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The Structure and Composition of Aleurone Grains in the Barley Aleurone Layer

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Summary. Cytochemical methods have been used in conjunction with light and electron microscopy to determine the nature of the inclusions in aleurone grains of barley aleurone layers. Two kinds of inclusions were found: (1) Globoids within globoid cavities which were not enclosed by a membrane: the globoids stained red with toluidin blue due to the presence of phytin, and with lipid stains; (2) Protein-carbohydrate bodies which stained green with toluidin blue. The characteristics of globoids and protein-carbohydrate bodies as seen in the electron microscope are described in detail using both glutaraldehyde- and permanganate-fixed tissues. The protein-carbohydrate body was identified by silver-hexamine-staining; this was not caused by carbohydrate but by some component which stained green in toluidin blue and which also occurred in cell walls in a thin band adjacent to the cytoplasm. The characteristics of both bodies are discussed in relation to apparent confusion in their identities in previous electron-microscope studies.

Introduction

The aleurone layer of barley seed consists of living tissue, usually three cells in thickness, surrounding the endosperm. This tissue has come to be of considerable interest in studies of plant hormone action since it was shown to be the site of production of hydrolytic enzymes appearing in de-embryonated seed after treatment with gibberellic acid (MacLeod and Millar, 1962; Paleg, 1963; Varner, 1964). In the course of cytochemical studies of enzyme induction in barley aleurone (Jacobsen and Knox, 1971) we found it necessary to characterize the structure of the aleurone grain.

Aleurone grains of various plant tissues have been reported to contain a variety of substances. The most extensively described aleurone-grain inclusion is the globoid originally described by Pfeffer (1872). It occurs in the aleurone grains of a variety of tissues and is a site of accumulation of phytin, the calcium and magnesium salt of *myo*-inositol hexaphosphate, probably bound to protein (Pfeffer, 1872; Poux, 1963; Sobolev, 1966; Lui and Altschul, 1967). The grains of various species have also been reported to contain lipid in the globoid (Salmon, 1940) or as free droplets (Poux, 1965), crystals of protein

called crystalloids (Hartig, 1856; Poux, 1965), and crystals of calcium oxalate (Haberlandt, 1914). There is little information in the literature bearing directly on the nature of barley aleurone-grain inclusions; however from the early studies Guillermond (1908) and Vazart (1960), it appears that there are two kinds of inclusions. One kind was identified as a globoid and the other as a proteinaceous granule.

In electron-microscope studies of barley aleurone tissue, two types of inclusions, based on electron density, have been found within aleurone grains (Paleg and Hyde, 1964; Eb and Nieuwdorp, 1967; Jones, 1969a, b). There is some evidence that one of these, the electron-dense inclusion in glutaraldehyde-fixed tissue, is a globoid, the density to electrons being caused by phytin (Eb and Nieuwdorp, 1967) but there is very little information available on the nature of the other, less dense inclusion. The purpose of this report is to describe the characteristics of barley aleurone-grain inclusions using light microscopy, and to identify the inclusions as seen in the electron microscope. In the process, we present evidence that some previous identifications of the globoid in electron micrographs of permanganate-fixed aleurone tissue (Eb and Nieuwdorp, 1967; Jones, 1969a, b) are incorrect.

Materials and Methods

Tissue Preparation

Aleurone tissue was obtained from seed of barley, *Hordeum vulgare* L. cv. Himalaya. The aleurone layers with attached seed coats were isolated from half seeds imbibed as described by Chrispeels and Varner (1967). Time of imbibition was usually 72 h but tissue imbibed for shorter times was used in several instances.

Light Microscopy

Layers were sectioned in both fixed and unfixed states depending on the histochemistry to follow. Fixation was done in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, or in some cases 4% formaldehyde (prepared from paraformaldehyde) in 0.05 M phosphate buffer pH 7.2 in both cases for 6 h at 2°C. After fixation, the layers were washed for 12–15 h in several changes of the fixing buffer at 2°C. The tissue was supported in a gelatin-antifreeze medium (either 8%, w/v, gelatin and 4%, v/v, glycerol for sectioning at −24°C; or 15% gelatin and 2% glycerol for −10 to −15°C for cryostat sectioning as described by Knox and Evans, 1966, 1968). 2-μ sections were cut, rapidly thawed on clean, dry slides and air-dried at room temperature before use. For fresh tissue, adhesion of the sections was improved by thawing onto slides which had been coated with 1% gelatin and air-dried.

Cytochemical Procedures

Toluidin Blue. Sections were stained in 0.02% toluidin blue 0 (Harleco, C.I. No. 52040) in acetate buffer. Originally we used 0.2% dye in buffer at pH 4.0 as recommended by Prescott (1964). However at this concentration and low pH, microchemical gardening of the phytin globoids occurred and precise localization

became problematical. The difficulty was overcome by staining in 0.02% toluidin blue at pH 5.0.

The slides were examined immediately after rinsing and mounting in buffer and also after dehydration in 95% ethanol (10 s), ethyl cellosolve (10 s) and xylene before mounting in Eukitt (O. Kindler, Freiburg). Absorption spectra of stained organelles were obtained using a Carl Zeiss UMSP1 Ultramicrospectrophotometer.

Phosphate. Phosphate localization was carried out by incubating sections in 0.12% lead acetate in 0.05 M acetate buffer at pH 5.0 for 10 min at room temperature (Gomori, 1952). The slides were rinsed in buffer and the lead phosphate was converted to lead sulphide by placing the slides for 2 min in water saturated with hydrogen sulphide (Bitensky, 1963). The sections were then dehydrated as above, or alternatively stained in naphthol yellow S for protein localization (see below).

Protein. Protein was localized by staining sections in naphthol yellow S (Deitch, 1955; Moss, 1967), Ponceau 2R (Flint and Moss, 1970), or mercuric bromophenol blue (Pearse, 1961).

Polysaccharides. These were localized using the periodic acid-Schiff reaction (PAS) (see Jensen, 1962). Controls were prepared by incubating sections in the Schiff reagent without previous hydrolysis in periodic acid.

Lipids. Sudan IV and Sudan black B both in 60% triethyl phosphate (Jensen, 1962) were used to localize lipids.

Electron microscopy.

Aleurone layers obtained from seeds which had imbibed water for three days, were cut into small pieces about 1 mm square and these were fixed mainly by two methods.

(1) 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 7.2) for 2 h at 21°, followed by a wash in phosphate buffer and post-fixation in 2% osmium tetroxide in 0.025 M sodium phosphate buffer (pH 7.2) for 2 h at 21°.

(2) 2% potassium permanganate in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at 4°C.

