THE INCORPORATION OF ^{3}H-PALMITIC ACID INTO *ORNITHOGALUM UMBELLATUM* LIPOTUBULOIDS, WHICH ARE A CYTOPLASMIC DOMAIN RICH IN LIPID BODIES AND MICROTUBULES. LIGHT AND EM AUTORADIOGRAPHY

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ABSTRACT

In the ovary epidermis of *Ornithogalum umbellatum* L. lipotubuloids were found, i.e. distinguished cytoplasmic domain with an agglomeration of half unit membrane-surrounded lipid bodies, entwined and held together by a system of microtubules (Protoplasma 75: 345-357; 77: 473-476).

Using light and EM-autoradiography with ^{3}H-palmitic acid (25 µCi/ml) it was found that lipotubuloids were the site of intense incorporation of this isotope. After extraction of lipids with lipid solvent the lipotubuloids were not labeled. Localization of autoradiographic grains after 15-h postincubation with isotope-free medium indicated a migration of the labeled substances from the lipotubuloids to the whole cells. Ultrastructural studies demonstrated that most autoradiographic grains after 2-h incubation were localized over the site of the microtubules adjoining closely the half unit membranes of lipid bodies. These observations suggest, that the surface of lipid bodies may be the active site in lipid synthesis and involvement of the microtubules in these processes is possible.

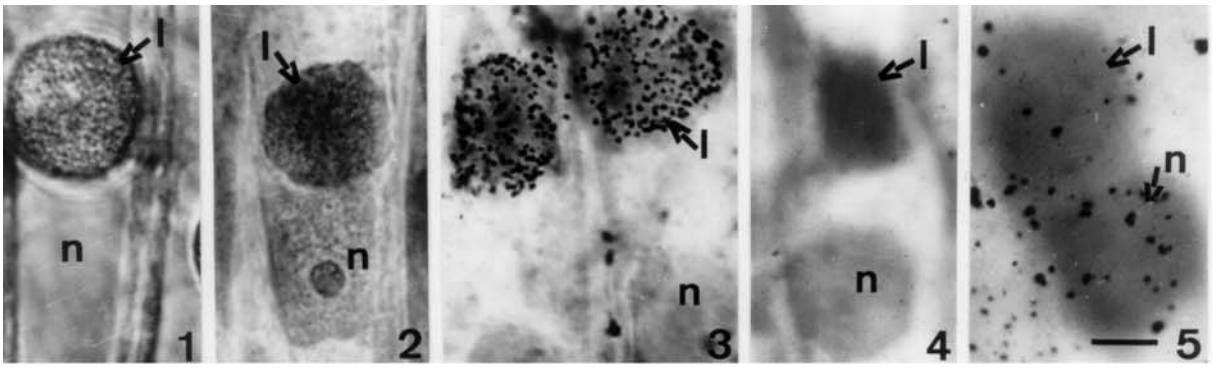
KEY WORDS: *Ornithogalum umbellatum*, lipotubuloid, lipid bodies, microtubules, EM autoradiography, ^{3}H palmitic acid.

