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Microscopy of soybean seeds: cellular and subcellular structure during germination, development and processing with emphasis on lipid bodies

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Microscopy of soybean seeds: cellular and subcellular structure during germination, development and processing with emphasis on lipid bodies

by

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INTRODUCTION

The soybean is a virtual storehouse of two nutritionally and economically important substances, protein and oil. With the advent of world food shortages and the demand for lower cost consumables, considerable research has been directed towards characterization of these two components with hope of increasing their utilization.

Research has dealt primarily with chemical and functional properties of soybean proteins and oil. In contrast, very little is known about ultrastructural characteristics of soybeans. Studies on soybean ultrastructure lag considerably behind similar research on wheat, corn, cottonseed and peanuts (Wolf, 1972). Although the profile of soybean structure based on optical microscopy was described more than 60 years ago, little additional work was done until the early 1960's when more sophisticated tools and techniques, such as the electron microscope, became readily available. Since then, a few investigators have filled in some of the details of soybean structure, but much more research is needed.

The soybean seed is made-up of four basic parts: a seed coat or hull (8%), the plumule and hypocotyl-axis (2%) and two large cotyledons (90%). The cotyledons are of primary interest, since they contain the storage tissue of the seed. Microscopy studies have revealed that the cotyledons are comprised of palisade-like cells that are filled predominantly with protein and oil. The electron microscope shows that most of the protein is packaged in subcellular inclusions referred to as protein bodies which are large (2 to 10 μm in diameter) and nearly

spherical in shape, while the oil is located in much smaller (approximately 0.2 to 0.5 μm in diameter) particles designated spherosomes (Wolf, 1972). Isolation, chemical characterization and electron microscopy studies have been directed mainly at protein bodies, whereas the spherosomes of soybeans have not been isolated nor characterized. Consequently, their structure, composition, susceptibility to enzyme attack and stability under various processing conditions are still unknown (Wolf, 1976). This is surprising when one considers that soybean oil is the most important domestic vegetable food oil. To grasp the significance of soybean oil on the U.S. economy, consider that in 1933 less than 500,000 lbs. of soybean oil were used in the manufacture of margarine and shortening; in 1971, the quantities were 1.0 and 2.4 billion lbs. respectively (Horan, 1974). In 1976, the domestic disappearance of soy oil totaled 7.5 billion lbs. by appearing in liquid cooking and salad oils, shortenings, margarine and other nonfood products (American Soybean Association, 1978). Because of its availability, versatility and price, soybean oil will no doubt maintain its dominance in the fats and oils market.

In addition to the lack of knowledge of soybean spherosomes, very little is known about the effects of processing variables on soybean ultrastructure. More knowledge of soybean structure might lead to more efficient processing. Ultrastructural modifications of soybeans during current processing practices — conditioning, cracking, flaking, defatting, desolventizing, toasting and

drying — has received little attention, even though these practices have been in use for over 30 years.

The integrity of subcellular components (i.e. spherosomes, protein bodies, cell walls and cytoplasmic network) subjected to a variety of physical and chemical treatments has never been fully investigated. For example, there is little information concerning the effect of heat on soybean ultrastructure, even though boiling of soybeans is a common practice in the preparation of soymilk to improve its flavor. Also, no study has been made of freezing on cellular and subcellular structure of the soybean seed. Studies on the stability of subcellular constituents to enzyme attack and to extraction by organic solvents and studies on ultrastructural changes during germination and developmental stages of soybeans lag considerably behind similar research on other seeds.

In view of the marginal and somewhat incomplete information available on soybean structure, a microscopy study was undertaken to better understand soybean ultrastructure by observing the mature seed, the germinating and developing seed, the processed seed and lipid bodies (spherosomes).

