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The ultrastructure of protein bodies isolated from *Pisum sativum* and *Iris pseudoacorus* L. seeds

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Abstract

Protein bodies of *Pisum sativum* and *Iris pseudoacorus* seeds have been isolated in sucrose gradient with addition of 50mM citrate buffer, pH 5. Their ultrastructure due to isolation procedure has been described. Two types of protein bodies are present in pea and iris seeds: simple and compex ones — with many inclusions. The method of isolation, used in this paper extracts partly proteins — probably albumins, and also substances present in globoids i.e. phytin and acid phosphatase.

INTRODUCTION

It is well known that almost all seeds accumulate storage proteins as so-called protein bodies or aleurone grains. Morphology of these protein bodies has been well described in many seeds with electron microscope (Morton et all., 1964; Yatsu, 1965; Horner and Arnott, 1966; Paulson and Srivastava, 1968; Jones, 1969; Briarty et al., 1970; Jacobsen et al., 1971; Lott and Vollmer, 1973a, b). Protein bodies have been also isolated from seeds e.g. peanuts (Yatsu and Altschul, 1963), wheat (Graham et al., 1963), pea (Varner and Schidlovsky, 1963; Konopska, 1973), soybean (Yokoyama et al. 1972), rice (Mitsuda et al., 1969). But the most of the reports concerns of biochemical data and there is only a little information bearing directly on the ultrastructure of protein bodies after the isolation procedure (Varner and Schidlovsky, 1963; Tombs, 1967; Yatsu and Jacks, 1968; Mitsuda et al., 1969).

The purpose of this report was not only to check whether the method of isolation allows to obtain pure fraction of protein bodies, what is of special value in the interpretation of biochemical results, but also to describe the ultrastructure of protein bodies isolated from mature cotyledon tissue of pea and from endosperm

of iris. This paper attemps to correlate the data derived from light microscope observations, cytochemical technique, biochemical investigations and electron microscope observations.

MATERIAL AND METHODS

Mature cotyledon tissues of *Pisum sativum*, Lagiewnicki variety and endosperm of *Iris pseudoacorus* L. seeds, were the source of protein bodies in the isolation procedure.

Protein bodies were extracted according to the Tombs method (1967) modified by Konopska (1973). The hard, outer parts of the seed coat were peeled off and the inner parts of the seed, after removing of the embryo, were ground. To remove the lipids, 200 mg of iris powder and 500 mg from pea were treated with 10 ml of cold acetone for 1 h. Every 10 min the acetone was changed. Then, a crude homogenate was decanted and to so-called acetone powder (Yokoyam a et al., 1972) 5 ml of 20% sucrose in 50 mM citrate buffer, pH 5, were added. The acetone powder was gently homogenized again and then, placed into refrigerator for 12 h. After that time the homogenate was centrifuged in sucrose gradient composed of 90% sucrose in citrate buffer, pH 5, and 70% sucrose in the same buffer at 22 300 g for 30 min in Janetzki K 70 rotor. Because it was impossible to obtain the clear layer of protein bodies, the supernatant was filtrated through Schott G2 and G3 filters to remove the remnants of cells. The supernatant was centrifuged again at 12 200 g for 30 min and the pellets of protein bodies were fixed for electron microscopy.

Fixation was carried out in: a) 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.0, for 2 h at 4°; b) 3.6 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, with addition of Ca^{++} and Mg^{++} ions at the total concentration 0.1—0.4 M (according to Poux, 1965 a), for 2 h at 4° followed by postfixation in 2% OsO_4 in the same buffer for 1 h at 4°; c) 3.6% glutaraldehyde in 20% sucrose with 50 mM citrate buffer, pH 5, with addition of Ca^{++} and Mg^{++} ions or without, for 2 h at 4°, followed by 2% OsO_4 in the same medium. The divalent ions were added to precipitate soluble phosphates present within protein bodies (Poux, 1965a; Gabara and Modrzejewski, 1971).

