Investigations on the elaioplasts of *Ornithogalum umbellatum* L.

I. Morphological, cytochemical and cytoenzymatic observations

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Investigations on the elaioplasts of *Malvaceae* (Wałek-Czernecka and Kwiatkowska 1961) and *Dahlia* (Kwiatkowska 1963) demonstrated that the elaioplasts of these plants are an agglomeration of OsO_4 -reducing granules, probably spherosomes. This supposition was confirmed by the detection in the elaioplasts of the hypocotyl and root of hollyhock of the activity of several hydrolases characteristic of spherosomes: acid and alkaline phosphatase, nonspecific esterases and lipases (Wałek-Czernecka, unpublished data; Kwiatkowska and Stepieński, in press). Some of these enzymes, namely acid phosphatase and nonspecific esterase as well as DNAase were also found in the elaioplasts of *Campanula* pollen grains (Górska-Bryllass, 1965).

The present study is a continuation of the earlier investigations. It was performed on such classical material as the elaioplasts of *Ornithogalum* — which most closely resemble the lipid bodies of *Vanilla* described by Wakker (1888) and considered as the prototype of elaioplasts of higher plants.

The elaioplasts of *Ornithogalum umbellatum* proved very convenient for studies because they preserve their structure better than the previously investigated ones after the tests and cytological staining so that the pictures obtained can be interpreted. Owing to this it was possible to determine accurately the localization of the enzymes within the elaioplasts, and consequently, to demonstrate the enzymic heterogeneity of the grains contained in the elaioplasts.

MATERIAL AND METHODS

The observations were made on elaioplasts occurring in the epidermis of the ovary and the stamina of *Ornithogalum umbellatum* L. flowers at various stages of development. The living epidermis cut off with a razor blade was examined in water.

The material was also fixed with osmium tetroxide vapour, Nawashin's or Regaud's fixative before staining with Haidenhein iron haematoxylin, acid Altmann fuchsine, pyronine, rosaniline and Victoria blue after Perner (1953).

The cytochemical and cytoenzymatic reactions were generally brought about after fixation overnight in cold Baker fixative or in 4 percent neutral formalin, and sometimes in fresh material.

The presence of lipids was revealed by staining the preparations with a mixture of Sudans III and IV dissolved in 70 percent alcohol or Herxheimer's mixture. Nile blue (1%) allowed to distinguish neutral and acidic lipids. It was applied to fresh (Lison 1960) or fixed (Pearse 1961) material. Phospholipids were detected by Baker's haemateine test. For confirmation of the results obtained, black Sudan B was used after McManus. The presence of unsaturated phospholipids was demonstrated by the PFAS test and free fatty acids by Fischler's method (Pearse 1961).

The lipids contained in the elaioplasts were extracted with alcohol, chloroform, carbon tetrachloride or pyridine after fixing in Bouin's fixative after Baker (Pearse 1961). The proteins remaining after extraction of lipids were revealed by the mercury—bromophenol blue reaction (Mazia et al. 1953).

For detection of mucilage and determination of its chemical character, the PAS reaction, ruthenium red, aniline blue, ClZnJ and resorcine blue were used.

Alkaline and acid phosphatase were revealed by Burstone's (1962) and Gomori's (Pearse 1961) methods, nonspecific esterase — by Holt's indoxyl method, lipases — by means of Tween 40 and Tween 80 after Gomori (Pearse l.c.). In some of the preparations these enzymes were detected jointly: after the indoxyl test for esterase, the preparation was washed repeatedly for one hour with 7.5 per cent sucrose and then incubated in the medium used for detecting alkaline, or acid phosphatase by Burstone's method with the use of Fast red violet LB salt or for the detection of lipase by Gomori's method.

VITAL EXAMINATION

Vital observations served to establish the origin of *Ornithogalum* elaioplasts and to follow the morphological transformations occurring in them with development of the flower.