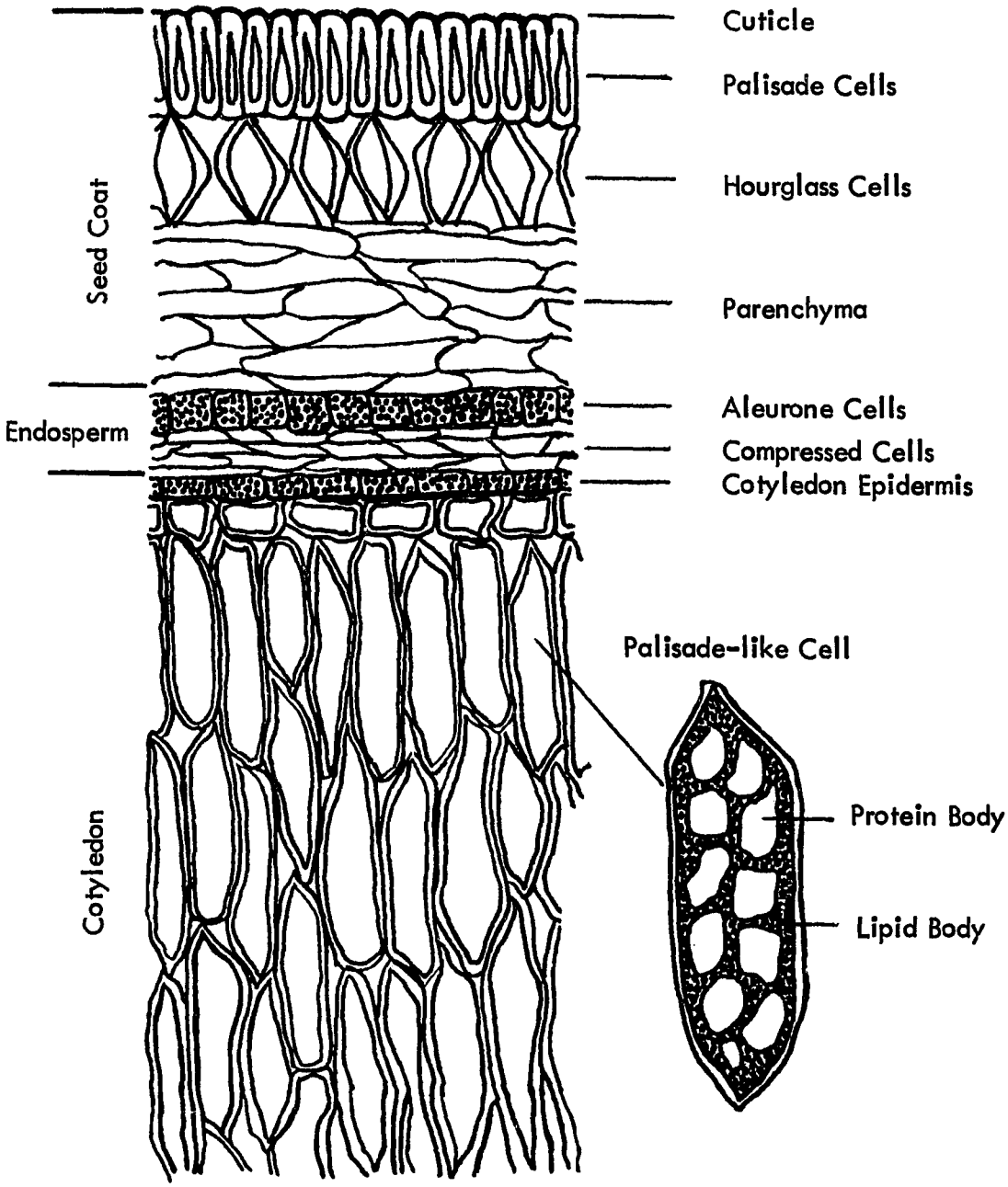
LITERATURE REVIEW

Soybean Seed Anatomy

The major anatomical parts of a mature soybean are seed coat or hull (8%), an embryo consisting of two large fleshy cotyledons (90%) and two minor structures, the plumule and hypocotyl-radicle axis (2%) (Wolf, 1972). A cross section of the hull and part of the cotyledon are shown in Figure 1. Proceeding inward the hull is made up of an epidermis composed of closely packed palisade cells (40 μm x 15 μm), rectangular in outline with the long axis at right angles to the outer surface. Under the epidermis is a single layer of cells which have the shape of an hourglass or capital "I". These hourglass cells are unique to soybeans and have often been used to detect the presence of soybean products in various foods and feed mixtures (Piper and Morse, 1923). The cells have unevenly thickened walls which are thin at the ends of the cell and considerably thicker in the central constricted portion of the cell. They form a strong supporting layer, and with their considerable intracellular space, they protect the embryo. Next, several rows of thin-walled, flattened cells make up the inner parenchyma tissue. These cells appear clear and seem to be lacking in cellular content.

Immediately under the seed coat is the endosperm tissue. Unlike many other seeds, soybeans are essentially devoid of endosperm, and it appears as only a thin layer surrounding the embryo. The outer layer of the endosperm is characterized by a well-marked single layer of small cuboidal aleurone cells (20 μm x 20 μm) filled with dense protein occurring in the form of small aleurone

Fig. 1. Cross section of mature soybean hull and part of the cotyledon.


Seed Coat
Endosperm
Cotyledon
Cuticle
Palisade Cells
Hourglass Cells
Parenchyma
Aleurone Cells
Compressed Cells
Cotyledon Epidermis
Palisade-like Cell
Protein Body
Lipid Body

grains, often referred to as protein bodies. Below the aleurone layer are several layers of parenchyma cells of the endosperm that appear flattened or compressed by the growth and enlargement of the embryo (Williams, 1950).

Inside the endosperm, one finds the two large cotyledons, which constitute the major storage tissue of the seed. In cross section the cotyledon is semicircular in shape and bounded by an epidermis of cuboidal shaped cells (10 to 15 μm x 15 μm), and like the aleurone cells of the endosperm, they contain small protein bodies. On the flat or adaxial side of the cotyledon, one to three rows of palisade cells (50 μm x 15 μm) lie just below the epidermis and merge with a more spongy type of parenchyma in the central portion of the cotyledon. The abaxial or curved portion of the cotyledon consists mainly of elongated parenchyma cells that do not form distinct layers. The remainder of the cotyledon is made up of thin-walled, elongated palisade-like cells (30 to 50 μm x 15 to 25 μm), which contain the economically important protein and oil. The proximate composition of soybeans and seed parts is shown in Table 1.

Table 1. Proximate composition of soybeans and seed parts (from Kawamura, 1967).

Fraction	Protein[a]	Fat	Carbohydrate	Ash
Whole bean	40.0	21.0	34.0	4.9
Cotyledon	43.0	23.0	29.0	5.0
Hull	8.8	1.0	86.0	4.3
Hypocotyl	41.0	11.0	43.0	4.4

[a] (N x 6.25).

Progress has been made in elucidating the gross structure of soybeans, but many of the fine details of the cotyledon palisade-like cells are still missing. For example, little is known about the cytological location of various enzymes (α-amylase, lipoxygenase and urease) and other minor constituents (saponins, isoflavones, oligosaccharides and phytate) and their relationship to the protein bodies and spherosomes (Wolf, 1976). Intracellular distribution of the compounds responsible for the characteristic beany and bitter flavors of raw soybeans likewise remain undetermined. However, it must first be determined whether these compounds pre-exist in the soybean, or whether they are formed by enzymatic and non-enzymatic reactions when the seed is crushed (Wolf and Cowan, 1971). A better understanding of the cellular and subcellular structure may shed some light on the distribution of these constituents and provide insights for new approaches to processing.

Current knowledge of some cotyledonary constituents is outlined in the following sections.

Subcellular Components

Protein bodies

The most obvious subcellular constituents in the soybean cotyledon cells, even at the light microscope level, are the protein bodies. Electron microscopy has been widely used by a number of researchers to identify and characterize soybean protein bodies (Bils and Howell, 1963; Saio and Watanabe, 1966, 1968;

Tombs, 1967; Webster and Leopold, 1977; Wolf, 1970). These subcellular structures appear spherical to irregular in outline and range in size from about 2 to 10 μm in diameter, with some occasionally being as large as 15 to 20 μm (Tombs, 1967; Wallis, 1913; Wolf and Baker, 1972). Wallis (1913) reported that protein bodies dissolved in caustic soda solution and in 10% sodium chloride. They appeared yellow when stained with iodine and picric acid and red with eosin. Tombs (1967), concluded that protein bodies accounted for about 70% of the total soybean protein, and that only one protein could be detected in them: glycinin, the major soybean protein. Glycinin is identical to the cold precipitated and 11 S proteins described by Wolf and Briggs (1959).

Komoda and co-workers (1968), isolated protein bodies from soybeans by sucrose density centrifugation. After soaking soybeans overnight in water, they homogenized them in 0.5 M sucrose, removed the cellular debris, diluted the homogenate to 0.25 M sucrose and centrifuged. The precipitate was believed to contain the protein bodies. Upon analysis they found total protein quite low (32 to 52%), but the isolated fraction was high in lipid (23 to 63%). In an isolation procedure by Tombs (1967), light and heavy fractions of protein bodies were obtained; the heavy fraction ($1.30 < \text{density} < 1.32$) was nearly 80% protein while the light (density < 1.30) protein body preparation was almost pure protein (98%). Protein bodies of such high protein content appear unique to soybeans (Wolf, 1972). Tombs reported that RNA, phytic acid and lipids were also present in the isolated fraction. The amount of neutral lipid

in the isolate was variable, but unfractionated protein bodies contained 10% lipid extractable with chloroform and methanol. However, only 1.5% lipid was found in the light protein body fraction, most of which was in the form of phospholipid. In still another isolation method, Saio and Watanabe (1966) homogenized soybeans in cottonseed oil and separated the protein bodies by differential centrifugation in cottonseed oil — carbon tetrachloride mixtures of varying densities. They found the protein body fraction contained 65% protein, 8.5% carbohydrate and 7.7% ash, while 17% of their preparation was unaccounted for.

Microscopically, Tombs (1967) and Webster and Leopold (1977) observed a clearly resolvable limiting unit membrane in protein bodies. However, Saio and Watanabe (1966) found no membraneous material. Membrane-bound protein bodies are prevalent in many other plant tissues and have been reported in a number of cotyledonary tissues similar to soybeans (Jones, 1969; Lott et al., 1971; Yatsu, 1965; Yoo, 1970). Tombs (1967) suggested that the membrane delimiting the soybean protein body (estimated at 75 Å in thickness), consisted of phospholipids. It was stable to diethyl ether and hexane extraction.