After both fixation procedures, the tissue pieces were washed in phosphate buffer, dehydrated in ethyl alcohol and propylene oxide, and embedded in the low-viscosity epoxy resin of Spurr (1969) employing Mixture A. Silver to gold sections were cut using a Reichert "Om U2" ultramicrotome.

Some tissue was fixed in formaldehyde followed by osmium, a mixture of formaldehyde and glutaraldehyde followed by osmium, and in osmium alone but results involving these fixations will be referred to only in brief.

Unless otherwise stated, sections of glutaraldehyde-osmium fixed tissue were stained in a saturated solution of uranyl acetate in 50% ethyl alcohol followed by lead citrate (Fiske, 1966); sections of potassium permanganate fixed tissue were stained with lead citrate only.

The silver hexamine test for carbohydrate was performed as described by Rambourg (1967). All sections were examined with a Philips EM200 electron-microscope.

Results

Light Microscopy

Cells stained with toluidin blue are shown in Fig. 1a. Whether unfixed, fixed in formaldehyde of glutaraldehyde, or imbibed for 16 or 72 hr, the appearance and distribution of organelles was the same.

a) Identification of Globoids. Bodies which stained intensely red occurred inside transparent areas which we shall refer to as globoid

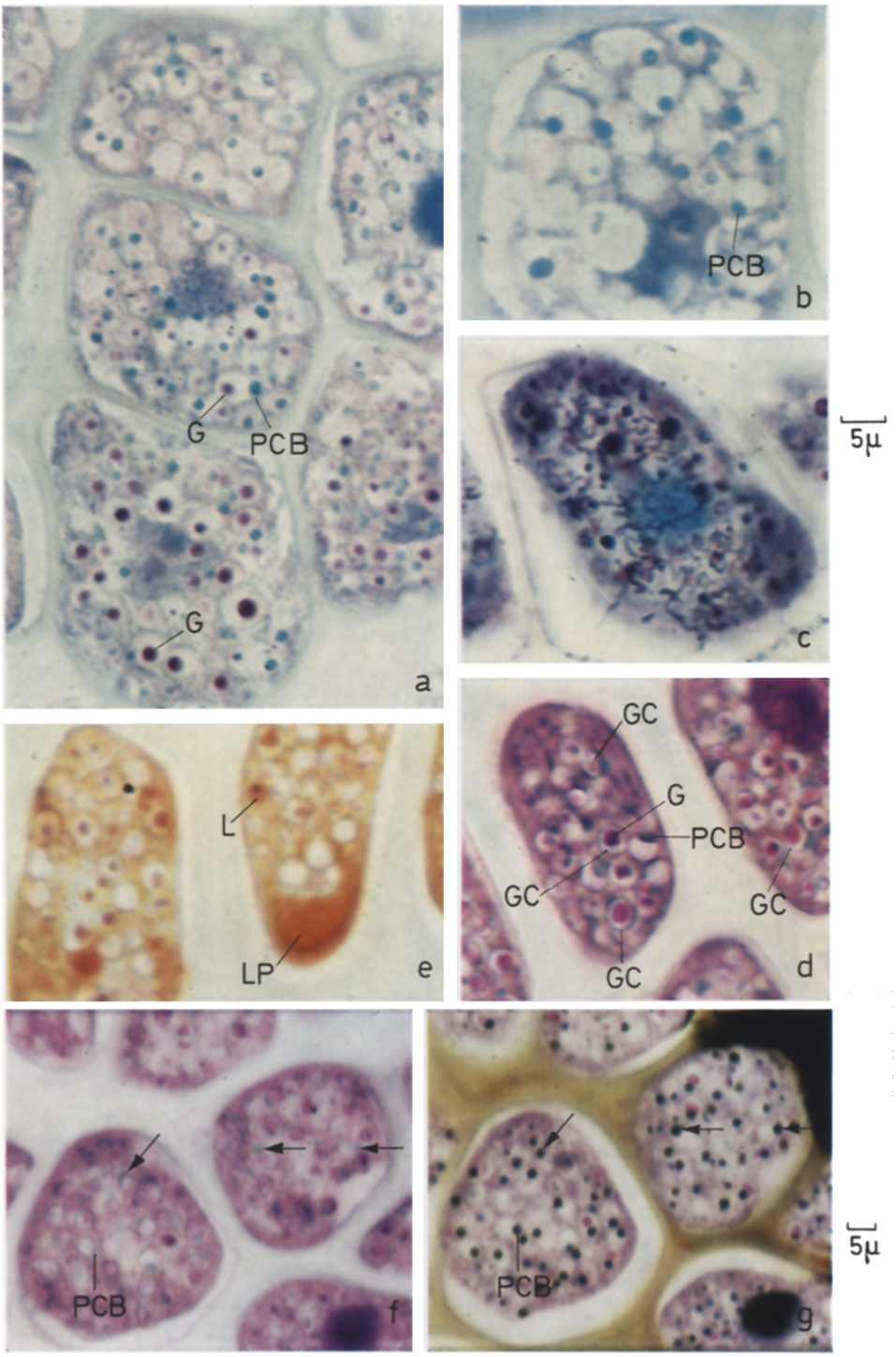

Fig. 1 a–g

cavities, and these in turn were contained by the aleurone grains. The globoid cavities are shown clearly in Fig. 1d where the tissue has not been differentiated and dehydrated after staining with toluidin blue. The globoids did not fill the cavities and the remaining volume had no stainable contents. In some tissue, the globoids filled the cavities completely. In differentiated and dehydrated tissue (Fig. 1a), it was difficult to distinguish globoid cavity from aleurone grain ground substance. Each aleurone grain usually contained one but occasionally two or three globoid cavities; each of these contained one red body which varied in size depending on the position of the cell in the aleurone layer. In the outer cells next to the seed coat (top Fig. 1a) the bodies were 0.5 μ or less in diameter and in some cells they were not present at all. The bodies increased in size and staining intensity in cells towards the inner surface of the layer (bottom Fig. 1a) where they ranged from 1 to 3 μ in diameter. The red bodies were most prominent and almost always present in aleurone grains of cells adjacent to the suture where they were numerous per cell, stained intensely, averaged 1.5 μ in diameter and usually filled the globoid cavity.