According to Raciborski (1895) elaioplasts appear in *Ornithogalum* as minute bodies which afterwards increase in size. It results from the pictures and descriptions given by Wóycicki (1929) that *Ornithogalum caudatum* elaioplasts are formed by agglomeration of minute granules which the author compares to Guilliermond's mitochondria without giving their closer characteristics.

Observations of ovary and stamen filament cells in *Ornithogalum umbellatum* gave results similar to those described by Wóycicki. In the young cells from the bud c. 1 mm long single granules c. $0.3\text{--}0.5\ \mu$ in size may be seen (plate I, 1) agglomerated in groups like in *Malvaceae* and *Dahlia* (Wałek-Czernecka and Kwiatkowska 1961; Kwiatkowska 1963). In the ovary epidermis the agglomerations form at the nucleus, one agglomeration occurring generally in each cell. Sometimes, however, the granules agglomerate in several places, and only after formation of groups join to form one body.

Further stages of elaioplast formation are presented in the photographs of cells from ovaries 1.5, 2, 5 and 7 mm long (Plate I 2—5). What is most striking here

is the rapid increase in size of the elaioplasts accompanied by the transformation of the irregular body into an almost ideally spherical one with the contour accentuated by a lighter homogeneous capsule (Plate I 5). As shown by the photographs the elaioplasts "grow" in such a way that the number of the granules contained in them increases. The size of the granules remains, however, always the same. If one observes the same living cell for a longer time, one can notice that some granules are detached from the elaioplast not yet definitively formed (i.e. not spherical) and are carried away by the cytoplasmic current. Other granules, on the contrary, join the elaioplast. The latter process distinctly prevails over the former and seems to be decisive for the increase of the elaioplasts. Evidence of granule formation outside the elaioplasts is found in the further stages of cell development in which the elaioplasts take on a spherical form and become surrounded with a homogeneous capsule. At this period no granules can detach themselves from the elaioplast, but in spite of this a constant increase of the number of shining granules within the cytoplasm is observed and even larger agglomerations, or smaller groups are formed.

In the ovary of older flowers (with drying perianth) a process of elaioplast desorganization occurs; the number of granules contained in them gradually decreases. At the sites where the granules disappear, places of low refractivity arise, called vacuoles by Raciborski (1895). The vacuole occurs either in the centre of the elaioplast (Plate I 6 and 7) or they are formed at several points simultaneously. The small vacuoles may merge in time (Plate I 8). The size of the vacuole increases with ageing of the flower. This process is accelerated if the cut inflorescences are kept in the dark.

At the next stage of ovary cells development fragmentation of the elaioplasts into smaller bodies occurs because of the detachment of bulges on the surface resembling yeast buds (Plate I 8). Hence probably the theories of Raciborski (1895) and Politis (1911) that elaioplasts multiply by budding. Soon after their formation, however, the "buds" disintegrate into single granules. There is every reason to believe that budding is one of the steps of fragmentation analogous to the phenomenon observed in *Malvaceae* and *Dahlia* (Wałek-Czernecka and Kwiatkowska 1961; Kwiatkowska 1963). At a time one or several buds may be formed at various points of the elaioplast. They are surrounded like the whole elaioplast by hyaline capsule and in the centre they have a vacuole. Before their disintegration this capsule disappears. In some cells the disintegration of elaioplasts occurs without "bud" formation.

FIXED MATERIAL INVESTIGATION

The chemical composition of *Ornithogalum* elaioplasts has not so far been determined. It was only certain that like all the elaioplasts known, they contain a protein component in which bead-like indefinite lipid bodies are embedded (Raciborski 1895).

The cytochemical tests most commonly applied in earlier studies of the elaioplasts of *Malvaceae* and *Dahlia* (Wałek-Czernecka and Kwiatkowska 1961; Kwiatkowska 1963) proved that *Ornithogalum* elaioplasts contain the same compounds which were found in the previously examined plants. They contain unsaturated lipids as proved by the positive PFAS test and darkening under the action of osmium tetroxide. Phospholipids can be identified in them by Baker's acid haematein test, free fatty acids — by Fischler's reaction and neutral lipids — by means of Nile blue. After the extraction of the lipids, a substance of protein character remains as indicated by the positive mercury—bromophenol blue reaction.