Protein bodies can be classified as: bodies containing amorphous protein only, bodies with globoid crystals (phytin) embedded in amorphous protein and bodies with both globoid crystals and protein crystalloids embedded in amorphous protein (Rost, 1972). According to the terminology of Lott et al. (1971), a region in a protein body consisting of protein in a definite crystalline arrangement is termed the protein crystalloid and spherical inclusions are termed globoids. An electron-opaque portion

of a globoid is called globoid crystal, and an electron-translucent portion is termed soft globoid.

Amorphous protein in the form of globules or shapeless masses has been found in the ova of gymnosperm and in algae and fungi (Bils, 1960). Protein bodies having a combination of crystalloid and amorphous protein were reported in the endosperm, embryo and perisperm of ripe, oily seeds of the genera, Zea, Ricinus, Juglans, and Bertholletia (Sharp, 1934). The well-known protein body of Ricinus, consists of a crystalloid, a protein ground substance, and a globoid of calcium and magnesium phosphate with certain organic constituents (Meyer, 1920). Jacobsen et al. (1971), working with the aleurone layer of barley, demonstrated that protein, phosphate (phytin) and lipid were present in the globoid of their protein bodies. Similarly, lipid, globoid inclusions and vestiges of internal and external membranes have been found as components of protein bodies in various tissues (Buttrose, 1971; Buttrose and Soeffky, 1973; Chamura, 1975; Tanaka et al., 1973). Paleg and Hyde (1964) reported that globoids viewed in barley protein bodies might be lipoidal in nature. Protein bodies of certain species have also been reported to contain crystals of calcium oxalate (Guilliermond, 1941; Vaughan, 1970).

In seeds of many members of the Leguminoseae, protein bodies appear structurally uniform when observed in the light microscope (Guilliermond, 1941; Rendle, 1888), and, despite certain electron microscopic data to the contrary, this view is still generally held. Tombs (1967), investigated soybean protein

bodies together with changes in their appearance upon germination and found that protein bodies were initially uniform in matrix but became more granular in appearance during the early stages of germination. Saio and Watanabe (1966) reported that protein bodies appeared homogeneous in structure in the cotyledons of water-soaked soybeans. However, Webster and Leopold (1977), found that the contents of protein bodies in dry cells of soybeans included granular or flocculent material, and following imbibition with water, showed an increase in electron-translucent area and a decrease in staining intensity. Problems in connection with studies on dry tissue, however, have been reported by a number of researchers (Perner, 1965; Swift and O'Brien, 1972; Yatsu, 1965). Dry tissues studied after vapor fixation (usually involves osmium tetroxide fumes), frequently result in poor infiltration, imperfect sections, and lack of resolution. The use of common aqueous fixatives to obtain adequate fixation of seeds often causes tissues to imbibe water creating a hydrated state and not the desired dry condition. Therefore, caution must be taken when interpreting the structural components of reportedly dry tissue.

Recently, Lott and Buttrose (1978), examined globoids in protein bodies of several legume seed cotyledons, and observed that soybean protein bodies contained globoid crystals. The globoids appeared small and rare. They found that while some cells contained protein bodies with inclusions, that often a neighboring cell would have protein bodies completely devoid of inclusions. With this cell-to-cell (or tissue-to-tissue) variation, it seemed understandable why soybean protein body

inclusions might have been easily overlooked or passed off as being artifacts due to sample preparation.

In the study by Lott and Buttrose (1978), energy dispersive X-ray (EDX) analysis of sections of electron-dense globoid crystals from soybean protein bodies revealed the presence of the elements P, K and Mg, with the highest reading for phosphorus. Their value obtained for phosphorus content of soybean cotyledons, fits well into the range of values (0.5 to 0.7% of seed dry weight) reported by Lolas et al. (1976) for 15 varieties of soybeans. Considering that soybean protein bodies have been found to contain from 0.5% to 0.8% phosphorus on a dry weight basis (Saio and Watanabe, 1966; Tombs, 1967), and that phytin accounts for over 50% of the total phosphorus of the seeds (Smith and Rackis, 1957; Lolas et al., 1976), there is the possibility that these inclusions or globoids found in soybean protein bodies are the sites of phytin. However, Tombs (1967), in his study on soybean protein bodies, writes, "Phytic acid interacts strongly with glycinin and is presumably protein bound in the body, but there is no evidence for its localization, unlike wheat where it appears to be present in specific areas of the protein bodies."

Starch granules

The presence of starch in mature soybeans has been under debate since the 1880's. In one of the few existing references describing the presence of starch, Meissl and Böcker (1883) reported that soybeans contained between 1 to 3%,

depending upon variety. The starch grains appeared as extremely small spheres, which occurred in groups or bundles of granules and were not in the same form as typical bean starch. Street and Bailey (1915) also found starch in soybeans, but only the equivalent of 0.5%. Many researchers, however, claim that mature soybeans are free of starch. Blondell (1888) and Prinsen (1896) were unable to detect starch in their soybean samples. They reported that thoroughly mature beans were practically starch-free, while immature soybeans contained considerable amounts. Bils and Howell (1963) microscopically observed the presence of numerous conspicuous starch grains throughout most of the period of seed development, but these structures ultimately disappeared in the final stages of maturation. Tada and Kawamura (1963) also reported that starch decreased to almost zero at maturity while immature soybean seeds contained 4 to 5%. Similarly, Wallis (1913) found a few starch grains in soybean cotyledons, but concluded that his preparations were of immature seeds and that mature cotyledons contained a negligible amount of starch. Piper and Morse (1923) suggested that the absence of starch was indicative of mature soybeans, but added that many varieties of soybeans had starch present in small quantities at maturity.

Recently, Wilson et al. (1978), isolated and identified starch in ten different soybean cultivars. The starch content ranged from 0.19% for the Hawkeye variety to 0.91% in the Marion cultivar. Yazdi-Samadi et al. (1977), found that mature seeds of Harosoy 63 contained 2.6 mg starch while Steele

contained 1.5 mg. These values represented 1.1% and 0.8% of the dry weight in Harosoy and Steele, respectively. Scanning and transmission electron microscopy of isolated starch and of intact seeds (Wilson et al., 1978), revealed that soybean starch granules were ovoid in shape, had smooth surfaces and ranged from 1.0 to 7.0 μm diameter with an average size of 3.0 μm. They also observed that the starch was concentrated toward the midline of the soybean cotyledon and practically absent near the periphery. They suggested that if microscopic examinations were confined only to sections near the periphery of the cotyledons, then the failure to find starch would be understandable. Possibly, this occurred when MacMasters and coworkers (1941) were unable to identify starch in soybean cotyledons by microscopic examination.

Calcium oxalate crystals

Wallis (1913) described small prismatic crystals of calcium oxalate, arranged in pairs end to end, which were scattered throughout the tissue of soybean cotyledons. He found that the crystals were easily overlooked, but became more visible when a cotyledon section was examined by polarized light. The double crystals measured about 24.5 μm long and 4 to 5 μm wide, and apparently dissolved in hydrochloric acid, but not in 20% caustic soda nor in acetic acid.

