Triglyceride Deposits in the Starchy Endosperm of Wheat [1]

K. D. HARGIN,[2] W. R. MORRISON,[2] and R. G. FULCHER[3]

ABSTRACT

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Triglycerides are stored in spherosomes (oil droplets bounded by a monolayer membrane) in the aleurone and scutellar cells of mature wheat, and were found in the aleurone-free pearled endosperm of Atou, Flinor (English), Waldron (hard red spring) and Edmore (amber durum) wheats. Analysis of Atou endosperm subfractions obtained by microdrilling showed triglycerides throughout the starchy endosperm with the normal endosperm glycolipids and phospholipids. Highest concentrations of triglycerides and glycolipids were found in subaleurone-enriched fractions. In sections of whole grains stained for microscopy with Nile Blue A, spherosomes appeared as bright fluorescent spots largely filling aleurone and scutellar cells in all four wheats. Small numbers of similar fluorescent spots were observed throughout the starchy endosperm, with greater numbers in the subaleurone region. All fluorescent spots were extractable with hexane. Milled aleurone-free endosperm from Atou exhibited similar spots that were hexane-extractable, the extracted lipids being triglycerides and other nonpolar lipids. Triglycerides therefore occur throughout the starchy endosperm of mature wheat as discrete oil droplets that may be normal spherosomes.

Altering the quantities and proportions of nonpolar and polar classes of the nonstarch lipid fraction of white flour can cause a substantial change in loaf volume (Morrison 1978). Flour lipid composition is determined first by wheat variety (genetic factors) and growing conditions but also by the distribution of lipids within the wheat grain and the transfer of germ lipids to starchy endosperm during milling to white flour (Stevens 1959). Little detailed information is found in the literature on any of these topics (Morrison 1978), and thus no sound basis for the control of flour lipid composition exists.

We have recently quantified all the principal acyl lipids in the germ, aleurone, and starch and nonstarch endosperm fractions of four wheats (Hargin and Morrison 1980a). Triglycerides and steryl esters are concentrated in the germ and aleurone, but appreciable amounts also are found in the aleurone-free starchy endosperm.

Triglycerides are stored in plants in spherosomes (oil droplets)

[1]Contribution 592 from Ottawa Research Station.

[2]Department of Food Science and Nutrition, University of Strathclyde, Glasgow G1 1SD, Scotland.

[3]Research Branch, Ottawa Research Station, Agriculture Canada, Ottawa, Ontario K1A 0C6.

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bounded by a half-unit or monolayer membrance of proteins and diacylphospholipids (Jelsema et al 1977, Wanner and Theimer 1978, Yatsu 1972). The aleurone and scutellar cells of wheat are packed with spherosomes, but so far spherosomes have not been observed in mature starchy endosperm (Ayre and Angold 1979; Colborne et al 1976; Jelsema et al 1977; Nougarède and Pilet 1964; Swift and Buttrose 1972; Swift and O'Brien 1972a, 1972b).

In this paper we present chemical and microscopic evidence showing that triglycerides occur throughout the starchy endosperm of wheat as discrete droplets that may be spherosomes.

MATERIALS AND METHODS

Atou and Flinor (English) wheats were obtained from K. Kingswood, RHM Research Ltd., High Wycombe, England, and Waldron (hard red spring) and Edmore (amber durum) wheats from B. J. Donnelly, North Dakota State University, Fargo.

Methods for the dissection of wheat kernels and for the quantification of lipids in each type of tissue are given in full in separate papers (Hargin and Morrison 1980a; W. R. Morrison et al 1975, 1980). Aleurone-free starchy endosperm was prepared by pearling manually degermed wheat until most of the pericarp and aleurone layer had been removed, cutting away the last of these tissues with a scalpel, and then washing the whole starchy endosperm in chloroform methanol (1:1) to remove any adventitious surface lipid. The starchy endosperm was then milled to pass a 75-μm mesh sieve. Additional subfractions of starchy endosperm were obtained from Atou and Waldron as described below.