Metachromatic staining (red) with toluidin blue is known to be a characteristic of globoids (Vazart, 1960). Since globoids of other plant tissues contain phytin and since polyphosphate compounds are known to stain metachromatically with toluidin blue (Wiame, 1947) it seemed likely that phytin was responsible for metachromasia in barley globoids. If the sections were immersed in 0.1 N acetic acid for 5 min, rinsed in 0.1 M acetate buffer pH 5.0 and then stained in toluidin blue, the bodies no longer absorbed the dye. The same effect could be produced by placing sections in 0.1 M acetate buffer pH 4.0

Fig. 1a–e. Light micrographs of aleurone tissue stained in various ways to demonstrate aleurone grain inclusions. a Stained in toluidin blue, dehydrated and mounted in Eukitt showing globoids (*G*) and protein-carbohydrate bodies (*PCB*). b Same as a, but the section was incubated in 0.05 M acetate buffer, pH 4, for 10 min before toluidin-blue staining and the globoids have been dissolved. c Stained in 0.2% toluidin blue at pH 4.0 showing redstaining tubules (microchemical gardening) arising from globoids. d Stained in toluidin blue and mounted in water without dehydration. Globoid cavities (*GC*) containing globoids (*G*) are clearly shown. e Stained in Sudan IV showing lipid (*L*) in the aleurone grains. Lipid pools (*LP*) occur above the sections. f and g Light micrographs showing that the protein-carbohydrate body which stains green with toluidin blue is also silver-hexamine positive. f Stained with toluidin blue and mounted in water for photography. The green-stained bodies are the protein-carbohydrate bodies (*PCB*). g The same section as in f, but subsequently silver-hexamine-stained, dehydrated and mounted in Eukitt. The same cells were rephotographed to show that the protein-carbohydrate bodies are silver-hexamine positive. The arrows point out identical bodies.

for 15 min (Fig. 1b). Sections incubated for 15 min in phosphate buffer pH 6.8 showed no loss of staining. Hence the component causing the metachromatic reaction was soluble, like phytin, in acid solution.

A marked decrease in staining intensity occurred when sections were treated with a solution of wheat-germ acid phosphatase or phytase. This was presumably due to the removal of phosphate groups from phytin which probably provide the negative charges necessary for metachromatic staining (Pearse, 1961).

If the pH of staining was 4.0, numerous small outgrowths accumulated around the red bodies (Fig. 1c). These outgrowths also stained red. This chemical gardening effect is characteristic of phytin (Jensen, 1962) and could be eliminated by staining at pH 5 or above. A similar effect was produced with a ferric-chloride solution but in this case, the outgrowths were long and tubular.

Absorption spectra of the red-stained bodies showed one peak at 555 nm and purified phytin set in gelatin, sectioned and stained as for tissue sections, also stained red and had an absorption maximum of 555 nm.

In addition, using selective filtration and centrifugation procedures similar to those of Lui and Altschul (1967) we have prepared a fraction from an homogenate of isolated aleurone layers which was greatly enriched in bodies which stained red in toluidin blue. It is known that Dowex-50 ion-exchange resin will complex the calcium and magnesium ions of phytin leaving phytic acid which is soluble (Sobolev, 1966). If some Dowex-50 resin was added to the enriched fraction suspended in water and the mixture left to stand for 30 min, there were no longer any bodies present which would stain red with toluidin blue. At the same time a phosphate compound appeared in the supernatant which co-chromatographed with purified phytin solubilized in the same way. The chromatography solvent system was N-propanol:ammonia (25% solution):water (50:40:10) (Sobolev, 1966) and the phosphate compounds were detected by the method of Rosenberg (1959).

Cytochemical methods showed that the red-staining bodies contained high levels of phosphate (Fig. 2d), leaving little doubt that these bodies are the globoids and that the red colour is due to the presence of phytin as well as perhaps free phosphate. Globoids contained protein (Fig. 2c) to which phytin could be bound. Where the globoid did not fill the globoid cavity phosphate was seen to occur in high concentration around the periphery of the globoid cavity and to a less extent in the ground substance of the globoid cavity.

Another distinguishing feature of globoids is that they are anisotropic. When viewed in polarized light, they show the typical "Maltese

cross" pattern which indicates that they are constructed in a highly ordered manner. None of the other bodies to be described were anisotropic before or after staining.

b) Protein-Carbohydrate Body. Also associated with the aleurone grains were bodies which stained green (absorption maxima 598 and 640 nm) with toluidin blue (Fig. 1a). They were quite uniform in size (1–1.5 μ in diameter, occasionally up to 2 μ) and number throughout the aleurone layer and were usually present throughout the layer irrespective of whether globoids were present or not. There appeared to be usually one green body associated with each aleurone grain. The green staining of the bodies was not due to dehydration of the tissue before mounting because the bodies were green in sections which were stained and mounted for observation in water (Fig. 1d). Presumably the staining characteristic of these bodies is due to some unusual component which possibly also occurs in the cell wall which also stained green. The wall staining was light except for a thin band adjacent to the cytoplasm which stained more intensely (Fig. 1a, d). In both fresh and glutaraldehyde-fixed tissue, both these bodies and the cell wall were stained red by the periodic acid-Schiff technique for detecting carbohydrate (Fig. 2a), but not by the Schiff reagent alone (Fig. 2b). They also contained a very high concentration of protein as shown by Ponceau 2R staining (Fig. 2c). The ground substance of the aleurone grain also contained high concentrations of protein but appeared to contain less than protein-carbohydrate bodies in terms of the total aleurone grain content. Exactly the same results were obtained with the other protein stains naphthol yellow S and mercuric bromophenol blue. Hence this organelle will be referred to as the protein-carbohydrate body.

c) Lipid. Aleurone cells stained heavily with Sudan IV (Fig. 1e) and Sudan Black B (not shown), indicating a high content of lipid. An aleurone grain inclusion which had about the same size, shape and occurrence as the globoids stained intensely, and by sequential staining of tissue with Sudan IV and then toluidin blue, it was not possible to distinguish globoids from the Sudan-positive inclusions. Therefore we concluded that it was the globoids which were Sudan positive. After immersion in ethanol-ether to dissolve lipids, the sections did not stain in Sudan IV but staining in toluidin blue was unchanged. Thus the globoids appear to contain all or most of the lipid of the barley aleurone grain. However, because cytoplasmic lipid was mobile during sectioning of the tissue (see below) the possibility that the globoids were contaminated with cytoplasmic lipid cannot be excluded.