Since this finding by Wallis, these polarizing crystals have received little mention in the literature concerning soybeans, and information on their occurrence and distribution is scarce. Piper and Morse (1923) and Bils (1960) only devoted

one and two sentences respectively in their publications, recognizing the existence of the crystals, but thus far there are no published photographs showing their structure, distribution or their polarizing characteristics in soybeans.

The taxonomic distribution of calcium oxalate crystals in plants is large and diverse. McNair in 1932 listed 215 plant families that were known to contain calcium oxalate crystals. The shape and location of calcium oxalate crystals within a given taxon is often very specific and some investigators have used these features for taxonomy (Chartschenko, 1932; Gulliver, 1864; Heintzelman and Howard, 1948; Jacard and Frey, 1928). Guilliermond (1941) reported the presence of calcium oxalate crystals in protein bodies from a variety of plant tissues. Similarly, Vaughan (1970), in summarizing the anatomical features of a number of oil seeds, noted the presence of druse or cluster (reference to crystal shape) crystals of calcium oxalate in the cytoplasm of many seeds and within the protein bodies of almonds and hazel nuts.

Little is known about the function of calcium oxalate crystals in plants though there has been much speculation. Many believe that oxalate is an end-product of metabolism, or that it may function as a storage form for either calcium or oxalate. Another major function attributed to calcium oxalate crystals is that of protection against foraging animals. The irritation and burning sensations of the mouth caused by eating crystal containing plants is well-known (Kingsbury, 1964) and may discourage foraging animals. Oxalic

acid is the only organic acid of plants that is toxic to live-stock under natural conditions (Kingsbury, 1964). Perirenal edema in swine has been linked to ingestion of plants that are high in oxalates (Osweiler et al., 1969). There have been reports of human deaths due to oxalate poisoning after ingestion of rhubarb leaves (Kallicate and Kauste, 1964; Tallqvist and Väänänen, 1960). However, Fasset (1973), in his investigation of the toxicity of oxalates, concluded that there is no hard evidence that oxalates in foods are toxic to man.

Lipid Bodies

Soybeans

Wallis (1913), in his article on the structure of the soybean, described the palisade-like cells of the cotyledons as being filled with protein bodies and numerous droplets of fixed oil. Following Wallis's observations, considerable attention was focused upon protein body isolation and chemical characterization, and by the late 1960's, their structural appearance and distribution had been examined by electron microscopy. The numerous droplets of "fixed oil," however, have remained an enigma. These intracellular particles have not been isolated nor characterized (Wolf, 1972), which may be partially responsible for confusion about their terminology. They have been referred to as lipid granules, droplets of fixed oil, lipid grains, lipid bodies and more recently as spherosomes (Bils and Howell, 1963; Saio and Watanabe, 1966; Tombs, 1967; Wallis, 1913; Webster and Leopold, 1977; Wolf and Baker, 1972, 1975).

According to Kahn (1959), the fat in soybeans occurs in submicroscopic droplets. Wolf and Baker (1972), utilizing a scanning electron microscope, examined the surfaces of freeze-fractured soybean cotyledons and observed that the oil was contained in small structures (0.3 to 0.5 μm in diameter), designated as spherosomes, a term used earlier by Saio and Watanabe (1966) to describe the interbody network interspersed with protein bodies in cotyledon cells of soybeans. Evidence from electron-microscopic studies of soybean native fat (Kahn et al., 1960) suggested that in situ, fat may be enclosed in membranous structures, which collapse as the fat is consumed during seed germination. Webster and Leopold (1977), found that soybean lipid bodies stain uniformly gray after glutaraldehyde-osmium tetroxide fixation and appear to be bounded by a thin electron-dense lamella. However, it could not be resolved as a distinct unit-membrane.

In dry soybean cotyledons, lipid bodies appeared round or oval and were distributed throughout the cytoplasm, typically in a single layer around protein bodies, and at the margins of the cytoplasm (Webster and Leopold, 1977). In imbibed cells, however, lipid bodies were frequently irregular in outline, often larger, and not as numerous along the plasma membrane. Tombs (1967), in a report on protein bodies of the soybean, found that lipid attached itself tenaciously to protein bodies in homogenates of whole soybeans, thus suggesting a strong lipid body-protein body interaction. Suryanarayana (1976) found that oil

globules in sectioned cotyledon tissue were conspicuously seen only after pretreatment with 50% ethanol or following progressive protein body fragmentation during germination, suggesting a tenacious attachment to the protein body mass. Furthermore, in freeze-fractured cotyledons, protein bodies were more readily seen when section surfaces were first washed with hexane. By this means the oil in the spherosomes, packed between protein bodies, was readily dissolved (Wolf and Baker, 1972). These investigators also observed a proteinaceous meshwork with indentations 0.3 to 0.5 μm in diameter on the interior surface of cell walls. They suggested that these indentations represent sites occupied by spherosomes.

Wolf and Baker (1975) concluded from their investigations that spherosomes functioned as the major storage sites for soybean oil. However, Kahn et al. (1960), believed that the membranes surrounding the native fat contained enzymes required for fat metabolism. Biochemically they found that endogenous as well as added lipase appeared to be preferentially adsorbed on the native fat bodies and detected enzymes such as RNase, protease, lipoxidase, peroxidase and ester phosphatase to be associated with this fraction.

These few reports contain the available information on the nature of lipid bodies found in the cotyledons of soybeans. In some respect, the terms lipid body, lipid granule and lipid grain, give a fairly clear and consistent view of not only their purported composition, but physical appearance as well, whereas spherosome, a term becoming more frequently used in the literature

(Saio and Watanabe, 1966; Taranto and Rhee, 1978; Wolf, 1972; Wolf and Baker, 1972, 1975; Wolf and Cowan, 1971), is not as readily understood. Although considerable attention has been directed towards characterization of spherosomes in a variety of tissues, there still is disagreement about their properties.

The controversy

The historical background of the spherosome, which is considered by some authors to be the direct precursor of the oil body (Frey-Wyssling et al., 1963), or the same as the oil body (Horner and Arnott, 1966; Yatsu et al., 1971), has been clouded by confusion and much controversy.

Almost a century ago, Hanstein (1882) described spherical bodies of dense material in the plant cell which he called "microsomes." These subcellular inclusions displayed a much higher refractivity than the surrounding cytoplasm and therefore showed up beautifully in darkfield microscopy. Ignoring Hansteins botanical term, Claude (1943), used the word "microsomes" for particles isolated from liver homogenates by centrifugation thus creating considerable terminological confusion. The botanists finally settled the problem by changing the name of their microsomes to spherosomes, a term which had been created by Dangeard (1922) to distinguish between spherical (spherosomes) and rod-shaped (mitosomes) "microsomes of the spherome." Earlier Dangeard (1919) had described the

spherome as consisting of "microsomes" which he reported as highly refringent spherules of an oily appearance that blacken, more or less, with osmic acid. From the very outset, there was controversy concerning spherosomes: one faction, Dangeard (1919), looked on spherosomes as organelles, and the other, Guilliermond (1921), considered spherosomes merely as products of cellular metabolism (lipids).