The enumerated tests are positive within the entire area of the elaioplast but the colour intensity in the particular structural elements varies: the granules stain most intensely, the substance between them, i.e. the elaioplast stroma — weaker, and the vacuoles — least intensely. It was found that the stroma contains, beside lipids and proteins, also mucilage similarly as the elaioplasts of *Malvaceae* (Wałek-Czernecka and Kwiatkowska 1961). This mucilage stains with aniline blue and coralline and gives a positive PAS test. The results of these stainings with simultaneous lack of staining in ruthenium and Congo red and in the solution of iodine in zinc chloride leads to the supposition that this is a callose mucilage. It should be mentioned, however, that I have not obtained absolutely unequivocal results with resorcline blue, considered as a most specific stain of callose.

The grains which in living elaioplasts seem equal may be differentiated after Baker's reaction for phospholipids, Mazia's tests for proteins. A small number is found to have larger dimensions and takes on a more intense colouring than the more numerous lighter ones (Fig. 5). On the other hand OsO_4 vapour, black Sudan B and red Sudan (III and IV) stain all the grains equally (Plate II 1).

In preparations stained with iron hematoxylin, acid fuchsin and the stains used by Perner in studies on spherosomes (rosaniline, Victoria blue, pyronine), the elaioplasts are found to contain two kinds of grains: intensely stained ones usually somewhat larger, and less intensely stained smaller ones (Fig. 6).

The author observed similar differences in staining of the granules in the elaioplasts of *Gentiana* and *Dahlia* (Kwiatkowska 1961, 1963).

CYTOENZYMATIC INVESTIGATIONS

Ornithogalum umbellatum elaioplasts at various stages of development — from the moment of granules aggregation to buds formation and disintegration — give a positive reaction for acid and alkaline phosphatase, nonspecific esterases and lipases. The final product of enzymic reaction occurs generally in the form of coloured grains. Observation under the microscope of live preparations in incubation medium for revealing nonspecific esterases by the indoxyl method allowed (partly already post vitam) to follow the gradual accumulation of indigo in some of the shining elaioplast granules. This process was most distinctly visible within small granule agglomerations. As the reaction advanced, the staining grains usually swelled, differing from the others by their dimensions. Similar experiments were made with

application of Burstone's method for acid phosphatase, and results very similar to those described above for indoxyl esterase were obtained.

The number of stained granules within the elaioplast is small. These granules are generally disseminated more or less regularly between unstained ones at various depths of the elaioplast (Plate II 2, 3, 6, 7). Less frequently the granules form one or more agglomerations of various size either on the periphery or deeper in the elaioplast (Plate II 4, 5, 8–11). Sometimes in the elaioplasts large irregular bodies are visible they may be the swollen or stuck together granules. These groupings are particularly frequent after the test for alkaline phosphatase (Plate II 10 and 11). All types of distribution of the stained reaction products may be found in one preparation, frequently even in neighbouring cells.

Successive finding of two enzymes in one section by application of methods so adjusted as to obtain final reaction products with contrasting colours — revealed that elaioplast granules are characterized by enzymic heterogeneity. For instance in elaioplasts in which nonspecific esterase was first revealed (blue staining in reaction), after Burstone's test for acid or alkaline phosphatase (giving a red colouring) completely red or blue granules may be distinguished as well as a certain number of red-blue granules. In the latter the two colours are either superposed or a part of the granule is red and the other blue. Sometimes the two-coloured granules are somewhat larger than the one-colored ones (Figs 1 and 2). In elaioplasts where lipase and esterase activity was revealed, the picture is similar: in some granules lipase occurs accompanied by esterase, the others either lipase or esterase may be found (Fig. 3).