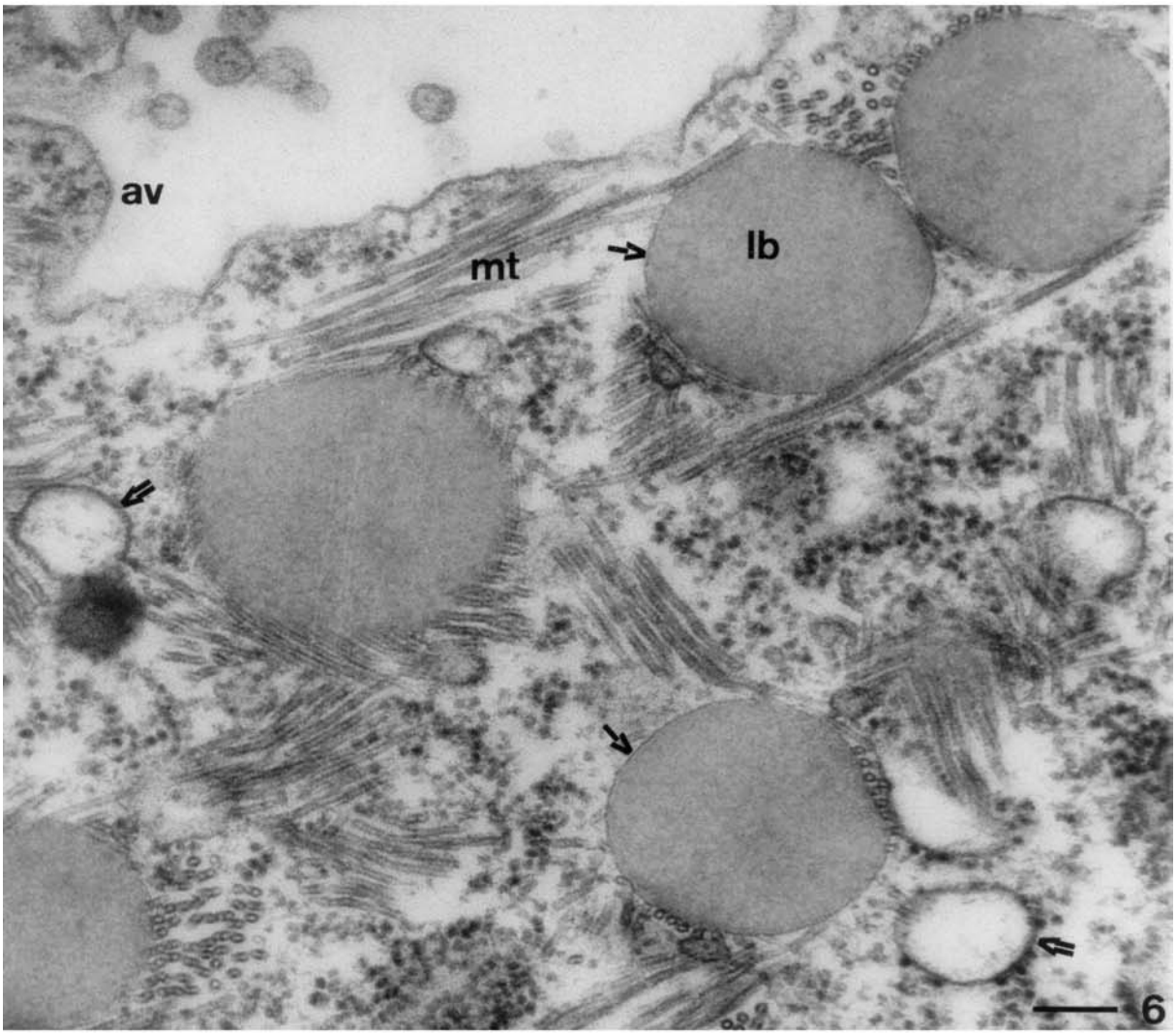
INTRODUCTION

In the epidermis of ovary and of other organs of *Ornithogalum umbellatum* large spherical structures of fatty nature were found (Raciborski 1895; Kwiatkowska 1966). In 1888 Wakker, who described analogous structures in *Vanilla*, called them "elaioplasten". Cytochemical and ultrastructural studies proved them not to be plastids, as the name introduced by Wakker could suggest. Similar non-plastidic "elaioplasts" were also observed in *Malva neglecta* and *Althaea rosea* (Wałek-Czernecka and Kwiatkowska 1961; Kwiatkowska 1972a), in 12 species of *Gentiana* (Kwiatkowska 1961), in *Dahlia variabilis* (Kwiatkowska 1963), in *Funkia sieboldiana*, *Clivia nobilis, Vanilla planifolia* (Kwiatkowska npb), and in *Haemanthus albiflos* (Tourte 1964).