The cytoplasmic staining was presumably due to the spherosomes which in barley appear to be lipid-containing (Jones, 1969b) and the

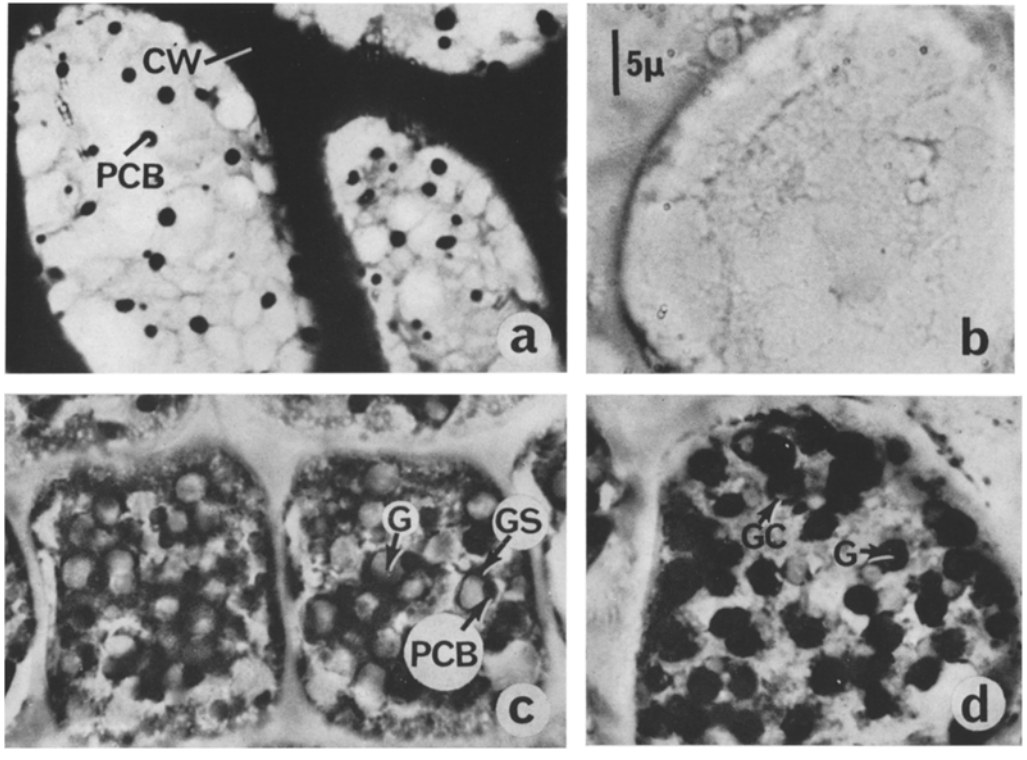

Fig. 2a–d. Light micrographs of unfixed tissue showing localizations of protein, carbohydrate and phosphate in aleurone tissue. a The protein-carbohydrate bodies (*PCB*) and cell walls are stained intensely by the periodic acid-Schiff reaction for demonstrating carbohydrate. b Control for a, showing that the Schiff reagent alone does not stain anything. c The protein-carbohydrate bodies (*PCB*) and the aleurone grain ground substance (*GS*) stained by Ponceau 2R demonstrating a high protein content. The globoids (*G*) are lightly stained and therefore contain some but relatively little protein. d Treated with lead acetate and then hydrogen sulfide demonstrating phosphate in the globoids (*G*) and around the periphery of the globoid cavities (*GC*). The ground substance of the globoid cavity also contains some phosphate. The rest of the aleurone grain is unstained

lipid which accumulated in pools above the plane of the section probably originated from the spherosomes.

Aleurone-Grain Fine-Structure

From light-microscope studies, it was not clear what relationship the protein-carbohydrate body bore to the aleurone grain because it was difficult to decide the limits of the aleurone grain. Therefore, we attempted to localize the carbohydrate-containing body using electron microscopy and the silver hexamine reaction but firstly it was necessary to understand aleurone-grain fine-structure as well as possible.

A typical glutaraldehyde-osmium(glut-Os)-fixed aleurone grain is shown in Fig. 3a. The aleurone grains were surrounded by spherosomes. Each grain was enclosed by a single unit-membrane and contained relatively electron-dense and electron-transparent inclusions. This description is generally in keeping with previous studies (Paleg and Hyde, 1964; Eb and Nieuwdorp, 1967; Jones, 1969a, b). However, we found no evidence that spherosomes were membrane-bound. Fig. 4 shows that while in glut-Os-fixed tissue the unit membrane of the aleurone grain was clearly defined, there was no such membrane associated with the spherosomes. However, spherosomes were bounded in places by thin, electron-dense lines which were most likely boundary staining artifacts.

The aleurone-grain membrane was not clearly visualized using the other four fixation techniques and glut-Os fixation was also superior in resolving the unit-membrane nature of the plasmalemma, the endoplasmic reticulum and of the membranes of the nuclei, plastids, mitochondria and Golgi bodies.

There was usually one or rarely there were two electron-dense bodies found in an aleurone grain. They were finely granular in appearance, not membrane bound, and had essentially the same electron density in all aleurone grains examined. It is these bodies which we will show to be the protein-carbohydrate bodies.

The electron-transparent inclusions were usually the larger of the two and grains contained from one to several. These areas contained no epoxy resin and were the areas from which globoids had been torn out during thin-sectioning. Occasionally in thickly sectioned, unstained glut-Os-fixed tissue, highly electron-dense globoid sections were retained in varying degrees of intactness (Fig. 3b) but these were dissolved out of the tissue after uranyl-acetate and lead-citrate staining to leave either a clear hole or a diffuse framework of the globoid supported on the parlodion-carboncoated grids. Fiske's lead citrate is strongly alkaline which could account for phytin dissolution. The clear areas have been