Moreover, to detect acid phosphatase activity in isolated protein bodies of iris endosperm, the Gomori lead method applied to electron microscopy was used.

After postfixation in osmic acid and washing in appropriate buffer, material was dehydrated in a graded ethanol series and embedded in Epon 812. Sections were cut with LKB ultratome and stained with uranyl acetate followed by lead citrate. The sections were viewed and photographed in a Tesla 513 A electron microscope with 80 or 100 kV.

Some thin sections after staining with toluidine Blue and mounting in canada balsam, were examined under a light microscope and photographed.

RESULTS

As indicate electron microscopy observations the fraction of protein bodies of pea nad iris revealed the presence of some mitochondria, fragments of endoplasmic reticulum, and clumps of unidentified material. Moreover, between protein bodies of pea and around them the clumps of material probably of cytoplasmic origin appear, linking protein bodies themselves (Fig. 2).

No differences in the ultramorphology of isolated protein bodies after all used methods of fixation were noticed.

Protein bodies of pea

The isolated protein bodies, approximately 3 μm in diameter, are generally oval or spherical in shape (Fig. 1). Among them the two types of protein bodies may be distinguished: the first, predominant type — composed of an granular protein mass (Fig. 2), and the second one — less frequently visible, with many electron--transparent areas various in shape and dimensions, embedded in granular protein (Fig. 3, 3b). Within some of these electron-transparent areas the electron-dense inclusions usually oval in shape may be present (Fig. 3). A high magnification of such protein inclusions seems to indicate that some of them are distinctly displaced (Fig. 3b), leaving empty holes. Only in a few cases, single cavity within protein mass may be seen (Fig. 3).

All two protein bodies types are enclosed within a definite single mebrane, well preserved (Fig. 3a). However in many cases this membrane may not be continuous or distinct and sometimes it may be limited to small patches.

Protein bodies of iris

Protein bodies isolated from endosperm tissue, 3—20 μm in diameter, are usually oval but sometimes irregular in shape (Fig. 4). The different types of protein bodies can be observed : simple-fibrillar in structure (Fig. 5) and predominant, complex ones, containing various number of electron-transparent areas within the main storage protein (Fig. 6—9). The main protein mass of protein bodies appears as a fibrillar structure; such texture is especially visible in Fig. 5, 6 and 8a.

Electron microscopy reveals the presence of a single unit membrane surrounding the protein bodies (Fig. 5 a, 8a), but in most of them there is no evidence of limiting membrane.

Electron-transparent areas within protein bodies appear as holes, oval or round in shape (Fig. 7, 8) . In a few cases a fibrillar protein material may be seen inside these regular areas (Fig. 8). Besides of these holes, electron-transparent cavities of irregular shape are frequently present in protein bodies of iris (Fig. 6). Inside cavities the electron-dense and round in shape inclusions, 1 μm in diameter, are visible (Fig. 6). They seem to be limited by a layer of electron-dense material (Fig. 6).

Moreover, electron-opaque bodies of 0.2—0.3 μm in diameter, are seen as attached to the surface of protein bodies (Fig. 8, 9). A high magnification of Fig. 8 allows to suggest that they are protein bodies component and membrane around them is formed during the releasing of these bodies from protein bodies membrane (Fig. 8a).

In spite of many tests it was impossible to demonstrate the presence of inorganic phosphates and acid phosphatase activity in protein bodies isolated from endosperm tissue of *Iris pseudoacorus*.

DISCUSSION

In agreement with an earlier statement (Gabara and Modrzejewski, 1971) protein bodies of iris are of two types : simple and complex ones. These two types of protein bodies seem to be characteristic also for pea cotyledons in spite of some reports excluding the complex protein bodies in Legumine family (Setterfield et al., 1959; Bain and Mercer, 1966; Opik, 1966; Tombs, 1967; Briarty et al., 1970).