O. umbellatum "elaioplasts" are the structures consisting of an agglomeration of osmiophilic granules containing triacylglycerides, free fatty acids and phospholipids (Kwiatkowska 1966). Single osmiophilic granules are entwined and connected to one another by a system of microtubules. Therefore a new name for "elaioplasts" has been proposed – i. e. lipotubuloids (Kwiatkowska 1971a, b, 1972b). It is not known, however, if this term can also apply to the above mentioned "elaioplasts", which are not plastids, in other plants since to date attempts to clearly show such labile structures as microtubules, together with osmiophilic granules, have failed (this may be due to serious problems with proper fixation). Microtubules in *O. umbellatum* are unusually stabile since they can be shown after the use of Palade (1952) solution containing only buffered OsO_4 (Kwiatkowska 1971a), while other non-stabile cytoplasmic microtubules require a fixative containing gluteraldehyde (Ledbetter and Porter 1963). Microtubules of nervous cells (neurotubules) are characterized by a similar stability. It seems possible that the stability of both of them results from the presence of polisaccharides (Tani and Ametani 1970; Kwiatkowska 1973a). In *O. umbellatum* lipotubuloids they form a 2.5 nm layer at the outer surface of a microtubule wall (Kwiatkowska 1973a).

Lipotubuloids of *O. umbellatum* are a distinguished cytoplasmic domain where in addition to osmiophilic granules and microtubules abundant ribosomes and membranes of the endoplasmic reticulum occur as well as a few mitochondria, Golgi structures and a single membrane surrounding bodies with granular contents (lysosomes, peroxisomes or glyoxisomes) (Kwiatkowska 1971a, b, 1973b).