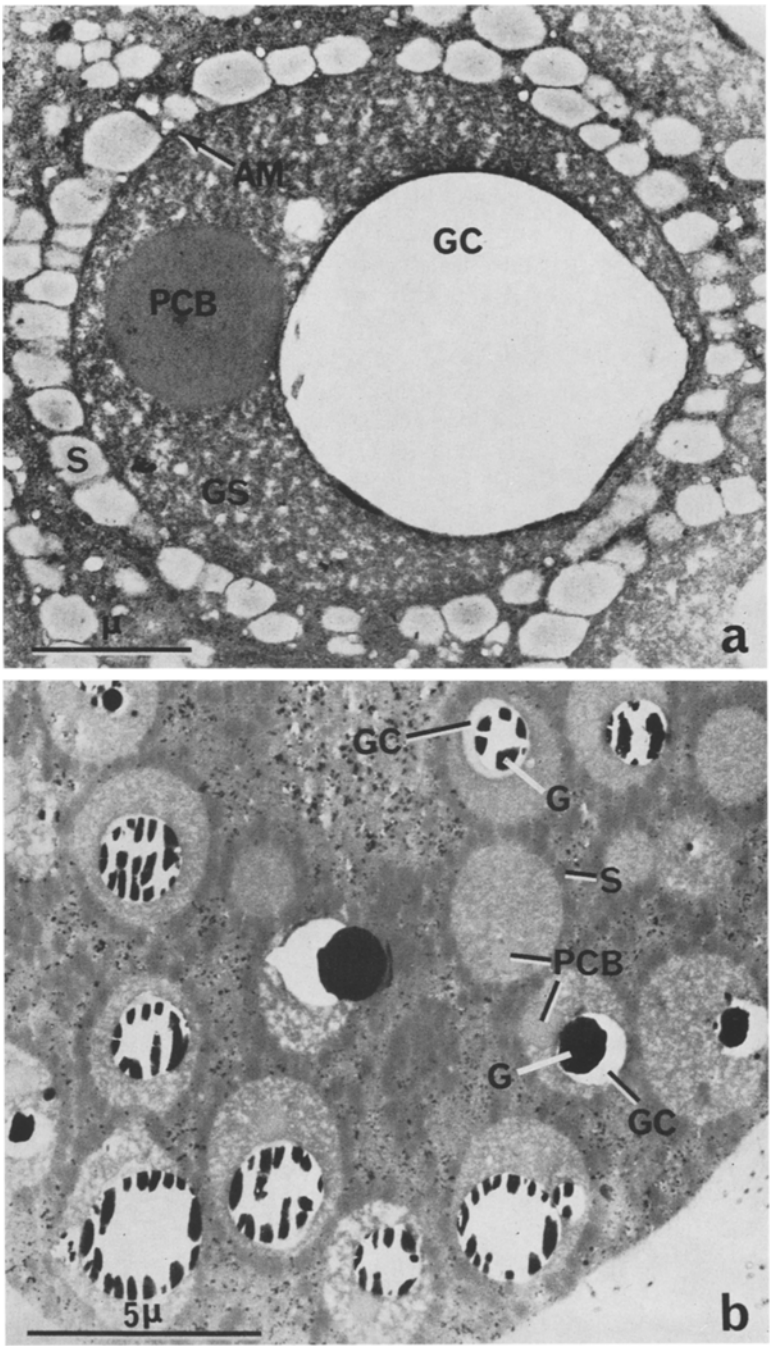

Fig. 3a and b

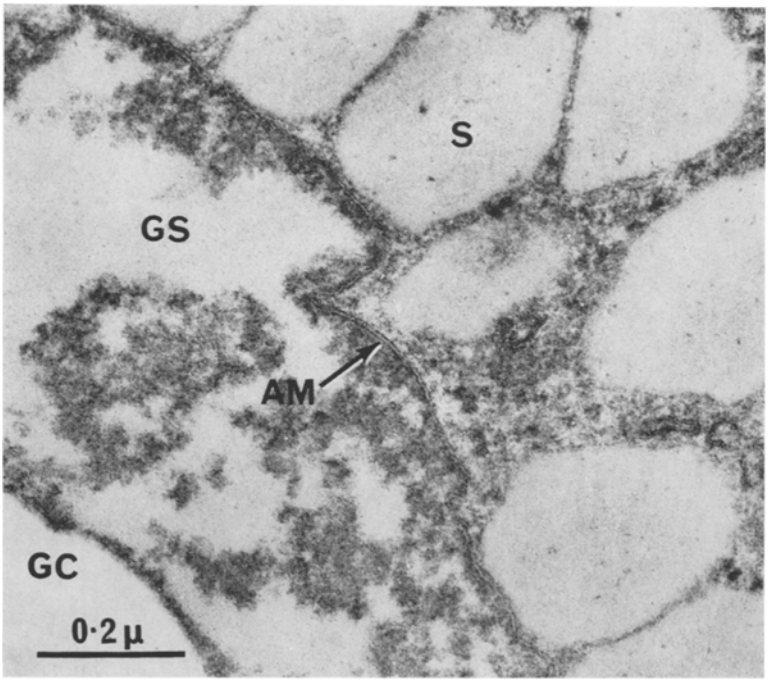

Fig. 4. A high-magnification electron micrograph of glutaraldehyde-osmium-fixed tissue showing that the aleurone grain membrane (*AM*) is a well-defined unit-membrane and that there is no unit membrane around the spherosomes (*S*) or the globoid cavity (*GC*). The ground substance of the aleurone grain is annotated *GS*

previously called internal cavities (Eb and Nieuwdorp, 1967); however, we shall refer to them as globoid cavities as this seems more appropriate.

At intermediate magnifications the globoid cavities seemed to be surrounded by an electron-dense, membrane-like boundary but at higher magnifications (Fig. 4) this boundary did not possess the fine structural characteristics of a unit membrane (parallel darklight-dark bands) and furthermore it varied considerably in thickness. Consequently,

Fig. 3a and b. Electron micrographs of aleurone tissue showing typical aleurone grains. a Glutaraldehyde-osmium-fixed tissue showing the aleurone-grain membrane (*AM*) enclosing a globoid cavity (*GC*) which contains no epoxy resin, the ground substance (*GS*) and a protein-carbohydrate body (*PCB*). The whole aleurone grain is surrounded by spherosomes (*S*). b A thick section of glutaraldehyde-osmium-fixed tissue which was not post-stained, showing globoids (*G*) retained within the globoid cavities (*GC*). Many of the globoids are shattered during sectioning while others are displaced from their original position. Other notations are the same as for a