Endosperm Subfractions

Both ends of a wheat kernel were removed with a scalpel, leaving a transverse section about 3 mm thick. This was held with fine-pointed tweezers under a microscope at ×6 or ×25 magnification and successive layers were abraded, using a parallel-sided dental burr fitted in a variable-speed microdrill. The pericarp, seed coat, and aleurone layers were removed from the dorsal side only, and the sample rinsed for 15 sec in chloroform methanol (1:1, by volume) to remove lipid released from broken aleurone cells. At this stage thin sections viewed at higher magnification showed that cutting had exposed the base of aleurone cells (now empty) or had just penetrated into the adjacent subaleurone layer.

The dorsal subaleurone cells and successive underlying layers of starchy endosperm (inner starchy endosperm layers I, II, III, and IV) were then removed, ending near the ventral subaleurone cells. Because of variations in kernel size and in the precise levels of cutting, the pooled subfractions used for analysis should be regarded as a series of slightly overlapping layers. Abraded endosperm was used directly for lipid analysis because it had a particle size similar to that of flour or of whole pearled endosperm milled to pass a 75-μm mesh sieve. From 400–500 mg of each subfraction was obtained, and this was sufficient for all analyses in triplicate when certain lipid classes were grouped together. The error in determinations was less than 1.5% of mean values for major lipid classes and less than 5% for minor lipid classes.

Fluorescence Microscopy

Plastic Embedding to Retain Lipids. A modification of Pease's (1973) method was used. Kernels were cut transversely into approximately 1-mm thick segments using a single-edged razor blade. Segments were then fixed in 6% glutaraldehyde in 0.025*M* potassium phosphate buffer (pH 6.8–7.0) at 4°C for 24 hr. Fixed tissues were subsequently transferred through 25% buffered glutaraldehyde to 50% stock unbuffered glutaraldehyde (Polysciences Inc.) for a minimum of 24 hr in each solution at 4°C. Finally, the segments were infiltrated for 12–18 hr at 4°C in a modified glutaraldehyde/urea/glycol methacrylate resin (Pease 1973) containing 35% water. The infiltrated segments were prepolymerized in plastic dishes under ultraviolet light (366 nm) for 18–24 hr and finally hardened in an oven at 60°C for 1–2 hr.

Embedded tissues were sectioned 1–2 μm thick, using glass knives, and affixed to glass slides.

Fluorescent Staining of Hexane-Soluble Lipids. Slide-mounted sections were stained for 30–60 sec in 0.01% aqueous Nile Blue A (J. T. Baker Chemical Co., CI 51180), then washed gently in water for 2–3 min, and allowed to dry until required for examination. The stained sections were mounted in a drop of water under a cover glass and examined using a Zeiss fluorescence microscope equipped with a IIIRS epi-illuminating condenser and standard FITC fluorescence exciter-barrier filter combination with maximum excitation at 450–490 nm. Control sections were viewed without staining or were extracted with hexane for 2–3 min before staining.

RESULTS

Lipid Analyses

The composition of the lipids in the aleurone tissues of the four wheats is shown in Table I. The high lipid content of Atou aleurone (19.40%) was calculated using an abnormally low aleurone dry weight and may be wrong. The other data show that Atou aleurone lipid is qualitatively and quantitatively similar to the aleurone lipids in the other three wheats.

The results, which are given in full elsewhere (Hargin and Morrison 1980a), show that aleurone lipids are mostly the reserve lipids found in spherosomes (triglyceride, sterylester) with some phospholipids, perhaps arising from the spherosome membrane (Jelsema et al 1977) and other membranes of the aleurone cells.

The aleurone glycolipids were tentatively identified as monogalactosyldiglyceride, monogalactosylmonoglyceride, digalactosyldiglyceride, and digalactosylmonoglyceride. The phospholipids were (in order) phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, with only small amounts of lysophospholipids and generally none of the *N*-acylphosphatidylethanolamines that are characteristic of starchy endosperm.