In all the cases described, there occurs in the elaioplasts beside the stained granules, a large number of colourless ones (Fig. 1–4).

It should be noted that the positive reaction to the enzymatic tests occurred also outside the elaioplasts in the granules dispersed in the cytoplasm. These granules correspond by their physical and chemical properties to sphaerosomes. When two enzymes were revealed jointly, the sphaerosomes showed an enzymic heterogeneity, i.e. some contained the one, and others the second enzyme. In others both the enzymes were present. This feature is also characteristic of sphaerosomes in other plants (Wałek-Czernecka 1965; Olszewska et al. 1965).

Searching for some connection between the morphological changes occurring in the elaioplasts and the localization of the activity of the enzymes contained in them, the author carried out cytoenzymatic reactions on elaioplasts at the stage of vacuole formation. As shown by the above described vital observations, vacuoles are formed owing to the disappearance of the granules. Therefore, at the site of vacuole formation an enhanced hydrolase activity could be expected.

The author observed that an increase of acid phosphatase and lipase activity occurs before the appearance of the vacuole in the elaioplast centre (Plate III 1, 2 and Fig. 4). At the next stage, beside the brown or red sediment formed in the reaction, a small area devoid of grains is visible (Plate III 2–4 and 6–8). In successive photographs this area is larger and larger; the reaction product is then to be found on the vacuole periphery.

Fig. 1—6. 1 — Elaioplast in which nonspecific esterase was revealed by the indoxyl method (reaction product marked by circles), and acid phosphatase by Burstone's method (site of activity marked by black dots). Unstained granules are grey. 2 — Jointly revealed nonspecific esterases (circles) and alkaline phosphatase (black dots). 3 — Elaioplast after a test for nonspecific esterase (circles) and lipases (black dots). 4 — Elaioplast at the stage of vacuole formation. Final reaction product of a test for lipases, localized chiefly in the centre (black area). Nonspecific esterases are distributed rather regularly (circles). 5 — Elaioplast after Baker's haematein test. The positive reaction is most intensive in the granules and capsules of the spheres formed under the action of the fixative, and weaker in the substrate and vacuoles. The most intensely stained granules are black. 6 — Elaioplast fixed with OsO_4 vapour and stained by Perner's method. Some of the granules are somewhat larger and more intensely stained (black). Magn. c. 1800 $\times$

It has been mentioned above that in some elaioplasts several smaller vacuols may occur. In these cases also products of lipase (Plate III 10 and 11) and acid phosphatase (Plate III 9) activity may be found at the edges of small vacuole. The author did not succeed however, in observing any relation between vacuole formation and alkaline phosphatase or nonspecific esterase localization. The activity of these enzymes was revealed at other site distant from the vacuoles (Plate III 12).

DISCUSSION

Observation of the stages of elaioplast formation in living cells of *Ornithogalum umbellatum* indicates that elaioplasts in this plant like in *Malvaceae* and *Dahlia* (Wałek-Czernecka and Kwiatkowska 1961, Kwiatkowska 1963) are formed

by way of agglomeration of the shining OsO_4 -reducing grains, in contrast to the elaioplasts of plastide origin formed e.g. in *Gaillardia Lorenziana* and *Iris versicolor* (Beer 1909; Faull 1936; Kwiatkowska 1962).

The granules forming the elaioplasts of *Ornithogalum* resemble spherosomes in so far that they contain proteins and lipids (chiefly phospholipids) and have a spherical shape, similar size and refractivity.

The results of cytoenzymatic studies show a further resemblance between the elaioplast granules and spherosomes. They contain a number of hydrolases found previously in onion spherosomes (Wałek-Czernecka 1963, 1964, 1965).

It is not clear, however, whether all the granules present in the elaioplasts are spherosomes.