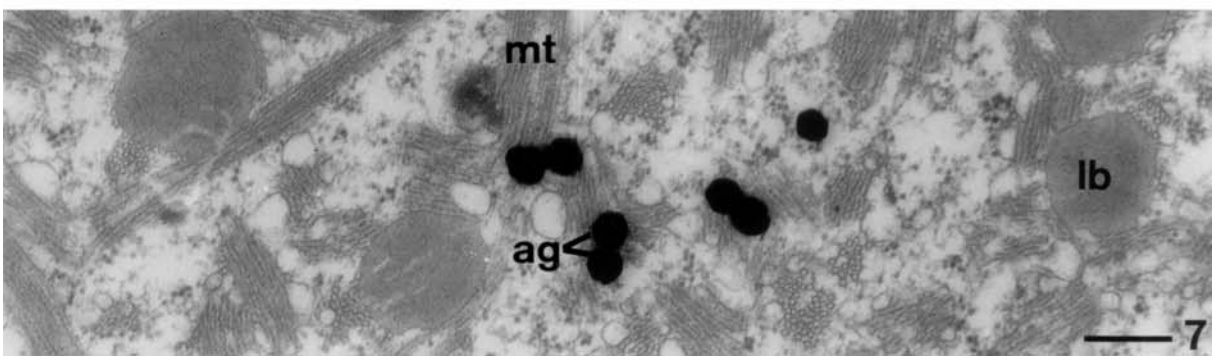
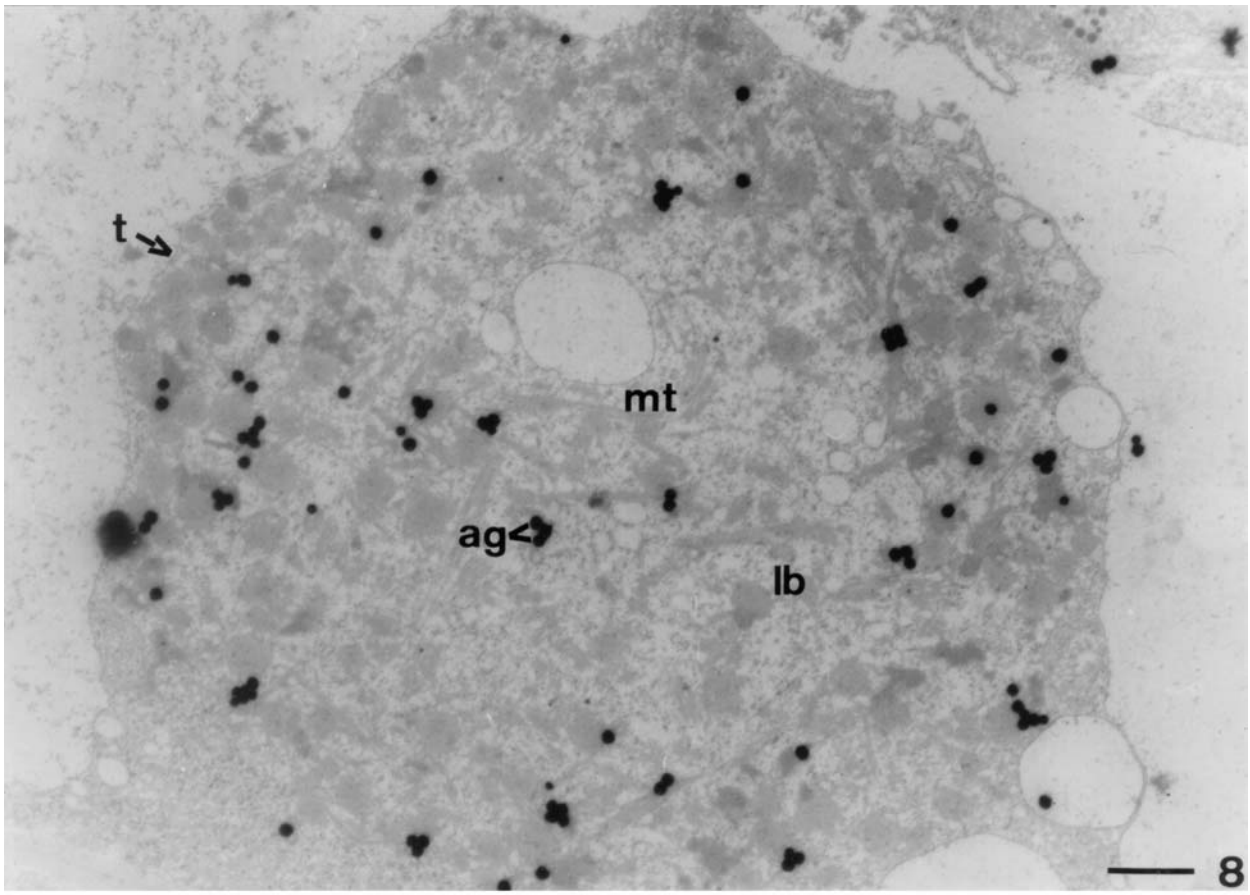
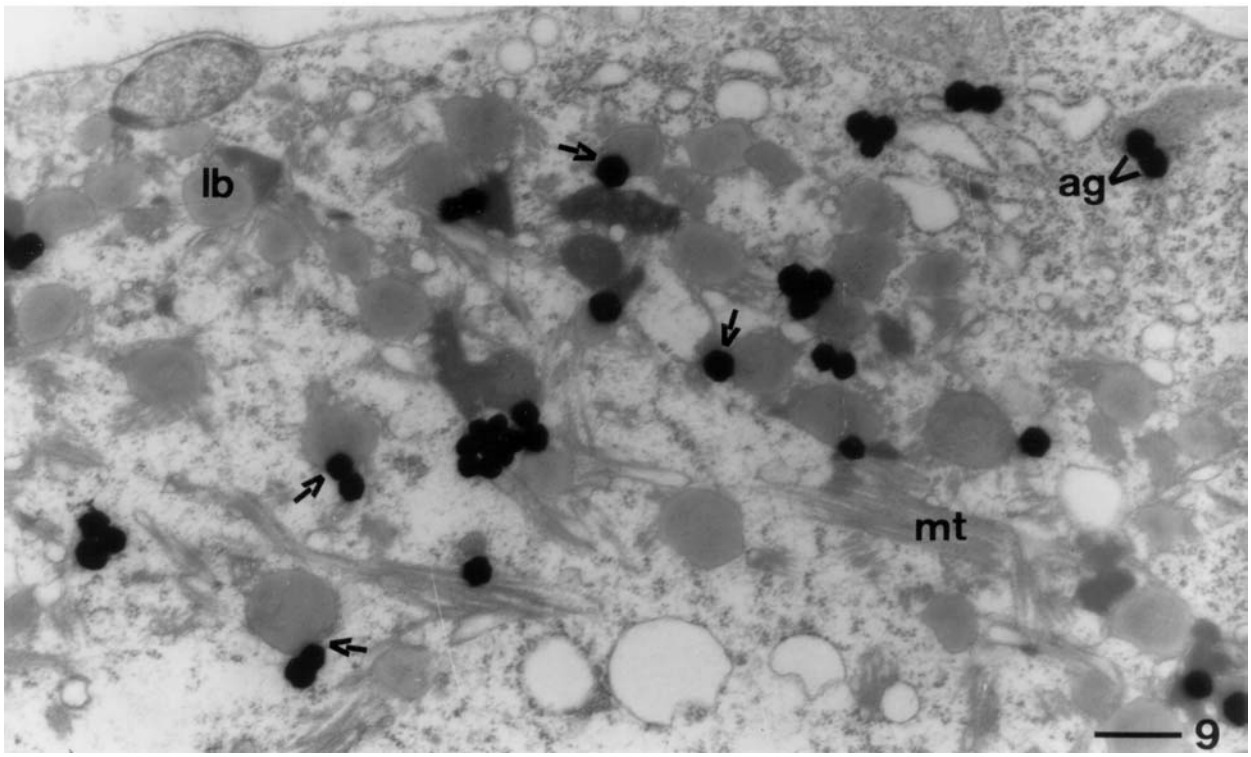