One of the most recent eruptions of this controversy was the objection voiced over the identification of "oil-droplets" from oleaginous tissues with "spherosomes" of nonoily tissues Sorokin (1967). Oil droplets were purported to be droplets of oil that were free in the cytoplasm without delimiting unit-membranes, whereas spherosomes were alleged to be composed of phospholipids and proteins and were bounded by unit-membranes. Many investigators (Perner, 1953; Walek-Czernecka, 1965) agreed with this distinction between oil droplets and spherosomes — both at the light and electron microscope levels. Yet, others continued to identify oil-droplets with spherosomes (Hrsel, 1966; Yatsu, 1965; Yatsu et al., 1971). Surprisingly, almost a century after cytologists first described these cytoplasmic particles, they are still the object of much controversy.

Spherosome and oil droplet are just two of the many names often used synonymously to describe these intracellular particles in a broad range of plant tissues. In the literature they have been referred to variously as lipid bodies,

oleosomes, lipid inclusions, oil globules, lipid vesicles and lipid granules (Bils, 1960; Bils and Howell, 1963; Mollenhauer and Totten, 1971a; Webster and Leopold, 1977; Yatsu et al., 1971). There seems to be as many discrepancies in their structural appearance and chemical composition, as there are names associated with them (Mishra and Colvin, 1970; Sorokin, 1967). To adequately understand the nature of these subcellular particles and their distribution, the findings of numerous researchers are reviewed.

Appearance

Light microscopy Sorokin and Sorokin (1966) found that in most cells of higher plants, spherosomes appeared as small spherical bodies which were conspicuous for their abundance and rapid motion when viewed under a light microscope. Yatsu et al. (1971), reported that spherosomes in epidermal cells of onion bulb scales were minute spherules, measuring 1.0 μm in diameter and characteristically prone to Brownian motion. Similar observations have been reported in a variety of tissues including the endosperm of tobacco seedlings (Matile and Spichiger, 1968), peanut cotyledons (Jacks et al., 1967), and in the guard cells of Campanula perisicifolia L. (Sorokin and Sorokin, 1966).

One of the most distinguishing characteristics of spherosomes when viewed with a light microscope is their highly refractile nature (Hanstein, 1882; Matile and Spichiger, 1968; Spichiger, 1969; Yatsu et al., 1971). In phase microscopy of living cells, Sorokin and Sorokin (1966), described spherosomes as appearing

as dark bodies surrounded by halos and as light bodies surrounded by dark rims. Under brightfield illumination, they observed that the rim of a spherosome often appeared red in unstained preparations, an optical phenomena that was not exhibited by other cytoplasmic particulates. Mollenhauer and Totten (1971a,b) found that typical droplets or vesicles of reserve lipid in pea and bean cotyledons appeared as small (0.1 to 2.0 μm in diameter), highly refractile, spherical bodies when studied by phase contrast. Holcomb et al. (1967), observed that spherosomes (0.5 to 3.0 μm in diameter) in sunflower stems, when viewed through brightfield and phase-contrast optics, were the most conspicuous bodies present in the cytoplasm of the cells. The spherosomes appeared as white, dark-rimmed or black spheres when observed through phase contrast optics. Under darkfield illumination they appeared as white, shining spheres against a black background and, except for the cell walls, were the only structures visible. Similarly, spherosomes from tobacco endosperm appeared as highly reflecting round particles, 1.0 to 3.0 μm in diameter, when viewed under darkfield illumination (Spichiger, 1969).

Sorokin (1967) studied spherosomes and reserve fat in tissues from avocados, coconuts and sunflowers and reported that considerable size differences were evident among the tissues. Sunflowers contained small (0.8 to 1.0 μm in diameter), spherical bodies, similar to bodies described as spherosomes in an earlier publication (Sorokin and Sorokin, 1966), while oil in avocados occurred in sizeable globules (5.0 to 8.0 μm in diameter). Contrary to spherosomes, which were considerably smaller, highly refractile and conspicuously black under phase

contrast, the oil globules displayed medium refractivity and appeared light. The lipid in coconut tissue was also distributed in oil globules, but size variations ranged from the limit of visibility to large (100 to 150 μm in diameter) spheres. Partially based upon this criterion, the authors differentiated between spherosomes and oil globules stating that the former retained a fairly constant size, 0.1 to 0.8 μm in diameter, while the latter exhibited a wide range of diameters. However, Yatsu et al. (1971) reported that spherosomes observed in onion and cabbage tissues were virtually indistinguishable from oil-droplets isolated from cottonseeds; all appeared as refractile spherules ranging in diameter from about 0.5 μm for cabbage to as large as 4.0 μm in cottonseeds. Similarly, Walek-Czernecka (1965) studying spherosomes in the epidermis of onion bulbs, reported that besides large spherosomes, measuring 0.8 to 1.0 μm in diameter, smaller ones occurred near the limit of the microscopes resolution power; a phenomena also observed by Url (1964).

Yatsu et al. (1971) concluded that spherosomes were lipid bodies based upon the known properties of spherosomes. Firstly, lipid droplets would form spherical shapes in an aqueous milieu. Secondly, since vegetable oils have a higher refractive index than water, lipid droplets should be refractile in water and would tend to exhibit Brownian motion more readily than protein bodies because of their lower densities (less inertia).

Not all authors are in agreement on the histochemical staining properties

of spherosomes. This lack of agreement is partially responsible for confusion and discrepancies related to their nature and presumptive function. Frey-Wyssling et al. (1963) found that spherosomes stained with all known fat dyes, such as Sudan Black, Sudan III, rhodanin B, Nile blue, indophenol blue; therefore, they concluded that these bodies were lipid. Likewise, Jelsema et al. (1977) demonstrated the lipophilic nature of spherosomes in aleurone layers of wheat by their histochemical staining with Sudan dyes. Yatsu et al. (1971) using the light microscope confirmed that spherosomes in cabbage and cottonseed, stained brown to black with osmic acid, indicating a composition of highly unsaturated fatty acids. Many others have demonstrated the reactivity of spherosomes with lipid stains (Balz, 1966; Matile et al., 1965; Semadeni, 1967; Walek-Czernecka, 1965).

Contrary to these reports, Sorokin (1967) and Sorokin and Sorokin (1966), using selective staining methods, demonstrated that spherosomes did not react positively to stains for neutral fats but gave positive reactions to phospholipid stains. Intact spherosomes readily stained with Sudan black B, a stain for phospholipids as well as neutral fat. However, the other Sudan dyes, Sudans III and IV and Oil Red O, which all stain neutral fat, did not stain the intact spherosomes. Similarly, in a study of Crambe abyssinica plants, Smith (1974), reported that spherosomes contained phospholipid and not neutral fat.

Holcomb et al. (1967) demonstrated the lipid nature of spherosomes in sunflower stems by extracting tissues with a 3:1 ether-alcohol solution. The spherosomes were no longer detected when extracted tissues were stained with

the lipid dyes Sudan IV or 3,4-benzpyrene. Furthermore, they found that the fluorescent lipid dyes, Nile blue and 3,4-benzpyrene, were excellent and selective stains for all sizes of spherosomes under ultraviolet light.