In confirmation with the earlier observations under light and electron microscopy (Setterfield et al., 1959; Varner and Schidlovsky, 1963; Gabara and Modrzejewski, 1971; Swift and Buttrose, 1973) the isolated protein bodies of pea and iris are surrounded by a single membrane. The existence of limiting membrane around protein bodies has been reported by many authors (St. Angelo et al., 1968; Ory and Henningsen, 1969; Mitsuda et al., 1969; Khoo and Wolf, 1970). Since it has been considered that protein bodies are formed as a result of protein accumulation in vacuoles or in endoplasmic reticulum (Bain and Mercer, 1966b; Englemann, 1966), their limiting membrane might be of vacuolar or endoplasmic reticulum origin.

Protein bodies of pea contain globulins (Varner and Schidlovsky, 1963), and in iris seeds they are composed of albumins and globulins (Gabara and Modrzejewski, 1971). But from biochemical results of Konopska (1974) results that only a slight quantities of albumins were found in protein bodies fraction of pea and iris. The protein of protein bodies are composed mainly of globulins making in pea 56.9%, and in iris 44%, and glutelins making respectively 26.1% and 34.3%. The method of isolation used in this paper extracts partly proteins especially distinctly from iris protein bodies and it seems obvious that albumins are these proteins (see also later).

The extraction of protein might be due to the homogenization medium at pH 5.0, which on the contrary to Tombs opinion (1967) does not prevent the solubility of protein as well as non-aqueous media do, for example, pure glycerol (Yatsu and Jacks. 1968). We must , of course consider another possibility, i.e. an influence of fixation technique. Although Lott et al., (1971) have claimed the only freeze--eching technique as the best for protein bodies, glutaraldehyde followed by osmic acid seems to be one of the best fixatives used to preserve the structure of protein

bodies (Yatsu and Jacks, 1968; Mollenhauer and Totten, 1971; Rost, 1972; Swift and Buttrose, 1973).

In an agreement with the results of Lui and Altschul (1967), Paulson and Srivastava (1968), Jacobsen et al., (1971), the electron-transparent holes within protein bodies of pea and iris, oval or round in shape, may correspond to globoids. The globoids of pea and iris are not delineated by membrane, what contrasts with the data of Poux (1963) who demonstrates that globoids in cotyledons of *Cucumis sativum* were membrane-bound.

Since it is known that globoids may consist of the globoid crystal, being the site of phytin storage (Lott et al., 1971), and usually surrounded by the soft globoid (Lott and Vollmer, 1973 b), and since the globoid inclusions which, because of bad penetration by Epon, tend to shatter are probably crystalline (Horner and Arnott, 1965), it seems likely that electron-dense inclusions within globoids of isolated pea protein bodies represent phytin in form of crystal described by Lott et al., (1971).

Following glutaraldehyde fixation the globoids are often characterized by the presence of electron-dense precipitate (Horner and Arnott, 1965; Jones, 1969; Jacobsen et al., 1971). There is some evidence that it occurs after chemical fixation (Lott and Vollmer, 1973a), and the precipitate formation is caused by phytin rich in phosphates and ions like Ca^{++} and Mg^{++} (Eb and Nieuwdorp, 1967; Jones, 1969; Lott et al., 1971).

Contrary to biochemical data (Konopska, 1973) demonstrating the presence of phosphates in the fraction of isolated protein bodies of pea and iris, and cytochemical results (Plate I, Fig. 11, 12; Gabara and Modrzejewski, 1971) localizing insoluble inorganic phosphates in globoids and soluble inorganic phosphates in protein mass, in the present paper there is no evidence of inorganic phosphates localization in pea and iris isolated protein bodies.

Because it is known that phytin seems to be easily removed after rinsing the sections in the acetate buffer, pH 5.0 (Jacobsen et al. (1971), the loss of phosphates in globoids might be due to citrate buffer at pH 5.0 used for isolation of pea and iris protein bodies. From that point of view the reduction in phosphates value should be taken into consideration in the discussing of biochemical results of isolated protein bodies by the method described in this paper. The same concerns acid phosphatase localized mainly within globoids (Poux, 1963, 1965 b; Gabara and Modrzejewski, 1971), or revealed biochemically in fraction of isolated protein bodies of pea and iris (Konopska, 1974) but not detected by Gomori technique in isolated protein bodies of iris.