The positive reaction to each of the enzymes tested was only observed in a small number of elaioplast granules distributed either regularly or in groups between the nonstaining ones. When the two enzymes are revealed jointly, the number of stained granules increases (Compare Plate II 2—4, and Figs 1—3). Some granules exhibit the colour indicative of the activity of one enzyme and others — that of the second one (e.g. nonspecific esterases and lipases; esterases and acid, or alkaline phosphatase) the third kind of granules contains two enzymes simultaneously.

The enzymic heterogeneity of these granules is one more feature showing their close relationship with spherosomes which as has been recently demonstrated, are equipped in varying enzymic sets (Wałek-Czernecka 1965; Olszewska et al. 1965).

The character of nonstaining (after one or two enzymic reactions) granules which are always fairly numerous in the elaioplasts, remains problematic. In the light of the facts presented, the possibility should be taken into account that these granules may either contain some other spherosomal enzymes (in onion spherosomes about ten have been found so far (Wałek-Czernecka 1965) or the enzymes present in them are inactive or else they represent degenerate spherosomes lacking enzymes. Neither is it excluded that, as has been earlier suggested, some of the granules are lipid droplets surrounded by a thin protein layer (Kwiatkowska 1962).

A solution of this problem may only be found after further morphological and enzymatic studies by means of an electron microscope. Up to date only one paper has appeared dealing with the elaioplast ultrastructure. *Haemanthus albiflos* elaioplasts are shown there as an agglomeration of unidentified granules each surrounded by a membrane (Tourte 1964).

The role of spherosomal enzymes in the elaioplasts is not clear. The enzymes in the *Allium cepa* spherosomes exert, according to Wałek-Czernecka (1962, 1963, 1965) a hydrolytic function similarly as the lysosomes of animal cells. Frey-Wyssling et al. (1963), on the other hand, associate spherosomes with the processes of lipid synthesis. Many earlier investigators believed that elaioplasts are the site of lipid formation (Wakker 1888; Raciborski 1895; Politis 1911; Wałek-Czernecka (personal communication) and others. If this view is considered in the light of Frey-Wyssling's suggestion, one might also attribute to the enzy-

mes present in elaioplast spherosomes a certain role in the process of lipid synthesis. One may suppose that elaioplasts are very active structures as manifested by the different localization of the hydrolases even in the elaioplasts of neighbouring cells. The occurrence of reaction products in the evenly distributed granules or in agglomerations of various kinds localized either in the centre or on the elaioplast periphery may be the result of displacement of spherosomes containing the appropriate enzyme to the sites where some definite processes occur.

It has been attempted to demonstrate the movement of the granules within the elaioplasts by means of a film camera by the slow-motion technique (Romer and Kwiatkowska, 1964). It appeared, however that *Ornithogalum* elaioplasts, even those which in direct microscopic observation seem motionless, are in constant movement. This movement is translational, translational-rotatory or rotatory and its speed varies. It was not possible on the basis of the available material and under the given conditions to establish the displacement of individual grains.

In one of the developmental phases of the elaioplasts, the author succeeded in linking the specific localization of lipase and acid phosphatase activity with the morphological changes occurring within the elaioplast. An accumulation of large amounts of final reaction products was observed at the site where disappearance of the granules occurred leading to the formation of vacuoles (see vital observations). This fact points to the contribution of the enzymes under consideration to hydrolysis processes and is in agreement with the observations of Wałek-Czernecka (1965) who demonstrated an increase of spherosomal hydrolase activity in the tissues of only temporary functional character, i.e. undergoing decomposition during the plant's lifetime.

SUMMARY

The elaioplasts of *Ornithogalum umbellatum* L. are formed by way of agglomeration of the shining Os O_4 -reducing granules 0.3—0.5 μ in size. In these granules the presence of proteins, neutral lipids, free fatty acids and unsaturated phospholipids has been demonstrated. These substances occur also in what is called the elaioplast stroma in which, moreover, the presence of mucilage probably of callose character has been ascertained.