Fig. 1. Intra vitam picture of lipotubuloid of *Ornithogalum umbellatum* ovary epidermis; l – lipotubuloid, n – nucleus. Fig. 2. OsO_4-fixed ovary epidermis. Fig. 3. Epidermis of ovary incubated for 2 h in ^{3}H-palmitic acid. OsO_4 fixation. Exposure: 6 days. The developed autoradiograph was cleared with H_2O_2 and stained by the Unna method. Fig. 4. Epidermis of ovary incubated for 2 h in ^{3}H-palmitic acid, fixed in Carnoy's fluid and lipids extracted by the Keilig





method. Stained by the Unna method. Fig. 5. Epidermis of ovary incubated for 2 h in ^{3}H-palmitic acid and then transferred to a non-radioactive medium for 15 h. Fixation and staining as in Fig 3. Bar = 10 µm (for Figs 1-5). Fig. 6. Fragment of a lipotubuloid of *O. umbellatum* epidermis fixed for 1 h in 1:1 mixture of 2.5% glutaraldehyde and 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4; postfixation in 1% OsO_4 for 1 h, followed by alcohol dehydration, epon-embedding, and staining according to Reynolds; mt – microtubules; lb – lipid body; av – autolytic vacuole. Single arrows point to half unit membrane of lipid bodies; double arrows point to tripartite unit membrane. Bar = 0.1 µm. Figs 7-9. Ultrastructural autoradiography. Incubation in ^{3}H-palmitic acid for 40 min (Fig 7) and 120 min (Figs 8, 9). Fixation and embedding as in Fig 6. Ultrathin sections were placed on grids, coated with Ilford emulsion using platinum loop. After 7 months of exposure the preparations were developed in a phenidone containing developer; t – tonoplast; ag – autoradiographic grains; lb – lipid body; mt – microtubules. Figs 7 and 9 – Bar = 0.2 µm; Fig 8 – Bar = 0.4 µm.

Lipotubuloids are not enclosed in their own membranes. Their greater part bordering to the tonoplast, because they intrude into vacuoles. The lipotubuloids move inside the cell as one unit with the progressive-rotary motion of autonomous nature (Kwiatkowska 1972b).