Since it has been proved that albumins of *Vicia faba* aleurone grains show activity of acid phosphatase (Morris et al., 1970), and since, as it is demonstrated in the present paper, proteins are partly extracted during isolation, especially of iris protein bodies in a consequences of what irregular electron-transparent cavities appear, the absence of acid phosphatase in isolated protein bodies of iris might be partly due to extraction of albumins. Moreover, the cytochemical re-

sults on electron microscopy level showing the presence of acid phosphatase frequently in amorphous protein mass (Poux, 1963) seem to support this conclusion.

There is no evidence of relation between the electron-dense inclusions (1 μm in diam), observed sometimes inside isolated protein bodies cavities, and electron--opaque bodies (0.2—0.3 μm) in diam), connected with protein bodies surface. Since it is known that globoids may fracture into many pieces (Jacobsen et al., 1971), all two types of protein bodies inclusions might represent component of iris globoid; if so. they should be the place of phosphates and acid phosphatase localization, as it has been demonstrated cytochemically in light microscope by Gabara and Modrzejewski (1971). In spite of an assumption no evidence of these components was noticed (see also above).

On the other hand, inclusions present within cavities may represent phospholipids since Konopska (1973) has revealed the presence of phospholipids in isolated fraction of protein bodies of iris. Hitherto, no evidence of phospholipids has been mentioned within globoids of protein bodies of any dry seeds, however lipid droplets were described in globoids of *Cucumis sativus* after 3 days of germination (Poux, 1965b).

Although electron-opaque bodies attached to the surface of protein bodies might represent the fragments of globoids it seems most probably that they are remnants of lipid droplets not removed by acetone treatment during isolation procedure. There are some reports concerning the presence of RNA in the fraction of isolated protein bodies (Yokoyama et al., 1972; Konopska, 1973). But as indicated from our observations, the method used for isolation of protein bodies does not allow to obtain their pure fraction, free of cytoplasmic remnants, so, it seems not very likely that this RNA is of cytoplasmic origin.

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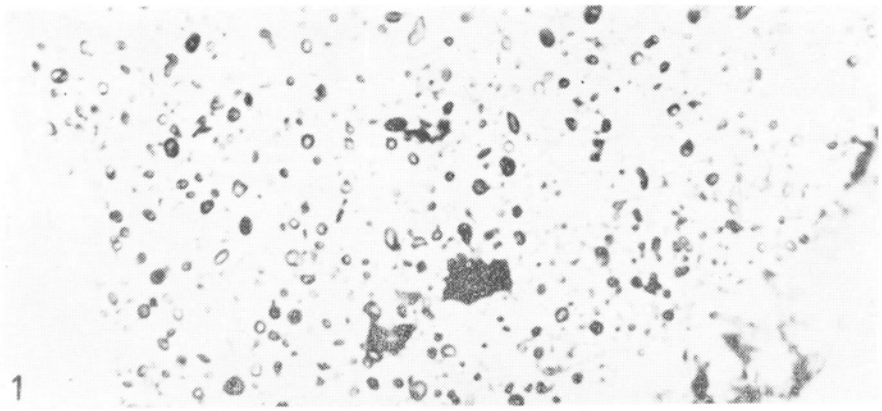
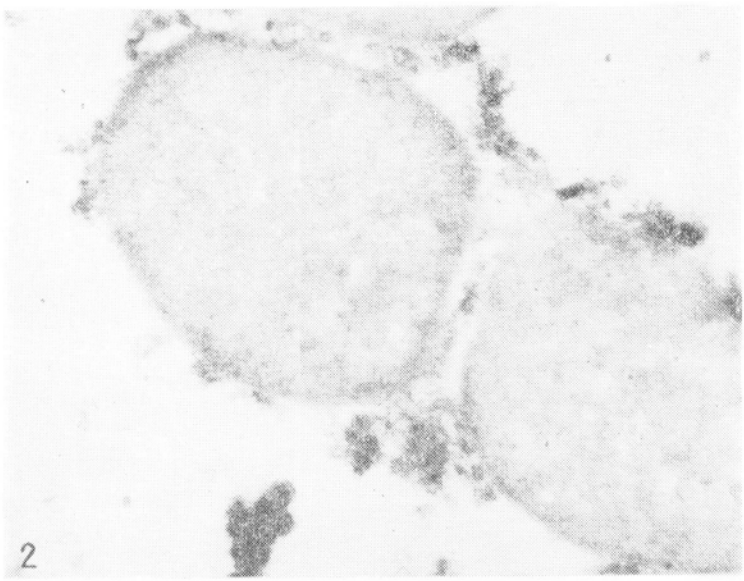