The composition of the nonstarch lipids in the aleurone-free starchy endosperms of the four wheats is also given in Table I. Edmore had more triglyceride, similar phospholipids, and less glycolipids than did the three hexaploid wheats.

Detailed analyses (Hargin and Morrison 1980a) showed the same distributions of glycolipids and phospholipids as in flour nonstarch lipids (Hargin and Morrison 1980b, Morrison 1978, Morrison et al 1975). The principal glycolipids were digalactosyldiglyceride and monogalactosyldiglyceride, with small amounts of monogalactosylmonoglycerides and digalactosylmonoglycerides. The principal phospholipids were the *N*-acylphosphatidylethanolamines; the diacylphospholipids consisted of phosphatidylcholine, phosphatidylethanolamine, and a few minor components. The lysophospholipids were lysophosphatidylcholine, lysophospha-

TABLE I
Lipid Composition of Aleurone and Endosperm (Nonstarch) Tissue in Four Wheat Varieties

Tissue	Atou	Flinor	Waldron	Edmore
Aleurone Lipid				
Percent aleurone dry weight	19.40	8.66	8.68	10.63
Grams per kernel	287	267	220	387
Composition, %				
Triglyceride	71.9	66.5	60.3	75.1
Nonpolar lipids[a]	7.7	8.4	12.0	7.9
Glycolipids	6.6	8.0	9.8	2.2
Phospholipids	13.8	17.1	17.9	14.8
Endosperm Nonstarch Lipid				
Percent endosperm dry weight	0.799	1.026	0.982	0.768
Grams per kernel	268	387	259	220
Composition, %				
Triglyceride	26.2	16.7	17.8	34.1
Nonpolar lipids[a]	19.5	19.1	15.4	13.3
Glycolipids	30.7	28.9	38.3	20.4
Phospholipids	23.6	35.3	28.5	32.2

[a]Includes sterylester, diglyceride, free fatty acid, monoglyceride and 6-0-acylsterylglycoside.

tidylethanolamine, and lysophosphatidylglycerol in the proportions found in starch lipids, and they could well be leaky starch lipids rather than partial hydrolysis products of nonstarch phospholipids.

The most interesting feature was that the nonstarch lipids contained triglyceride, although at lower levels than in flour. Because the method used to prepare aleurone-free endosperm precluded any possibility of triglyceride or other lipids being derived from aleurone, germ, or pericarp, the triglyceride must have been in the starchy endosperm.

Information on the distribution of lipids was obtained by analysis of subfractions of Atou endosperm (Table II). Endosperm sections were washed in chloroform/methanol to remove residues of aleurone lipids before microdrilling, and this may have removed some nonstarch lipids from the exposed surface so that the quantities of lipids found in Subaleurone plus Inner Endosperm I and in Inner Endosperm I (prepared on separate occasions) may have been lower than the true values, but the other subfractions would not have been affected.

Subfractions II and III from the central inner endosperm had least lipids. On the dorsal side, subfraction I had a little more phospholipid, and when subaleurone tissue was present all lipids increased, particularly triglyceride, digalactosyldiglyceride, and phospholipids. Values for sterylester, triglyceride, monogalactosyldiglyceride, monogalactosylmonoglyceride, and digalactosyldigylceride were also higher in subfraction IV than in the central fractions, possibly indicating the presence of some ventral subaleurone material. Total starchy endosperm would be mostly inner endosperm, but the subaleurone must have contributed a significant proportion of its triglyceride and digalactosyldiglyceride.

These observations indicate the existence of gradients of most lipids across the starchy endosperm, with particularly marked concentrations of triglyceride in the dorsal and ventral subaleurone regions. Previous work has demonstrated gradients of starch, protein (nitrogen), ash, and vitamin B_1 across the endosperm (MacMasters et al 1971).