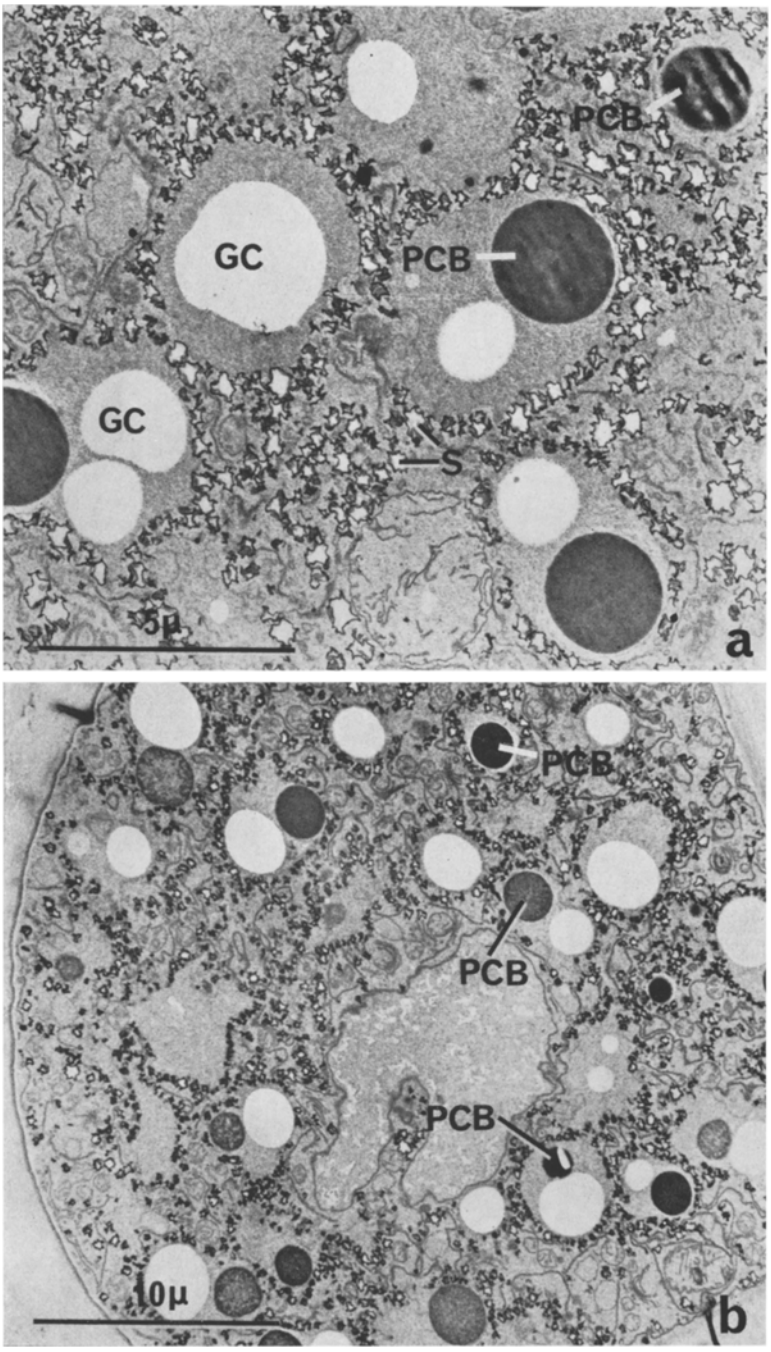

Fig. 5a and b

we conclude that the globoid cavity is not membrane-bound which contrasts with the work of Poux (1965) who concluded that globoids in the cotyledons of *Cucumis sativus* were membrane-bound.

In potassium-permanganate-fixed tissue (Fig. 5a), aleurone grains contained electron-dense and -transparent bodies as in glut-Os-fixed tissue but the spherosomes were malformed as is usual for this fixation of imbibed tissue (Paleg and Hyde, 1964). There was no evidence of unit membranes surrounding spherosomes as reported by Paleg and Hyde (1964) and Jones (1969a) even in lead-citrate-contrasted sections. However, spherosomes were surrounded by a wide electron-dense border and a possible interaction between potassium permanganate and lipid has already been discussed by Eb and Nieuwdorp (1967). Based on their frequencies of appearance per aleurone grain in electron-microscope sections, the electron-dense and -transparent bodies are the same as those in glut-Os-fixed tissue. In permanganate-fixed tissue, there were 0.56 electron-dense bodies and 0.96 transparent bodies per aleurone grain (261 grains counted) and in glut-Os-fixed tissue, the frequencies were 0.47 and 0.95 per aleurone grain, respectively (330 grains counted). However, in permanganate-fixed tissue, the density of the electron-dense bodies varied from medium to very dense, much more dense than in glut-Os-fixed tissue but no matter how densely stained, the bodies were always granular in appearance in contrast to the globoids which are uniformly electron-opaque and amorphous in both glut-Os-fixed (Fig. 3b) and permanganate-fixed tissue (Jones, 1969b, Fig. 1a). These bodies were often rippled in appearance, presumably due to sectioning (Fig. 5a and 5b); this is reminiscent of the shattered globoids seen in glut-Os-fixed tissue, but the bodies were never fractured into many pieces like globoids.

The globoids were never visualized as electron-dense bodies after permanganate fixation. They appeared to have been dissolved out of the grains during processing for electron microscopy to leave epoxy-resin-filled, electron-transparent globoid cavities. In a few cases, remnants of the globoid were retained but only at the peripheral regions as illustrated by Eb and Nieuwdorp (1967) in their Fig. 4. Although Jones (1969b) has shown some micrographs with globoids retained

Fig. 5a and b. The appearance of aleurone grain inclusions in permanganate-fixed tissue. a The protein-carbohydrate bodies (*PCB*) have the same shape and frequency as in glutaraldehyde-fixed tissue but vary in electron density. The globoid cavities (*GC*) appear as they do in glutaraldehyde-osmium-fixed tissue but contain epoxy resin. The aleurone grains are surrounded by spherosomes (*S*). b The protein-carbohydrate bodies (*PCB*) have a fine granular appearance regardless of their electron density and some of them tend to section very unevenly

