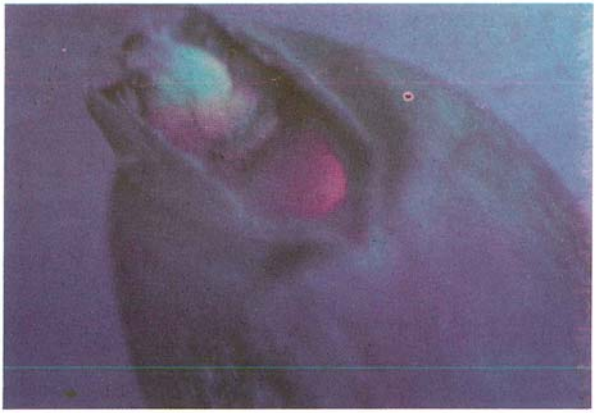
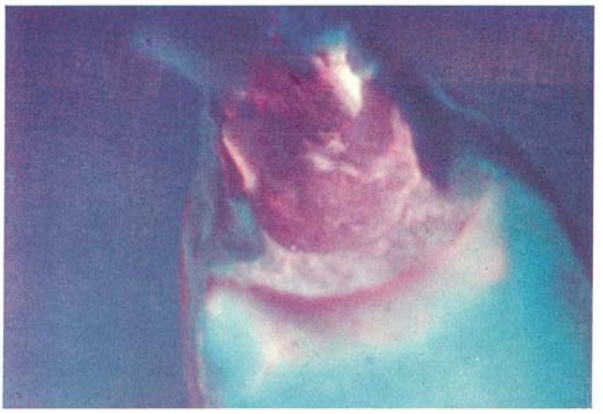


Figs. 1 and 2, page 126. Dorsal side of longitudinal wheat halves after 1 and 2 days of germination, respectively, viewed by reflected light. (20×)

Figure 2



Figs. 3 and 5 inclusive, page 126. Halves of germinating wheat, longitudinal, after 2, 4, and 8 days of germination, respectively. In Fig. 5 the shoot is seen along the half of the kernel. (20×)

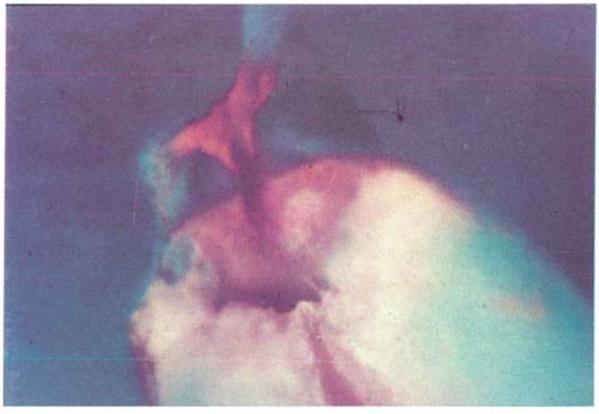


Figure 4

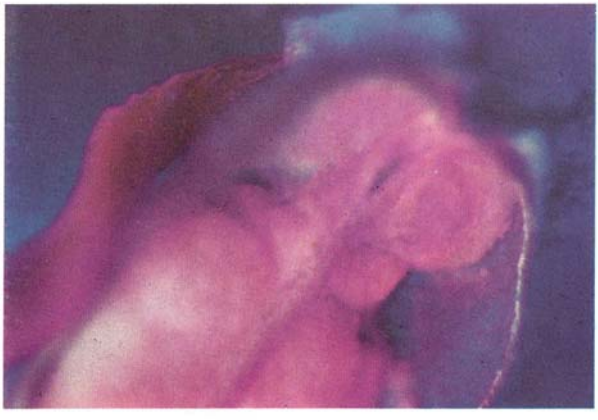


Figure 5

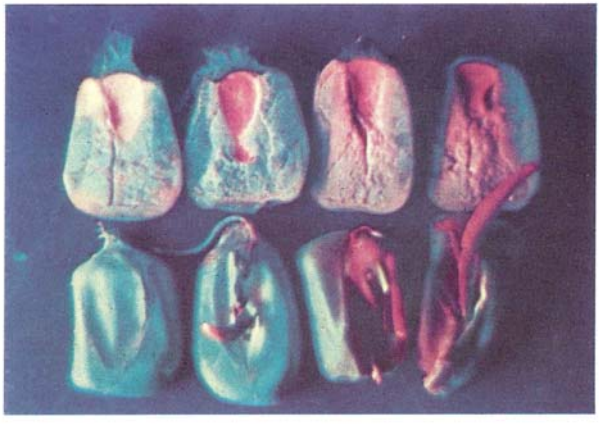


Fig. 6, page 126. Halves of germinating corn, longitudinal, taken with ordinary camera, employing extension tubes; viewed with reflected ultraviolet light. Upper row, exposed interior; lower row, dorsal side showing emerging shoots. From left to right: after 1, 3, 6, and 8 days of germination.