Material comparable to the combined subfractions I-III from Atou endosperm was prepared from Waldron. This contained almost the same amounts of triglyceride, other nonpolar lipids, glycolipids, and phospholipids as did aleurone-free pearled endosperm. The results confirm the presence of triglyceride in the inner endosperm of Waldron, but unfortunately lack of material prevented study of lipids in the subaleurone region.

Fluorescence Microscopy

When applied to sections of endosperm tissues embedded in glycol methacrylate/urea/glutaraldehyde resin (Pease 1973), Nile Blue A induced intense fluorescence in the seed cuticle, the aleurone cells, and in most cells of the starchy endosperm (Figs. 1–9). With the exception of the cuticle, all Nile Blue-positive structures were absent if hexane extraction preceded staining (Fig. 3), which strongly suggests that the fluorescent structures were composed largely of neutral lipid.

As expected, the aleurone cells of all four wheat varieties contained much higher concentrations of stainable lipid than did the starchy endosperm cells (Figs. 1,2,4, and 5), and the deposits in the aleurone layers formed a network surrounding the numerous protein bodies in each cell (Figs. 4 and 5). Similarly, the lipid-rich germ was also intensely fluorescent after Nile Blue A staining, and the fluorescence was most notable in the scutellum (Fig. 9).

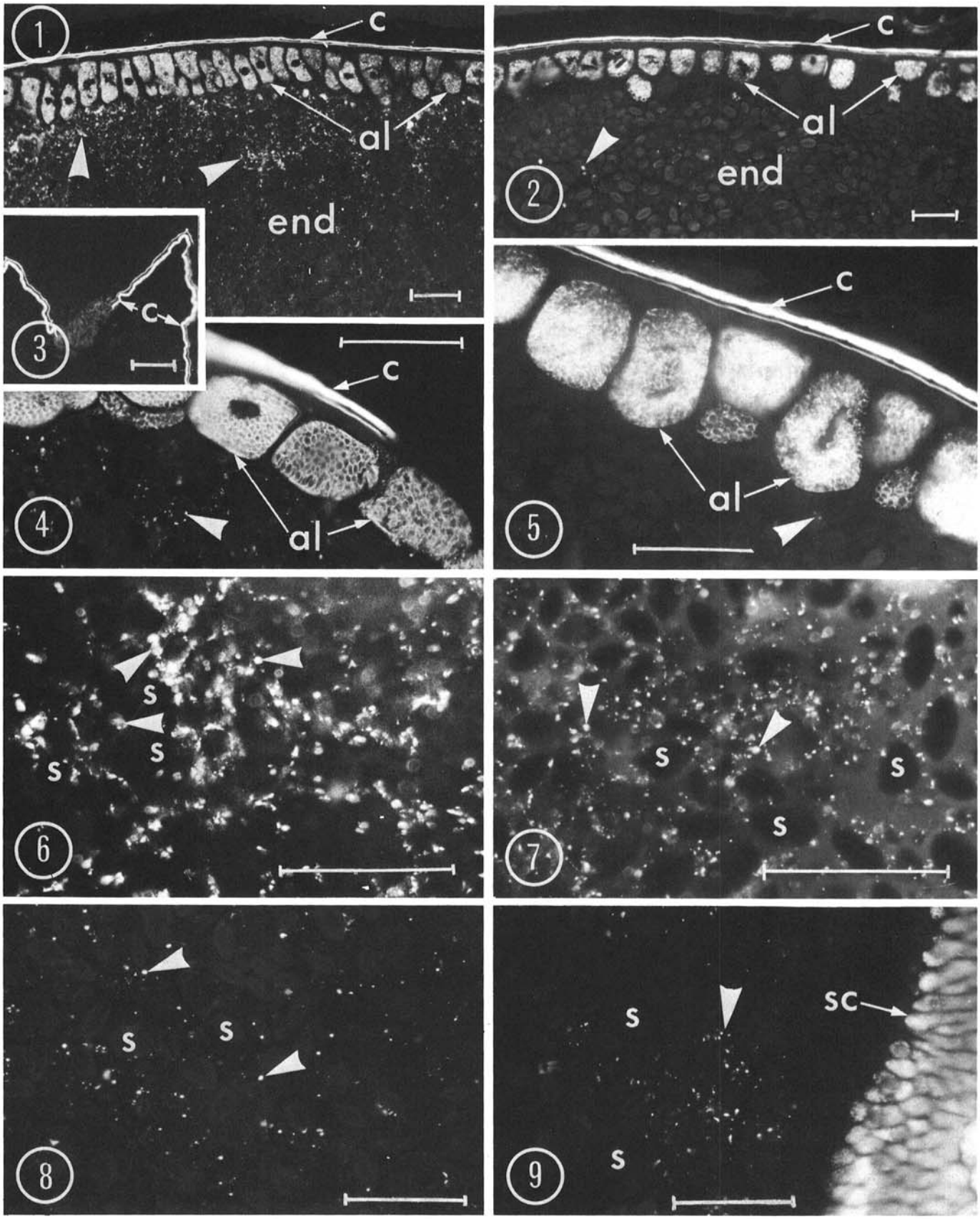
Although we could not detect significant differences in the intensity or distribution of Nile Blue A fluorescence in the aleurone cells of the four varieties, differences that supported the quantitative data on triglyceride contents were evident in the starchy endosperm cells. Edmore (Figs. 1 and 6) invariably contained more spherical fluorescent structures in the starchy endosperm, and particularly in the subaleurone layer (Fig. 1), than did any other variety, whereas Flinor (Figs. 2 and 9) typically had the lowest concentration of fluorescent deposits. Such differences between varieties were evident even at higher magnifications (Figs. 4 and 5). Atou (Fig. 7) and Waldron (Figs. 4 and 8) were intermediate between Edmore and Flinor in the apparent frequency of fluorescent endosperm deposits. In all cases, the starchy endosperm structures were roughly spherical (or were aggregates of spherical deposits), were 0.2–2.0 μm in diameter, and were dispersed throughout the protein matrix. In only a few isolated instances were fluorescent structures observed over starch grains or in other unusual positions, which suggests that mobilization or shifting of the hexane-soluble deposits during embedding or sectioning was minimal. Changing the direction of sectioning did not alter the pattern of lipid deposits in any of the samples.