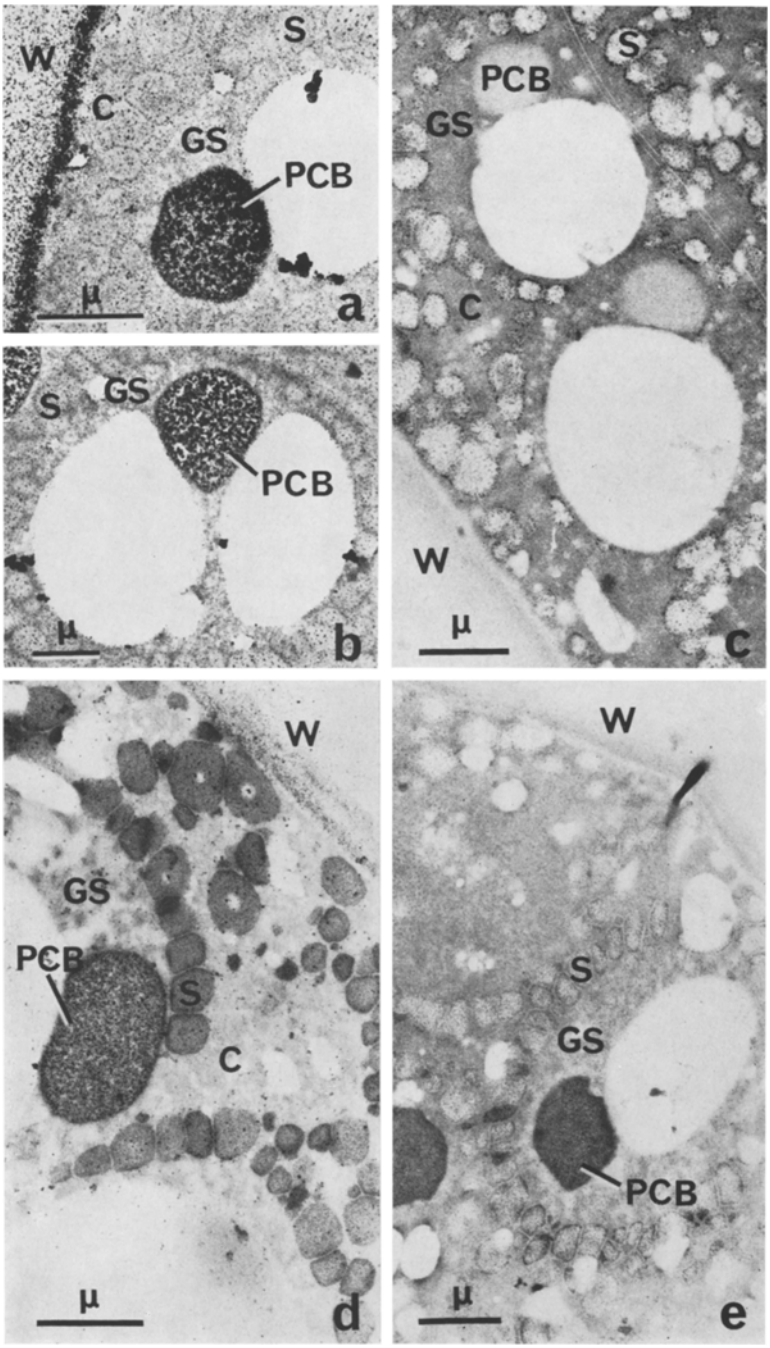

Fig. 6a–e

after permanganate fixation, he too appears to obtain the same electron-transparent image as we do in most cases.

Localization of the Protein-Carbohydrate Body

In glut-Os-fixed tissue, both the electron-dense body and the cell wall, especially a thin strip adjacent to the cytoplasm, stained heavily with silver hexamine whether or not the tissue had been pretreated with periodic acid or chromic acid (Fig. 6a, b). The cytoplasm and spherosomes also stained but much less intensely. Staining of the cell wall, the electron-dense body and the cytoplasm was eliminated if the sections were pretreated (blocked) with alkaline iodoacetate (Pickett-Heaps, 1967) but the spherosome staining was unaffected (Fig. 6c). Bisulfite (Pickett-Heaps, 1967) and chlorous acid (Rappay and Van Duijn, 1965) were ineffective blocking agents and caused disruption to the sections. If the tissue was blocked and then oxidized with periodic acid for 30 min or chromic acid for 1 h, the electron dense body and a thin layer of cell wall around the cytoplasm were again positive to silver hexamine although they were not as intensely stained as originally (Fig. 6d). This would have been taken as a positive test for carbohydrate had it not been for the fact that 0.05 N HCl also caused the same zones to become silver-hexamine-positive again (Fig. 6e). None of these treatments caused silver staining of the bulk of the cell wall which presumably should have stained because of its carbohydrate content. Treatment of blocked tissue with 0.05 M KCl at pH 6.8 and 0.05 M $K10_4$ at pH 6.8 for up to 2 h did not reintroduce staining so that the effects of periodic, chromic and hydrochloric acids were presumably due to a reversal of the iodacetate blocking at low pH and not

Fig. 6a–e. Silver-hexamine staining of glutaraldehyde-osmium-fixed tissue. a, b Section stained with silver hexamine only, showing dense silver deposition over the protein-carbohydrate body (*PCB*). Some silver has been deposited over the ground substance of the aleurone grain (*GS*), the spherosomes (*S*) and the cell wall (*W*), in particular a thin band immediately adjacent to the cytoplasm (*C*). c Iodoacetate-treated tissue stained with silver-hexamine. Staining of the cell wall (*W*), cytoplasm (*C*), the aleurone grain ground substance (*GS*) and the protein-carbohydrate body (*PCB*) is almost completely blocked but the treatment has no effect on spherosome (*S*) staining. d, e Iodoacetate-treated (blocked) tissue subsequently treated with chromic acid (d) and hydrochloric acid (e) which reintroduced staining to the protein-carbohydrate body (*PCB*) and the layer of cell wall (*W*) adjacent to the cytoplasm although the staining was not as intense as in controls (a, b). The cytoplasm (*C*), spherosomes (*S*) and ground substance (*GS*) of the aleurone grains appeared essentially as in blocked tissue, but spherosomal staining in d and e is heavier than shown in c because it varied from experiment to experiment

liberation of new aldehyde groups. In permanganate-fixed tissue, there was no silver hexamine staining before or after periodic- or chromic-acid treatment.

Although a carbohydrate-containing body could not be demonstrated by electron-microscopy methods, the silver staining of the electron-dense inclusion was used to demonstrate that this body was in fact the protein-carbohydrate body. Glutaraldehyde-fixed tissue was sectioned as for light microscopy and the sections were stained with toluidin blue, mounted in water and an area showing green-stained protein-carbohydrate bodies was photographed (Fig. 1 f). The section was then stained with silver hexamine and the same area was again photographed (Fig. 1 g). The green-stained bodies were the only organelles to stain with silver-hexamine showing that the electron-dense, silver-hexamine-positive aleurone grain inclusion is the protein-carbohydrate body. Using glut-Os-fixed tissue gave exactly the same results but the green toluidin-blue staining of the osmicated protein-carbohydrate body was not as clear as in non-osmicated tissue. The bodies were silver-hexamine-positive in fresh tissue (unfixed) also so that both green staining and silver deposition are attributable to some natural component(s) of the protein-carbohydrate bodies.

On the basis of frequency of appearance in electron-microscope sections, we have stated that electron-dense bodies in permanganate and glutaraldehyde-fixed tissues are the same bodies, the protein-carbohydrate bodies. This was supported by looking at permanganate-fixed tissue with the light microscope. The electron-dense body in permanganate-fixed tissue is electron-dense, even without lead staining, presumably because of the affinity of permanganate for the contents of the body. Since permanganate is coloured, it was possible to see the body in the light microscope. Fresh tissue was sectioned for light microscopy and the sections were stained in permanganate on the slide. The densely stained bodies were the protein-carbohydrate bodies. It is of interest to note here that permanganate prevented green-staining of the bodies as well as the cell wall with toluidin blue.