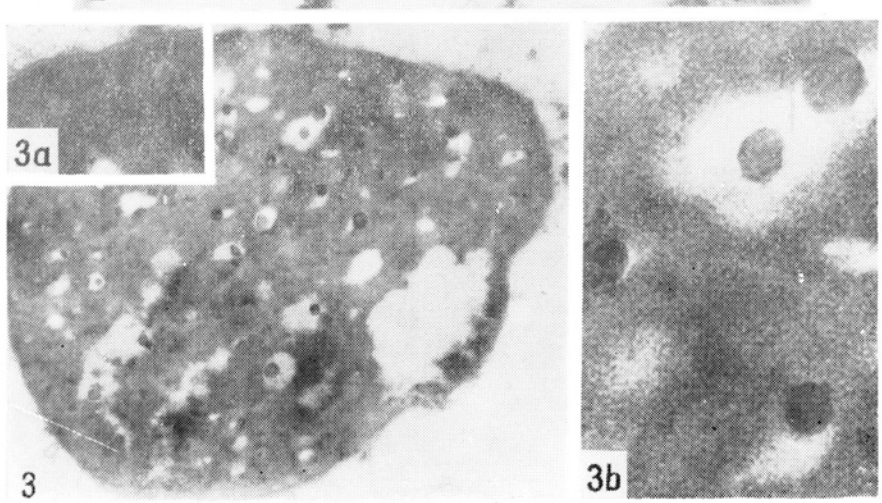


Fig. 1—3. Protein bodies isolated from mature cotyledons of *Pisum sativum*, Lagiewnicki variety

Fig. 1. Light microscopy. 720×.

Fig. 2. Electron microscopy. Simple protein body. Note clumps of material around them. 40 000×.

Fig. 3. Electron microscopy. Complex protein body. Irregular cavity inside protein mass, and many electron-transparent areas with electron-dense material inside them. 38 500×.

Fig. 3a. Enlargement of Fig. 3. A distinct single membrane is visible. 175 000×.

Fig. 3b. Enlargement of Fig. 3. Note granular protein mass nad electron-transparent area with inclusions. One of these inclusions is distinctly disclocated. 175 000×.