In some tissues, spherosomes have been reported to stain with specific reagents for protein (Perner, 1952; Jarosch, 1961). The presence of proteins on spherosomes has been suggested because of their reaction with the fluorochromes, berberine sulfate and Janus green, (Drawert, 1953) and because of their coloration with crystal violet (Sorokin and Sorokin, 1966). Frey-Wyssling and Mühlethaler (1965) reported that a protein ghost, indicative of a proteinaceous stroma, remained after lipid extraction of spherosomes. Sorokin and Sorokin (1966) tested spherosomes for the presence of carbohydrates by using the PAS reaction. They found that while starch grains in the chloroplasts stained brilliantly, and cell walls stained moderately, spherosomes failed to stain.

Electron microscopy There is disagreement on which of the cytoplasmic bodies visible in the electron microscope represent spherosomes (Drawert and Mix, 1962, 1963; Frey-Wyssling et al., 1963; Hanchey et al., 1968; Hohl, 1960a; Peveling, 1962). According to Frey-Wyssling et al. (1963), spherosomes were identified as irregular sacs containing a characteristic granulation, which was referred to as the stroma of the spherosome. After the publication of Frey-Wyssling et al. (1963), other authors described spherosomes as certain vacuolar areas within the cytoplasm of the cell. Vacuoles measuring 0.3 to to 3.0 μm in diameter in cells of cotyledonary tissue of Gossypium hirsutum

were described as spherosomes by Yatsu (1965). In other micrographs of the same tissue, Engleman (1966), identified large transparent spaces in the cytoplasm of the cells as spherosomes. Jacks et al. (1967) observed spherosomes in peanut cotyledons and described them as spherical to irregular in outline, very much like vacuoles. In another report, Jensen (1965a,b) described spherosomes of the synergids and egg cells of cotton as electron-translucent bodies. However, they contained a small electron-dense core. Finally, a number of researchers described spherosomes as small spherical bodies, characterized by a relatively electron-dense stroma (Jelsema et al., 1977; Matile et al., 1965; Yatsu and Jacks, 1972; Yatsu et al., 1971).

According to Yatsu et al. (1971), these conflicting reports on the structural appearance of spherosomes may be due to the fixative used. When seeds of Phaseolus vulgaris L. were fixed in osmium tetroxide only, spherosomes appeared irregular and stellate in outline and contained an electron-opaque matrix, presumably caused by the high content of unsaturated fatty acids and their reactivity to osmic acid (Mishra and Colvin, 1970). Similar observations have been reported for a variety of other tissues (Jacks et al., 1967; Mann and Engleman, 1972; Yatsu, 1965; Yatsu et al., 1971). However, spherosomes in doubly-fixed (aldehyde-osmic acid) tissues appeared spherical, similar to spherosomes as seen in the light microscope (Yatsu et al., 1971; Mann and Engleman, 1972; Mollenhauer and Totten, 1971b). This observation lead Yatsu and coworkers (1971) to the conclusion that the highly osmiophilic

round bodies observed in the electron microscope (often referred to as "lipid bodies") and the highly refractile, osmiophilic spherical bodies (spherosomes) seen in the light microscope were one and the same. The matrices of spherosomes from cabbage, after double-fixation, were uniformly electron-dense with no apparent internal structure and ranged in size from 0.4 μm to slightly over 2.0 μm in diameter (Yatsu et al., 1971). Matile et al. (1965) found that glutaraldehyde-osmium tetroxide fixation produced spherosomes in the endosperm of tobacco that were characterized by an electron-dense stroma and that measured 1.0 μm in diameter. Using the same fixatives, Mishra and Colvin (1970), described spherosomes in Phaseolus vulgaris L., as ovoid, osmiophilic organelles 0.5 to 1.0 μm in diameter, with little internal structure. Evert and Murmanis (1965) found that all sizes of lipid bodies (similar in size to spherosomes) were preserved in typical spherical form and appeared black in phloem parenchyma cells of Tilia americana fixed in buffered glutaraldehyde followed by osmium tetroxide. However, Sorokin and Sorokin (1966) observed spherosomes in doubly-fixed tissue as containing an electron-permeable medullary zone occupying three-fourths of the central region of the spherosome surrounded by a cortex of osmiophilic material.

Drawert and Mix (1963), correctly, identified spherosomes with "stem-formige" bodies observed in tissues after osmium fixation, but apparently other workers (prior to double-fixation techniques) could not or would not identify a

star-shaped or irregular-shaped body with the neat, spherical body seen in the light microscope. In their quest for trim, round bodies (the counterpart of the neat, refractile spherosomes observed in the light microscope), many microscopists apparently ignored the star-shaped bodies and sought a round body. Consequently, many bodies labeled "spherosome" in the literature were not spherosomes, but simply spherical organelles such as microbodies or round vesicles (Yatsu et al., 1971).

Furthermore, spherosomes in potassium permanganate (a fixative primarily used prior to the introduction of double-fixation), appeared irregular and stellate in outline, similar to those observed in osmic acid treated tissues but displayed an electron-translucent matrix (Jacks et al., 1967; Mishra and Colvin, 1970). Potassium permanganate fixation in pepper grass tissue caused spherosomes to appear as crenated or collapsed, irregularly shaped bodies, not at all suggestive of their shape as seen in the living cell (Mann and Englemen, 1972). They described the spherosomes as having an electron-permeable center encircled by a thin gray cortex and an electron-dense rim. These structures tended to collapse since permanganate is not a good lipid fixative, therefore, the lipids were removed by the solvents used in the preparation of the specimen for microscopical examination. The authors suggested that a description of the morphology of lipid bodies based upon this fixative alone was not very reliable, and that fixation with osmic acid, which is more lipophilic than permanganate, enhanced the reliability in determining the morphology of such bodies.

Lott et al., (1971) observed that glutaraldehyde-potassium permanganate fixation often caused shrinkage and clearing of oil vacuoles in cotyledons of Cucurbita maxima, however, they appeared more electron-dense and not shrunken after using osmium tetroxide in place of the permanganate.

Based upon the three previously mentioned fixatives (glutaraldehyde, osmium tetroxide, potassium permanganate), Mishra and Colvin (1970), presented evidence for marked variation of the internal structure of spherosomes in the cytoplasm of Phaselous vulgaris. In summary, three kinds of spherosome-like bodies were observed based upon their internal structure: those with a homogeneous interior; those with a less electron-dense center, and those with a more electron-dense center. The authors concluded that the simplest assumption consistent with all observations was that these types of structures constituted a group of similar organelles which appeared differently in different tissues with different methods of preparation.

Irrespective of fixative used, Mollenhauer et al. (1969) reported the existence of two structurally different lipid vesicles in bean and pea cotyledons. The larger ones, referred to as simple vesicles, were spherical, measured 0.5 to 3.0 μm in diameter and were dispersed singly throughout the cell cytoplasm. The second type or composite lipid vesicles were elongated, usually small (0.1 to 1.0 μm in diameter) and firmly associated into sheet-like aggregates. The author's acknowledged that simple lipid vesicles were similar to those bodies

reported as spherosomes, but they preferred to use the term lipid vesicles to describe these intracellular particles.