Discussion

The red metachromasia of globoids with anilin-blue dyes was noticed many years ago, firstly by Meyer and then by Guilliermond and Beauverie (see Salmon, 1940). Guilliermond and Beauverie proposed that the metachromasia was not due to phytin but to a protein substance which was similar to "metachromatin". Using Sudan dyes, Salmon (1940) described the occurrence of a lipid substance in globoids of *Cucurbita pepo* cotyledons and inferred that this was responsible for the metachro-

masia. More recently Vazart (1960) concluded that the metachromasia of barley-aleurone globoids was due to ribonucleic acid at least in part. It is now clear that the phytin is responsible for the metachromasia. We have also sectioned and stained other plant tissues which contain phytin. These include wheat aleurone tissue, castor-bean endosperm and cucumber and cotton cotyledons. All contained red-staining globoids apparently within aleurone grains. It is noteworthy that the globoids are the only sites of phytin deposition in the cells examined. Hence toluidin-blue staining emerges as a useful aid for determination of phytin localization in plant tissues, although it is not necessarily specific.

The total phytin content of aleurone layers varied and probably reflected the nutritional status of the plant but there was uniformity in phytin distribution in the aleurone layers. The phytin content of cells immediately adjacent to the suture was highest and that of the cells away from the suture was much lower. This probably reflects the way in which phosphate is supplied to the seed. In analogy with wheat where this occurs via vascular tissue running parallel to and just beneath the suture (Frazier *et al.*, 1956; Frazier and Appalanaidu, 1965), the aleurone cells of barley adjacent to the suture would be very close to the phosphate supply of the seed. Because the phytin content of aleurone cells falls off often very rapidly away from the suture and because the inner cells of the bulk of the aleurone layer contain more phytin than the outer cells, it would appear that phosphate is supplied to most of the aleurone cells via the starchy endosperm rather than via adjacent aleurone cells.

There have been very few studies on aleurone grain composition but studies on peanut seeds (Dieckert *et al.*, 1962) and castor-bean seeds (Sobolev, 1966) show that the main components of the aleurone grains of these tissues are protein, phytin, phospholipids, RNA and some carbohydrate. We have presented evidence showing that at least phytin, protein, carbohydrate and lipid are present in barley aleurone grains and that they tend to be localized in discreet particles. Carbohydrate is confined to the protein-carbohydrate body, phytin and lipid to the globoid and although protein is widespread in the aleurone grain, it is in very high concentration in the protein-carbohydrate body and it seems likely that the bulk of the storage protein occurs in this body and not in the ground substance of the aleurone grain. The phosphate associated with the periphery of the globoid vacuole is probably either free phosphate or solubilized phytic acid.

It seems very likely that what we have described as the protein-carbohydrate body is the crystalloid of Pfeffer (1872). Although the body in barley aleurone cannot be described as a crystal, the general appearance of it in glut-Os-fixed tissue, its size and occurrence is very

similar to what Poux (1965) has called crystalloids in glut-Os-fixed cotyledonary tissue. The protein-carbohydrate body is very rich in protein and Pfeffer originally described the crystalloid as being proteinaceous.

The compound(s) causing the protein-carbohydrate bodies to stain green with toluidin blue and to be silver-hexamine-positive are natural component(s) of the bodies but their identities are unknown. However, there is some evidence that the same compound causes both phenomena. Green staining with toluidin blue was confined to the protein-carbohydrate body and the cell wall, with a thin band of the cell wall adjacent to the cytoplasm being most densely stained (Fig. 1a, b, d). Silver-hexamine staining showed the same localization (Fig. 6a). Iodoacetate blocked silver-hexamine staining and also blocked toluidin-blue staining. Likewise permanganate interfered with silver-hexamine staining and also blocked toluidin-blue staining. If there is only one compound involved, the chemical group responsible for staining cannot be an aldehyde as the protein-carbohydrate bodies are not Schiff-positive. It also seems unlikely that sulfhydryl groups are involved because although iodoacetate blocks staining, the blocking is reversible in acid conditions and this would not be expected if the effect of iodoacetate was to form thioether bonds with sulfhydryl groups. Some polyphenols and lignin stain green with toluidin blue (Feder and O'Brien, 1968); this explains perhaps why the cell walls stain green but whether this has any bearing on the staining of the protein-carbohydrate body is unknown.

This combined study of light and electron microscopy has allowed us to evolve a fairly clear picture of the barley aleurone grain and to interpret structure in the light microscope. In the light microscope, it is difficult to define the limits of the aleurone grain at least in toluidin-blue-stained tissue. The globoid often does not fill the globoid cavity and the tendency is to identify the globoid cavity as the entire aleurone grain. Even so at the light-microscope level it was still difficult to localize the protein-carbohydrate body with certainty. It is now clear that it also is an inclusion of the aleurone grain. Sometimes the limits of the aleurone grain are clear and the analogy with the structure as seen in the electron microscope is obvious.

The staining characteristics of the protein-carbohydrate body and the fact that it is difficult to preserve globoids in electron-microscope sections of permanganate-fixed tissue especially, has led to difficulty in the literature in identification of aleurone-grain inclusions. Paleg and Hyde (1964) did conclude tentatively that this body contained protein but other reports (Eb and Nieuwdorp, 1967; Jones, 1969a) have mistaken it for the globoid. The distinguishing characteristics

Table. *Summary of the characteristics of globoids and protein-carbohydrate bodies in barley aleurone tissue*

	Globoid	Protein-carbohydrate body
Light microscope		
Toluidine-blue staining	Red (chemical gardening)	Green
Acid (pH 3) then toluidin blue	Not stained, phytin solubilized	Stained green
Polarized light	Anisotropic	Isotropic
Content	Protein, phosphate (phytin), lipid	Protein, carbohydrate
Diameter	Up to 3 μ in inner cells	Uniform, 1–1.5 μ, occasionally 2 μ
Frequency/aleurone grain	Usually 1, occasionally 2 or 3	1 or rarely 2
Electron microscope		
Glut-Os-fixed	Amorphous, electron opaque, usually shattered during sectioning or removed from section	Granular, medium electron density, doesn't shatter, silver-hexamine-positive without periodate oxidation
Permanganate-fixed	Mostly dissolved but amorphous and electron opaque when retained, shatter during sectioning	Granular, varying electron density, sometimes rippled or shattered during sectioning

of both bodies are listed in the Table. In glut-Os-fixed tissue, the electron density of the protein-carbohydrate body is quite low and there is no chance of confusing it with the globoid which is electron opaque. In permanganate-fixed tissue the globoid, when it is retained, is again electron opaque (Jones, 1969b) and the protein-carbohydrate body is also commonly very electron dense. The mistake has arisen because the globoid is often not preserved, and because the protein-carbohydrate body often appears torn and broken like the globoid, as well as being electron dense. However, the other distinguishing features such as frequency of occurrence and size relative to the globoid cavity leave little doubt about the identity of the protein-carbohydrate body.

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