Osmiophilic granules of *O. umbellatum* lipotubuloids are surrounded by half unit membranes 2-3 nm thick and are filled with nearly homogenous osmiophilic material which allows their recognition as lipid bodies (lipid droplets), (Kwiatkowska 1973b), similary as in other plants (Wanner et al. 1981; Hayashi et al. 2001). Analogous structures are called by other authors oil bodies (Leprince et al. 1998; Napier et al. 1996; Huang 1996; Chen et al. 1999; Wu et al. 1999), oleosomes (Wanner and Theiner 1978) or spherosomes (Yatsu and Jacks 1972).

During the development of *O. umbellatum* ovary and its transformation into a fruit the lipotubuloids significantly increase their dimensions and, at the final growth stage, in their central zone, the lipid bodies begin to disappear due to the activity of hydrolytic enzymes, consequently forming lipotubuloid autolytic vacuoles (Kwiatkowska 1971b). In the cells of ovarian epidermis which achieved its final dimensions the lipotubuloids disintegrate into single lipid bodies, which gradually vanish. This disintegration is preceded by the disappearance of the microtubules entwining lipid bodies (Kwiatkowska 1971a).

Autoradiographic studies have shown that lipotubuloids are a very active site of ^{3}H-palmitic acid incorporation and synthesised lipids are metabolised by a cell. Ultrastructural autoradiography suggested that microtubules might cooperate in synthesis or accumulation of lipids.

MATERIAL AND METHODS

Ovarian epidermis from fully developed flowers of *Ornithogalum umbellatum* L. was the subject of investigations. The cells of the tissue were in the phase of an intense elongation growth and were characterized by the presence of a single lipotubuloid 20-30 µm in diameter (Kwiatkowska 1971b).

Light microscope autoradiography

The carpels together with stems were immersed for 2-h in ^{3}H-palmitic acid (25 µCi/ml, spec. ativity: 50 mCi/mmol). In order to analyse secondary isotope localization some carpels after incubation in the radioactive palmitic acid were transferred to a Petri dish padded inside with water-moistened filter paper and placed in darkness for 15-h. The epidermis cut off from the ovary was fixed with 1% OsO_4 or with Carnoy's fluid (ethanol: acetic acid 3:1 v/v) for 1 h. From Carnoy fixed preparations the lipid material was extracted by the Keilig method (Pearse 1961). After fixation, the tissue sections were attached to gelatine-coated slides and subseqently covered with damp cellophane and strongly squashed. After drying, the preparations were washed in tap water and then coated with Ilford L_4 emulsion in gel form. The exposure period was 6 days. After development, the autoradiograms were stained by the Unna method. Prior to staining the OsO_4-fixed preparations were treated with 10% hydrogen peroxide for 15 min. In thus prepared tissue sections the lipotubuloids stained red and conseqently could be easily distinguished from cell nuclei which were similar in shape and size but stained blue.

Electrone-microscope autoradiography

The carpels of *O. umbellatum* plants shedding blossoms were incubated in ^{3}H-palmitic acid (25 µCi/ml, spec. activity 50 mCi/mmol) for 40 and 120 min. Contiguous epidermis sections were fixed for 1-h in freshly prepared 1:1 mixture of 2.5% glutaraldehyde and 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4 at 4˚C postfixed in 1% OsO_4 for 1-h, followed by alcohol dehydration and epon-embedding. For autoradiographic investigations ultrathin sections were placed on grids. Single grids with preparations were coated with Ilford emulsion using the platinum loop (Haase and Jung 1964). After 7 months of exposure the preparations were developed in a phenidone containing developer (Lettre and Paweletz 1965) and contrasted according to Reynolds (1963).

The distribution of autoradiographic grains over structural elements of lipotubuloids (± S.E.) was estimated on the basis of analysis of 50 electronographs.

RESULTS

Light-microscope observations

Figure 1 shows a spherical lipotubuloid in a living *O. umbellatum* epidermis cell. Figure 2 presents an analogous lipotubuloid accompanied by a cell nucleus after the epidermis was fixed with 1% OsO_4.