Because analysis of lipids extracted with hexane from microscopic sections was not possible, flour prepared from aleurone-free Atou endosperm was used as an alternative material for study. The flour contained numerous fluorescent spherical structures (Fig. 10) comparable to those seen in central endosperm sections (Fig. 7). Flour defatted briefly with hexane contained almost none of these structures (Fig. 11). The lipids in the hexane extract were almost entirely triglyceride and other nonpolar lipids (Table II) similar to the nonpolar lipids in the aleurone layer (Table

TABLE II
Distribution of Lipids in the Endosperm of Atou Wheat Kernels[a]

Lipid Class	Aleurone	Subaleurone Plus Inner Endosperm I	Inner Starchy Endosperm Fractions				Total Starchy Endosperm[b]	Hexane Extract[c]
			I	II	III	IV		
Nonstarch Lipids								
Sterylester	670	20	18	17	16	41	15	6
Triglyceride	13,940	205	50	40	30	99	222	155
Other nonpolar lipids	1,250	238	205	192	181	203	179	107
Monogalactosylglycerides	460[d]	59	36	42	36	70	44	5
Digalactosyldiglyceride	110[d]	170	71	86	65	115	173	18
Phospholipids	2,970[e]	347[e]	234[e]	208[e]	218[e]	215[e]	215[e]	11
N-acylphosphatidylethanolamine	···	84	NM[f]	NM	NM	NM	71	5
N-acyllysophosphatidylethanolamine	···	72	NM	NM	NM	NM	50	6
Diacylphospholipids	2,430	31	NM	NM	NM	NM	30	1
Lysophospholipids	260	120	NM	NM	NM	NM	50	1
Total nonstarch lipids	19,400	1,039	614	585	546	743	848	302
Nonstarch lipids, as FAME[g]	17,690	709	397	376	342	490	640	250
Starch lipids, as FAME	···	NM	533	586	567	573	438	NM