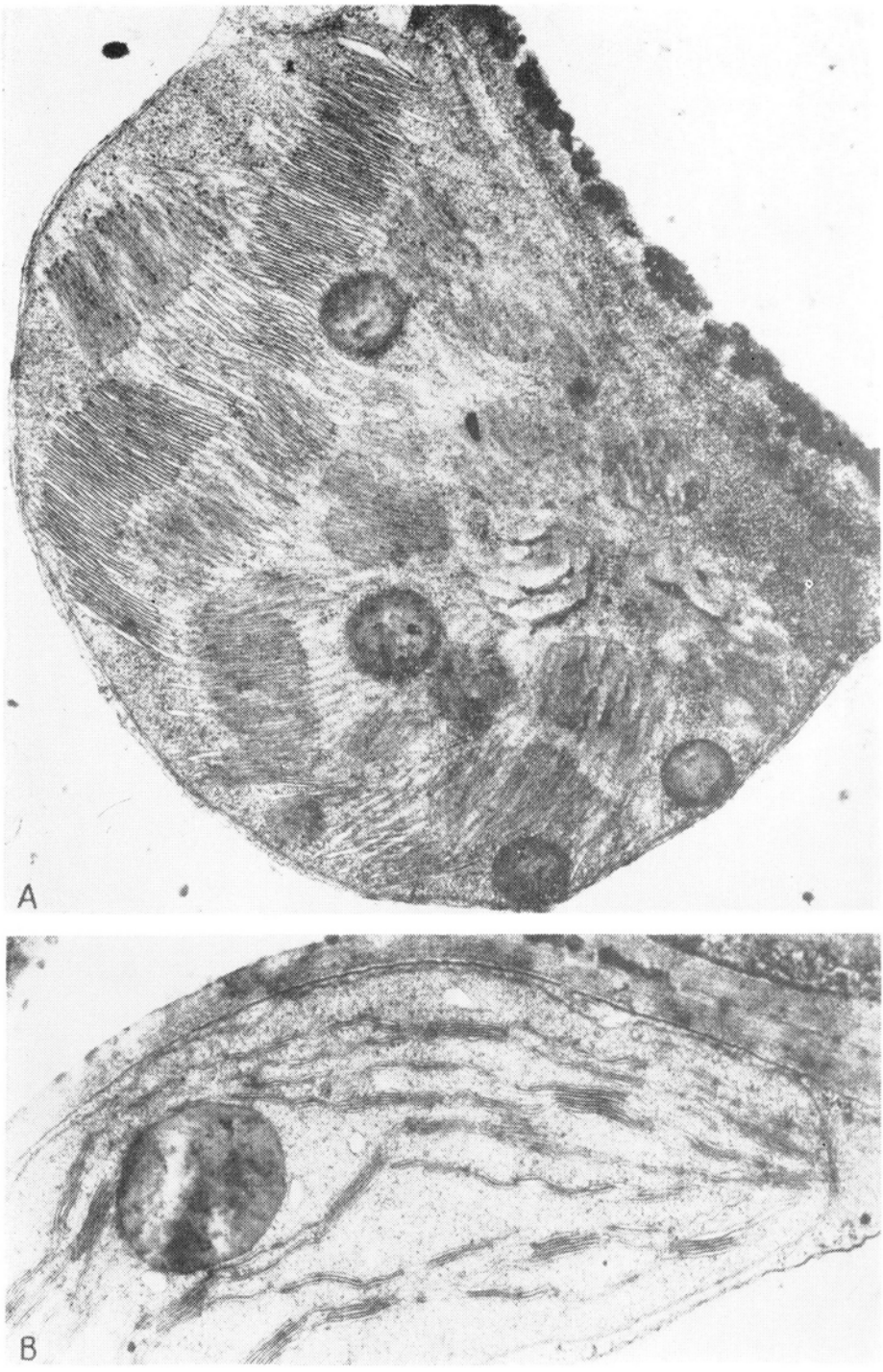


Fig. 4. Ultrastructure of chloroplasts of the chlorophyll-deficient chimera of *A. platanoide* at the end of summer time.

A — Ultrastructure of a chloroplast from unmutated part of the crown, 21000×;
B — Ultrastructure of a chloroplast from yellow spots of variegated leaves, 33600×.