Autoradiographic analysis reveals that ^{3}H-palmitic acid is intensively incorporated in lipotubuloids (Fig. 3). Following lipid extraction with organic solvents no labeling is observed in lipotubuloids (Fig. 4). After 15-h postincubation in darkness selective labeling of lipotubuloids disappears and autoradiographic grains are dispersed on the wall cells (Fig. 5).

Electronmicroscope observations

Figure 6 presents a fragment of lipotubuloid with a few lipid bodies and microtubules. Lipid bodies (0.04-0.5 µm in diameter) are surrounded by half unit membrane ca

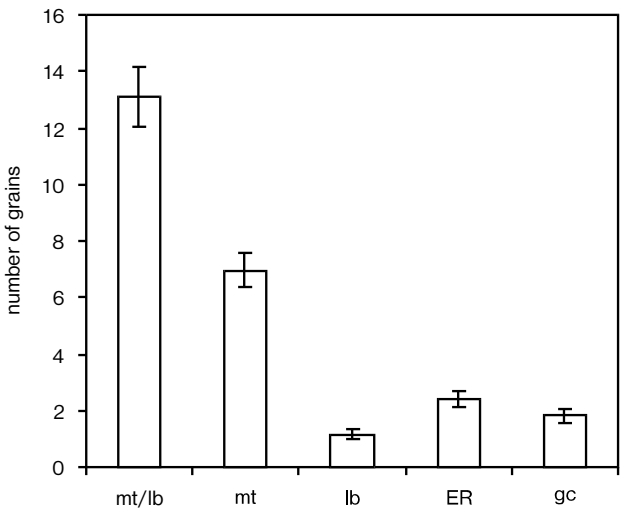


Fig. 10. Distribution of autoradiographic grains over structural elements of lipotubuloids after 2-h incubation in ^{3}H-palmitic acid (± S.E.); mt – microtubules strands; mt/lb – microtubules/lipid bodies surfaces; lb – lipid bodies insid; ER – endoplasmic reticulum; gc – ground cytoplasm.

2-3 nm thick, which is much thinner than that of endoplasmic reticulum vesicles (ca 6.5-7 nm). Lipid bodies interiors are filled with nearly homogenous, osmiophilic substance. Microtubules, running in various directions and connecting the lipid bodies, closely adhere to a half unit membrane surrounding these bodies. Numerous ribosomes and polyribosomes as well as rough and smooth ER vesicles are present in cytoplasm. A fragment of an autolytic vacuole can also be seen.

The analysis of ultrastructural autoradiograms indicates that following 40-min incubation in ^{3}H-palmitic acid 80% of autoradiographic grains are localized over microtubules placed between lipid bodies (Fig. 7). After 120 min incubation autoradiographic grains are mainly present above a half unit membrane and/or the site where microtubules adhere to lipid bodies (ca 52% of grains) (Figs 8-10); quite many of them (27%) are still over microtubule strands running between lipid bodies; some of them (10%) are near endoplasmic reticulum vesicles. A few of autoradiographic grains can be seen over cytoplasm of lipotubuloids (7%) and over lipid body insides (4%).

DISCUSSION

The present and earlier observations clearly indicate that one of the most characteristic components of lipotubules are osmophilic granules which correspond to lipid bodies as they are surrounded by a half unit membrane. A similar structure of lipid bodies was described in a previous paper (Kwiatkowska 1973b), in which, as in the present experiment, *O. umbellatum* epidermis was fixed in glutaraldehyde and OsO_4 mixture. The difference in thickness of lipid bodies and ER membranes was not visible in electronograms of lipotubuloids fixed only with OsO_4 according to Palade (1952) (Kwiatkowska 1971a, b, 1972b).