[a] Milligrams of lipid per 100 g of dry fraction.
[b] Pearled kernels, some subaleurone missing, milled to pass a 75-μm mesh sieve.
[c] Total starchy endosperm extracted with hexane for 10 min.
[d] Tentative identification.
[e] Includes a small amount of digalactosylmonoglyceride (tentatively identified).
[f] NM = Not measured.
[g] FAME = fatty acid methyl esters.



Figs. 1–9. Fluorescence micrographs of transverse sections of wheat endosperm tissues embedded in glycol methacrylate/urea/glutaraldehyde and stained with Nile Blue A solution. Large arrows indicate endosperm lipid deposits. All scale bars represent 50 μm. al = aleurone layer, c = cuticle, end = starchy endosperm, s = starch, sc = scutellum. **1,** Edmore wheat showing Nile Blue A-induced fluorescence in the cuticle, aleurone layer, and endosperm. Deposits increased in frequency in subaleurone region (adjacent to aleurone layer); **2,** Flinor wheat; comparable to Fig. 1 but showing reduced frequency of deposits in the endosperm; **3,** the groove region of Edmore wheat after hexane extraction. The cuticle remains fluorescent; **4 and 5,** higher magnification of Waldron (Fig. 4) and Flinor (Fig. 5) showing typical intense fluorescence patterns surrounding the protein bodies in each aleurone cell. Subaleurone deposits are more evident in Waldron than in Flinor; **6-9,** central endosperm tissues of Edmore (Fig. 6) Atou (Fig. 7), Waldron (Fig. 8), and Flinor (Fig. 9), showing typical spherical structure of the fluorescent deposits and varying concentrations among the four varieties.

II) and in spherosomes (Jelsema et al 1977). The fluorescent lipid structures in the endosperm clearly resemble spherosomes, but no boundary membrane has been seen, and membrane phospholipids and proteins have not been isolated.

DISCUSSION

Nile Blue A has been known for some time as a lipid stain for bright-field microscopy (Gurr 1960), but to our knowledge its cytological use as a lipid-specific fluorochrome has not been recorded (although fluorescent components were noted by Gurr). The stain has a high affinity for hexane-soluble lipid and cuticular waxy lipid, but membrane-bound glycolipids and phospholipids (which are still present in hexane-extracted tissue) are evidently not detected. Nile Blue A has considerable potential for localizing nonpolar lipids in cereals because the method is rapid, nonextractive, and extremely sensitive.

The fluorescent spherical structures found in aleurone and scutellar cells were undoubtedly spherosomes. The evidence presented in this paper indicates that the fluorescent structures in starchy endosperm may also have been spherosomes, but they were not isolated and thus no direct information on the nature of their core and membrane lipids was obtained.

Hexane extraction removed the bulk of the spherosome lipids that gave a fluorescent reaction with Nile Blue A, but hexane would also extract nonpolar lipids from other sites. The hexane-extracted lipids from endosperm flour (Table II) were consistent with spherosome core lipids without the polar membrane phospholipids (Jelsema et al 1977), and coextraction of other low polarity endosperm lipids would account for the *N*-acylphospholipids and perhaps also for the rather high levels of diglyceride, free fatty acid, monoglyceride, and acylsterylglycoside.