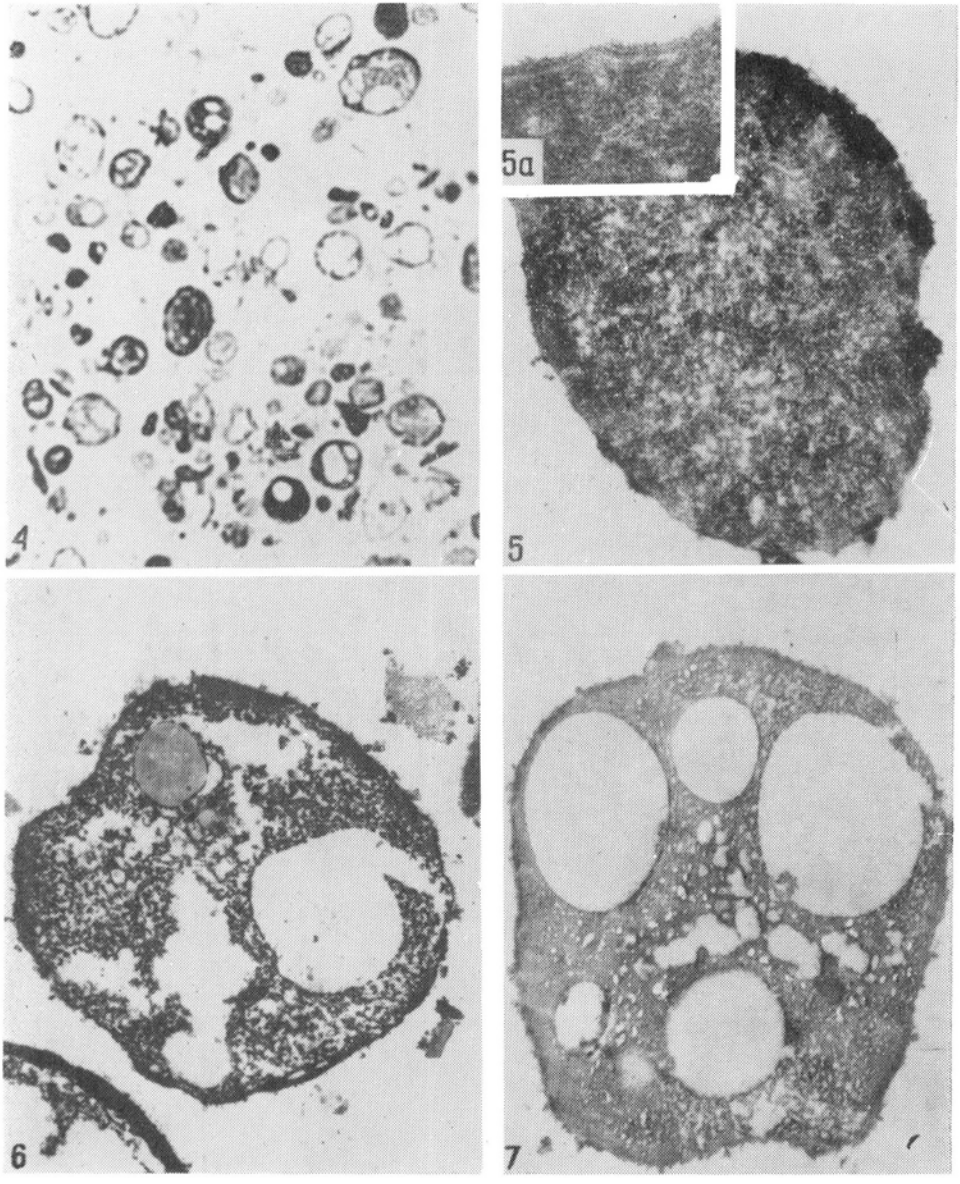


Fig. 4—7. Protein bodies isolated from dry *Iris pseudoacorus L.* seeds

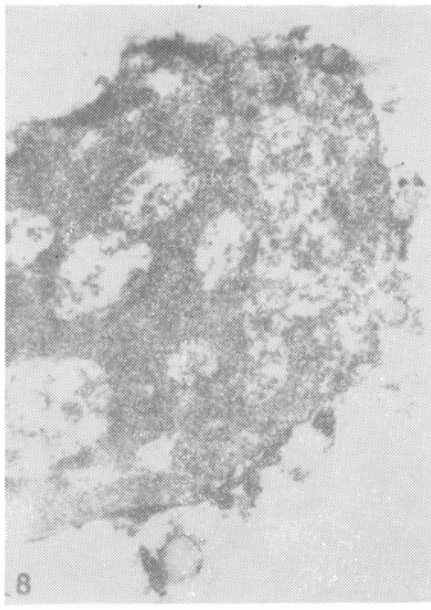
Fig. 4. Light microscopy. 720×.