Several authors' papers show that lipid body membranes contain phospholipids and alkaline protein-oleosin, molecular mass 15-24 kDa (Qu et al. 1986; Napier et al. 1996), which prevent lipid bodies coalescence during seed imbibitions (Leprince et a. 1998). Oleosin is characteristic of both angiosperm and gymnosperm (Wu et al. 1999). Apart from oleosin in sesame-oil bodies three minor proteins of higher molecular mass were also identified (Chen et al. 1998) and termed Sops 1-3. Sop 1, named caleosin, exists in diverse seed oil bodies and is a putative calcium binding protein (Chen et al. 1999).

The present ultrastructural autoradiographic studies suggest, that the surface of the lipid bodies surrounded with half unit membrane may be the active site in the lipid synthesis. These observations comply with the hypothesis assuming that lipid bodies originate from the triacylglyceride accumulation in the lypolytic central layer of the endoplasmic reticulum membrane being the site of fat synthesis and separated halves of ER membrane cover lipid bodies (Frey-Wyssling et al. 1963; Wanner et al. 1981; Huang 1996; Napier et al. 1996; Chen and Tzen 2001).

Another very characteristic component of lipotubuloids are microtubules running in various directions entwining and connecting lipid bodies.

The fact that a considerable majority of autoradiographic grains after 40-min incubation in ^{3}H-palmitic acid is localised over the microtubule strands of lipotubuloid, adjoining the lipid bodies or running in between, leads to the assumption that lipotubuloid microtubules are associated with lipid metabolism. Biochemical studies prove that a number of enzymes, the ones of fat metabolism among them, are connected with microtubule proteins (Luduena 1979). A 90-kD phospholipase D has been shown recently to bind and decorate microtubules in plant cells in a cosedimentation assay (Gardiner et al. 2001; Gardiner et al. 2003; Dhonukshe et al. 2003). Thus it may be assumed that ^{3}H--palmitic acid may form a complex with these enzymes close to the microtubules, which is suggested by their labeling. Massive occurrence, following 2-h incubation in ^{3}H--palmitic acid, of autoradiographic grains on the periphery of the lipid bodies in the site of microtubules adjoining their surface leads to the assumption that bound fatty acids are transported along the microtubules to the sites of their incorporation into lipid bodies.

The micrographs presented demonstrate that lipotubuloids are not only a site of lipid material deposition. They constitute a cytoplasmic domaine in which a very intense synthesis of lipids, or at least combinning of fatty acids with glycerophosphate, occurs. The lipids synthesised inside the lipotubuloids are used up by the epidermal cells, which is proved by the disappearance of the lipotubuloid selective labeling after 15-h postincubation in the dark. These lipids are metabolised by a cell and resulting compounds are incorporated into different synthesis processes. Earlier studies showed that after postincubation autoradiographic grains scattered over a cell did not disappear completely as a result of lipid extraction (Kwiatkowska 1972c). On paraffined crossections of *O. umbellatum* ovarian epidermis following 8-h postincubation the radioactive substance non-extractable with lipid solvent was localized mainly in the outer layer of the cell wall just beneath the cuticula (Fig. 6, Kwiatkowska 1972c). Thus a hypothesis might be put forward that components of a cell wall or cuticula are the main cell elements synthesised from radioactive molecules. Their intensive synthesis coinciding with the period when lipotubuloids are biggest is evident since it is the time when cell growth is most intensive (when the latter process stops lipotubuloids disappear). The highest surface area of epidermal cells increment (ca 2400%) falls to the relatively short period from coming into flower to its drying up lasting, depending on the weather, 3 to 5 days (Kwiatkowska 1971b).

The above data clearly indicate that lipotubuloids are an important element of *O. umbellatum* ovarian epidermis playing a significant role in cell metabolism. Research on these unusual structures, not being the focus of interest to date, may elucidate the function of both lipid bodies and microtubules, and their possible functional relationship.

It seems certain that autonomous progressive-rotary motion of lipotubuloids inside the whole cell (1972b) greately facilitates both infiltration into them of components necessary for lipid synthesis and out of them substances distributed inside a cell. The motion of *O. umbellatum* lipotubuloids will be the subject of further investigation.

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