Cytoenzymic reactions revealed acid and alkaline phosphatase, nonspecific esterase and lipase activity in the elaioplasts. The enzymes are localized in a part of the elaioplast granules. When two enzymes were jointly dedected (e.g. esterase and lipase; esterase and acid, or alkaline phosphatase), the elaioplast granule population was found to be heterogeneous: some granules contained only one the others both the enzymes.

The conclusion was drawn that the elaioplast granules showing hydrolytic enzyme activity correspond to spherosomes.

The character of the granules present in the elaioplasts but giving no positive enzymatic reaction could not be defined.

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Plate I

Development of *Ornithogalum umbellatum* L. elaioplasts

1. Small agglomerations of granules in a cell of the stamen filament from bud c. 1 mm long.
 - 2—4. Elaioplasts in epidermis of ovary in bud. 1.5, 2 and 5 mm long
 5. Spherical elaioplast surrounded by homogeneous capsule (7-mm bud, ovary epidermis)
 - 6 and 7. Elaioplasts from ovary of developed flowers. In the centre of the elaioplasts vacuoles are visible
 8. Elaioplast from ovary surrounded by dried perianth. Several minute vacuoles and the bulge termed "bud" are visible; next to them a small elaioplast formed from a detached "bud"
- Magn. c. 1500×

Plate II

Localization of hydrolases in elaioplasts of *Ornithogalum umbellatum*

1. Elaioplast treated with OsO₄ vapour for 5 min. All granules are more or less intensely blackened
2. Elaioplast after a test for lipase (Gomori's method). A part of the granules reacts positively
- 3—5. Elaioplasts with nonspecific esterase activity revealed by indoxyl method
6. Elaioplast after a test for acid phosphatase after Burstone
- 7—11. Examples of varying distribution of final reaction products after a test for alkaline phosphatase according to Burstone method. Magn. c. 1800×

Plate III

Localization of hydrolases in elaioplasts with vacuoles

- 1—4, 10 and 11. Elaioplasts after test for lipase. Reaction product in the peripheral part of the vacuoles (in 10 and 11 several minute vacuoles are visible, in the remaining figures one vacuole occupies the centre)
- 5—9. Analogous pictures after test for acid phosphatase after Burstone
12. Elaioplast with alkaline phosphatase activity revealed. Colour reaction products are seen at a distance from the central vacuole. Magn. 1800×

Plate I

Plate II

Plate III

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Z badań nad elajoplastami *Ornithogalum umbellatum* L.

I. Obserwacje morfologiczne, cytochemiczne i cytoenzymatyczne

Streszczenie

Elajoplasty *Ornithogalum umbellatum* L. tworzą się przez aglomerację lśniących ziarenek osmotycznie czynnych wielkości ok. 0,3—0,5 μ . W ziarenkach tych stwierdzono obecność białek oraz tłuszczów obojętnych, wolnych kwasów tłuszczowych i fosfolipidów o charakterze nienasyconym. Substancje

te znajdują się także w tzw. podłożu elajoplastów, w którym stwierdzono ponadto obecność śluzów prawdopodobnie o charakterze kalozowym.

Reakcje cytoenzymatyczne ujawniły w elajoplastach aktywność kwaśnej i zasadowej fosfatazy, esteraz niespecyficznych i lipaz. Enzymy te zlokalizowane są w części ziarenek elajoplastów. Przy łącznym ujawnianiu dwu enzymów (np. esterazy i lipazy; esterazy i kwaśnej, bądź zasadowej fosfatazy) stwierdzono zjawisko heterogenności enzymatycznej w populacji ziarenek elajoplastów: niektóre ziarenka zawierają tylko jeden z enzymów badanej pary, a w innych ziarenkach obydwie enzymy występują obok siebie.

Wysunięto wniosek, że ziarenka elajoplastów wykazujące aktywność enzymów hydrolitycznych odpowiadają sferosomom.

Charakter znajdujących się na terenie elajoplastów ziarenek, które nie dają pozytywnych reakcji na badane enzymy, nie został określony.

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