It is obvious from the evidence presented thus far, that there is considerable variation in the appearance of spherosomes depending upon the type of fixative employed in their preparation, and more recently, the problem has been compounded by the existence of two distinct types of lipid vesicles found in some tissues. Nevertheless, researchers have had little trouble, for the most part, distinguishing these intracellular particles from other cellular structures. Yatsu et al. (1971) observed that spherosomes appeared as the most intensely stained (osmiophilic) subcellular particles in the cell; they stained so intensely that they stood out in stark contrast from the rest of the cytoplasm (hyaloplasm) and other organelles. Furthermore, mitochondria and plastids, although stained, were not as conspicuously osmiophilic as spherosomes when viewed at low magnification with the electron microscope. Mishra and Colvin (1970), reported that spherosomes in seeds of Phaseolus vulgaris L. were clearly distinguishable from mitochondria, Golgi vesicles and lipid droplets. In another report, Jones (1969) found that he could differentiate between spherosomes and microbodies in barley aleurone layers, in contrast to the experience of Frederick et al. (1968). The distinction between spherosomes and microbodies was made on the basis of permanganate fixation. The author reported that spherosomes appeared crenate when fixed in potassium permanganate, while microbodies retained a smooth,

circular appearance, a characteristic observed in doubly (glutaraldehyde-osmium tetroxide) fixed material.

Membrane

The presence of a true morphological membrane surrounding the spherosome, a characteristic researchers have often used to distinguish these intracellular particles from other inclusions, is debatable.

Frey-Wyssling and coworkers (1963) described spherosomes in the coleorhiza of corn seedlings as coated by a typical unit-membrane with its characteristic three strata. They concluded that their conspicuous unit-membrane, which prevented any confluence of the oil droplets, was not simply a phase boundary because olive oil suspended in agar showed no apparent membrane or limiting boundary associated with it. Likewise, Peveling (1962), reported that the spherosomes in the epidermal cells of onion bulbs were surrounded by a continuous membrane described as trilaminar and measuring 70 to 88 Å in thickness. Similar observations in a variety of plant tissues have been reported by other researchers (Buttrose, 1971; Mühlethaler, 1955; Paleg and Hyde, 1964; Perner, 1957; Strugger, 1960).

The findings of Sorokin (1967) in Campanula tissue agree with those of Frey-Wyssling et al. (1963), that a limiting unit-membrane is evident at the boundary of spherosomes. In contrast to spherosomes, Sorokin reported that reserve oil-droplets do not possess a limiting membrane and easily coalesce

into larger aggregates. However, in a more recent publication, Yatsu et al. (1971), compared spherosomes from nonoily tissues with oil bodies from oil-bearing seeds, and concluded that the two structures were identical.

Working with Ricinus communis, Schwarzenbach (1971), observed that although immature spherosomes (prospherosomes) were bounded by unit-membranes, the mature spherosomes were not. The author indicates that the spherosomal membrane undergoes differentiation during seed maturation resulting in a separation of the unit-membrane into an inner and outer layer. Lipid synthesis it is claimed takes place between the inner and outer layer of the membrane. The inner half of the unit-membrane eventually disappears leaving the outer half of the unit-membrane to form the limiting membrane of the spherosome.

This evidence suggesting a half-unit-membrane surrounding the spherosome (oil body) is further substantiated by numerous workers, who describe an unusual single-line-membrane surrounding the spherosome in contrast to the usual tripartite membrane (Hohl, 1960a,b; Jelsema et al., 1977; Matile et al., 1965). Yatsu and Jacks (1972) described spherosomes in peanut (Arachis hypogaea L.) seeds as bounded by unusual single-line "membranes" which measure 2 to 3.5 nm in width, contrasted to the well-known tripartite unit-membrane which measures 6 to 8.5 nm in overall thickness. Morphologically, they interpreted the single-line-membrane of spherosomes as a barrier whose two sides differ in polarity. The external, polar surface (protein) of the membrane, they claimed, contacts the aqueous ground plasm (hyaloplasm) of the cell and the interior

nonpolar surface (lipid) contacts the storage oil. Thus a barrier exists with dissimilar sides, each of which is compatible with its respective environment. In contrast, a unit-membrane is composed of a nonpolar diffusion barrier sandwiched between polar portions that are in contact with and wetted by the polar milieu on either side of the membrane. Yatsu and Jacks (1972) did acknowledge, however, that when juxtaposed externally (from the side addressing the hyaloplasm), two single-line spherosome membranes adjoin to form a thicker single-line, but when apposed internally (the sides that contact stored lipid), two single-line-membranes touch to form a tripartite structure resembling a unit-membrane. They concluded that an atypical, single-line-membrane representing one-half of a unit-membrane delimited spherosomes in peanut cotyledons. Corroboration of this interpretation was shown biochemically by demonstrating the presence of membrane structural protein in their isolated peanut spherosomes (Yatsu and Jacks, 1972). Cabbage and cottonseed spherosomes were reported bound by the same type of single-line-membrane (Yatsu et al., 1971). These spherosomes were similar to the lipid bodies of corn embryo cells described earlier by Trelease (1969), namely lipid droplets bounded by single-line-membranes 25 to 40 Å in thickness. Single-membranes were also reported for lipid bodies in pea seeds (Yoo, 1970) and lipid storage vacuoles in the cotyledons of Cucurbita maxima (Lott et al., 1971).

Mollenhauer and Totten (1971a,b) described lipid vesicles in bushbean and

pea cotyledons, to be clearly separated from the cytoplasmic ground plasm by a thin interfacial structure. In their electron micrographs, the interfacial structure appeared relatively dense in contrast to its surroundings and therefore, they assumed it to be a bounding membrane. Their interpretation was supported by the following observations. In tissue sections, a membrane, or at least a dense residue, was visible at the lipid-cytoplasm interface, and lipid droplets which were closely packed together, as they are in most oil seeds, did not fuse.

Alot of confusion concerning the interpretation of a spherosomal membrane arose from differences in their appearance after use of various fixatives. Following osmium tetroxide fixation of corn and tobacco seedlings, no structure was observed that could be interpreted as a morphologically distinct membrane surrounding the spherosome. However, some kind of phase boundary was assumed to exist between the lipoidal spherosome and its aqueous surroundings (Mann and Engleman, 1972). After permanganate (a fixative immiscible with lipid) fixation of identical tissue, a rim appeared which was markedly more electron-dense than the interior of the spherosome. Mann and Engleman concluded that although they could not distinguish a "true membrane," they did observe the presence of some type of molecular orientation or concentration at the phase boundary of the spherosome. Mishra and Colvin (1970) found that a limiting membrane or border was often indistinct in spherosomes from Phaseolus vulgaris L. that had been fixed in glutaraldehyde followed by osmium tetroxide. When tissue was fixed in potassium permanganate, they reported a distinct outer membrane was

always apparent. Ory et al. (1968) demonstrated that isolated spherosomes from castor beans, which were first immobilized in agar, hexane-extracted and subsequently fixed in osmium tetroxide, showed the presence of electron-dense membranes, although they were unable to distinguish the classical tripartite membrane as seen by other researchers. Similarly, after hexane extraction of isolated peanut spherosomes, the doubly-fixed (glutaraldehyde-osmium tetroxide) material contained electron-translucent bodies surrounded by electron-dense membranes. Chemical analysis of the spherosomal preparation indicated that an amount of protein and phospholipid was detected that was sufficient to account for limiting membranes. The existence of a spherosomal membrane was chemically indicated by the presence of phospholipid in preparations of isolated spherosomes from tobacco seedlings (Matile and Spichiger, 1968). However, chromatography did not reveal phospholipids in isolated spherosomes from mature pepper grass embryos (Mann and Engleman, 1972).