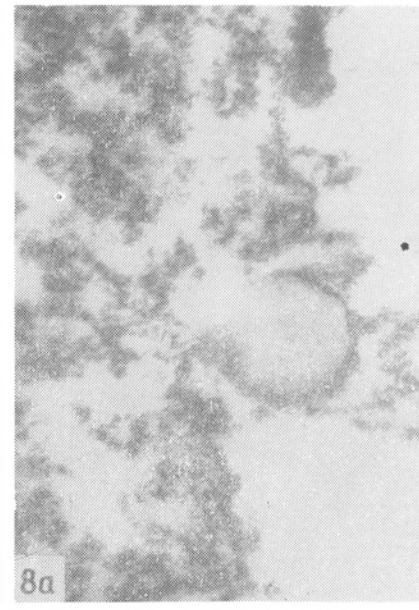
Fig. 5. Electron microscopy; simple protein body. 11 600×.

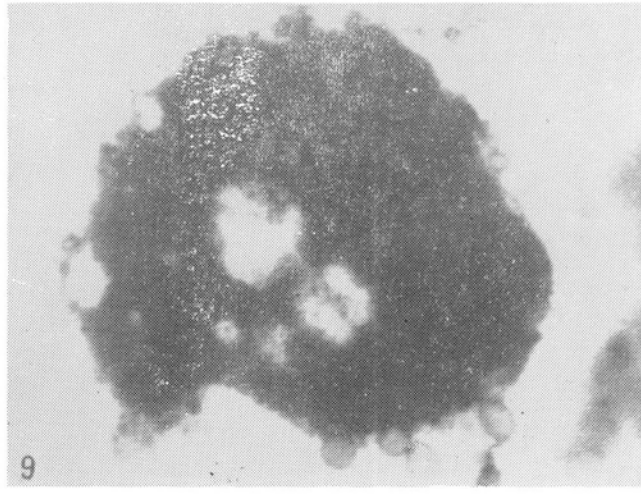
Fig. 5a. Enlargement of Fig. 5. Note a distinct single membrane. 48 720×.

Fig. 6. Electron microscopy; complex protein body with fibrillar texture of protein mass. Many cavities within protein mass. Note electron-dense inclusion inside one cavity. 7280×.

Fig. 7. Electron microscopy; complex protein body. Many electron-transparent holes are seen. 8900×.







Figs 8 and 9. Protein bodies isolated from dry *Iris pseudoacorus* L. seeds

Fig. 8. Electron microscopy; complex protein body. Note a fibrillar protein mass with electron--transparent holes and electron-opaque bodies attached to protein body surface. 12 320×.

Fig. 8a. Enlargement of Fig. 8. Limiting membrane around protein body, continuous with the membrane of electron-opaque body; fibrillar structure of protein mass is seen. 56 000×.

Fig. 9. Electron microscopy. Many electron-transparent areas, within some of these a fibrillar material is present. Electron-opaque bodies are in close contact with protein body surface. 35 530×.

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Ultrastruktura ciał białkowych izolowanych z nasion Pisum sativum i Iris pseudoacorus L.

Streszczenie

Ciała białkowe z nasion grochu i irysa izolowane były w gradiencie sacharozy z dodatkiem 50 mM buforu cytrynianowego o pH 5.0. Opisano ultrastrukturę ciał białkowych po ich wyizolowaniu.

W nasionach grochu i irysa występują dwa typy ciał białkowych: proste i złożone, zawierające inkluzje. Zastosowana w niniejszej pracy metoda izolowania ciał białkowych powoduje częściową ekstrakcję białek, prawdopodobnie albumin, a także zanik substancji występujących w globoidach, jak fityny i kwaśnej fosfatazy.