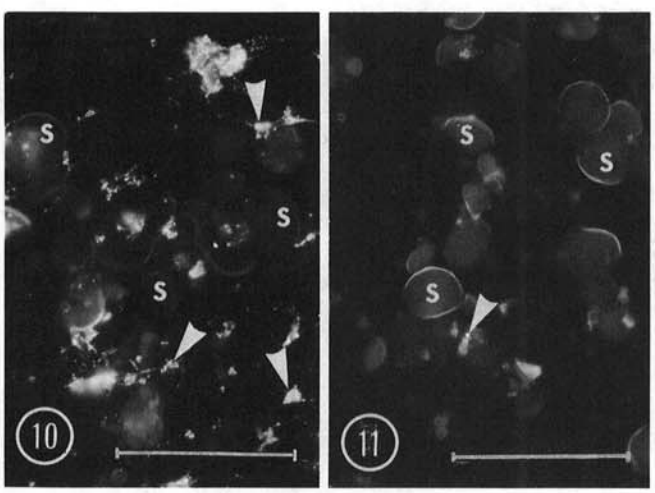
Spherosomes accumulate in developing aleurone and scutellar cells, and their sterylesters and triglycerides provide important reserves during the early stages of grain germination (Ayre and Angold 1979; Buttrose 1963, 1971; Colborne et al 1976; Drapron et al 1969; I.N. Morrison et al 1975, 1978; Nougarède and Pilet 1964; Swift and Buttrose 1972; Swift and O'Brien 1972a, 1972b; Tavener and Laidman 1972a, 1972b). Spherosomes have been observed in starchy endosperm cells at early stages of development (Angold,[4] Ayre, and Angold 1979) but not at maturity, although triglyceride is actively metabolized in starchy endosperm during germination (Tavener and Laidman 1972a, 1972b).

Simmonds (1972a, 1972b) has described two types of osmiophilic lipid inclusions, from 1–3 μm in diameter, in flour and dough. Type I inclusions are derived from remnants of membranes and organelles and show typical bilayer structure in transmission electron microscopy. The lipids would be expected to consist of glycolipids and phospholipids. Type II inclusions are extractable by chloroform and petroleum ether and appear to be similar to the structures visible under fluorescence described in this paper. Simmonds considered type II inclusions to be lipid-rich material, possibly derived from aleurone and scutellar spherosomes, which form oil droplets during dough mixing. In the present study, lipid structures viewed by fluorescence in starchy endosperm appeared to survive unchanged after milling to flour; Simmonds' type II inclusions possibly consisted of endosperm oil droplets as well as of lipids expressed from germ.

Other authors (Adams et al 1976; Barlow et al 1973; Crozet 1977; Crozet and Guilbot 1974; Crozet et al 1966, 1974; Seckinger and Wolf 1967, 1970) have described osmiophilic inclusions, which they regard either as remnants of membranes and organelles or as artefacts formed when expressed aleurone or germ lipids coalesce into droplets on wetting of flour or on exposure to aqueous osmium tetroxide.

In flour milled from whole Atou wheat a substantial part of the flour sterylester and triglyceride is derived from the germ, but no germ phospholipids and none of the aleurone lipids are transferred (Hargin and Morrison, 1980b). The proportion of triglyceride in flour is thus determined by the quantity present in the starchy endosperm and by the amount transferred from the germ during milling. The former determines the lowest level of triglyceride and is probably a varietal character modified by environmental factors during grain development. The latter might be controlled to a limited extent by the miller and could, of course, be raised to any desired level by the addition of a suitable oil or fat to a bakery recipe.

[4]R. E. Angold, personal communication, 1979.



Figs. 10 and 11. Samples of Atou flour before and after hexane extraction. Samples were prepared by suspending a small amount of each flour in 1–2 drops of Nile Blue A solution under a cover glass on a microscope slide. Unextracted flour (**Fig. 10**) contained a considerable number of intensely fluorescent lipid deposits (arrows) interspersed among the starch grains (s) and other components. Hexane-extracted flour (**Fig. 11**) contained very few of the typically spherical deposits.

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