Some authors have reported that spherosomes do not appear to have any surface membrane (Buttrose, 1971; Drawert and Mix, 1962; Jacobsen et al., 1971). However, most researchers agree that these organelles are delimited by some type of boundary, whether it be a unit-membrane or the so-called single-membrane (half-unit membrane).

Cellular distribution and association

Mishra and Colvin (1970) observed that the distribution of spherosome-like bodies in the cytoplasm of Phaseolus vulgaris L., varied from one tissue to

another. These bodies tended to be less numerous in the more mature portions of the root tips and were not observed at all in root tips of plants grown in water cultures for three to five weeks. In four day old seedlings, two distinct categories of distribution in the cell were observed, depending on the tissue. In root and shoot apices, the spherosomes were irregularly distributed in the cytoplasm without any particular association with other structures. In contrast, in cotyledonary leaves, the spherosomes were almost always in close proximity to the plasmalemma and the cell wall. Spherosomes in aleurone layers of wheat showed characteristic associations with the plasma membrane at the cell surface and firmly surrounded protein bodies like a halo, imparting a bumpy appearance to their surface (Jelsema et al., 1977). Due to the close spatial association of lipid and protein bodies observed both in situ and in isolated preparations, Jelsema and coworkers suggested that this might represent a functional relationship facilitating the interaction of enzymes and their substrates. Similar observations of spherosomes clustered around protein bodies and associated with the plasmalemma have been reported in other tissues (Buttrose, 1971; Chamura, 1975; Jacobsen et al., 1971; Jones, 1969; Morrison et al., 1975; Nieuwdrop, 1963; Ogawa et al., 1975; Paleg and Hyde, 1964).

In a study of ultrastructure and chemistry of seed lipids, Mollenhauer et al. (1969) described two structurally different lipid vesicles (simple and composite) in bush bean (Phaseolus vulgaris) and pea (Pisum sativum) cotyledons. The larger, simple vesicles appeared dispersed singly throughout the cell

cytoplasm, while the smaller composite lipid vesicles were found associated with the plasma and plastid membranes. The data suggested either that the plasma and plastid membranes become similar (in the sense that they bind or attract the composite lipid); or that two classes of composite lipid vesicles exist such that one class binds to the plasma membrane, and the other binds to the plastid membrane. In another study, lipid bodies in pea seeds were found closely packed against each other and confined to regions near the cell surface or near plastids in radicles of dormant, 80 minute, 8 and 12 hour embryos (Yoo, 1970).

Sorokin and Sorokin (1966) reported that in tissues from Campanula persicifolia L., spherosomes were distributed throughout the tissue and appeared singly, in twos or in more numerous groupings. Bead-like chains or layers of spherosomes were observed in barley aleurone cells (Paleg and Hyde, 1964).

Occasionally, lipid vesicles or spherosomes have been reported closely associated with mitochondria (DeRobertis et al., 1965; Fawcett, 1966; Stein and Stein, 1968), endoplasmic reticulum (Stein and Stein, 1967a, 1967b, 1968; Vigil, 1970) or other lipid vesicles (Mollenhauer, 1967), but most often they are free in the cell cytoplasm (Mollenhauer and Totten, 1971a).

Chemical composition

Evidence of the heterogeneity of spherosomal composition has already been discussed from purely morphological investigations. Likewise, chemical analysis of the material composition of spherosomes appears to be extremely variable.

Spherosomes from tobacco endosperm were extremely rich in lipid, with the amount of lipid exceeding that of protein by a factor of more than one thousand (Matile and Spichiger, 1968; Spichiger, 1969). As far as lipid content is concerned, spherosomes of peanut cotyledons resemble the oil droplets of tobacco endosperm (Jacks et al., 1967). However, Semadeni (1967) reported that spherosomes isolated from corn seedlings contained only twice as much lipid as protein.

Spichiger (1969) found that isolated spherosomes from resting (dormant) seeds of tobacco consisted of 99% of their dry weight as lipid, predominantly triglycerides with some polar lipids (approximately 1%). Mann and Engleman (1972) also reported that triglycerides accounted for most of the lipid in extracts of spherosomes from tobacco seedlings. According to Yatsu et al. (1971), cottonseed oil droplets contained 98.8% lipid, while cabbage spherosomes contained somewhat less, 81.0%. Analysis for phosphorus in the oil-droplets from cottonseed was 0.015% and 0.007% in the spherosomes of cabbage corresponding to 0.38% and 0.16% phospholipid, respectively. Protein content of the isolates varied considerably, having 0.63% and 4.0% for cottonseed and cabbage, respectively.

Jelsema et al. (1977) found that light spherosomes (density < 1.0) and heavy spherosomes (density > 1.18) isolated from aleurone layers of wheat, were composed primarily of triglycerides. Light spherosomes were richest in triglycerides while a higher amount of protein was evident in the heavy spherosomes. Analysis of the two fractions revealed the presence of three major

phospholipids: phosphatidylcholine accounted for nearly two-thirds of the total phospholipids and the remainder was approximately equally divided between phosphatidylethanolamine and phosphatidylinositol. Similarly, Allen et al. (1971) reported that simple lipid vesicles from bush bean and pea cotyledons contained primarily triglycerides, whereas the smaller, denser, composite lipid vesicles, in addition to triglycerides contained three phospholipids: phosphatidyl-ethanolamine, phosphatidylcholine and phosphatidylinositol.

Besides the extremely lipid rich spherosomes of peanut cotyledons and of tobacco endosperm, some with a protein content of over 50% have been isolated from Gossypium cotyledons (Yatsu and Altschul, 1963). Although Paleg and Hyde (1964) did not determine the composition of their isolated spherosomes from barley aleurone cells, they suggested that they were probably a mixture of lipid and protein. Furthermore, Buttrose and Soeffky (1973) reported that spherosomes in coleoptile cells of ungerminated rice grains sometimes contained nonlipid inclusions, however, their composition was not analyzed.

Function

The variability in morphology and composition of spherosomes does not contribute substantially to an understanding of their role in the plant cell. Aspects of the heterogeneity emphasized thus far may be used to support any of the suggestions that they are (a) sites of lipid synthesis (b) structures for lipid storage or (c) sites of reserve protein storage.

Frey-Wyssling et al. (1963) questioned whether the spherosomes had a special structure and function or were simply inert bodies of ergastic material. They concluded that spherosomes of growing corn seedlings were not only the precursors of oil droplets within the cells, but were also special organelles containing the enzyme for the final step in fat synthesis. The authors suggested that the granulated stroma of the spherosome was either responsible for the translocation of fat molecules synthesized by the ground plasm across the unit-membrane and for their accumulation in the central portion of the spherosome, or it was itself involved in fat synthesis. Semadeni (1967) found spherosome-like po: :les of corn contain all the enzymes necessary for lipid synthesis, and concluded that this points to the possible conversion of these organelles to lipid bodies. Tavener and Laidman (1968) reported similar results.

Spherosomes have also been described by several workers as the principle site of oil storage in seeds. Yatsu (1965), concluded that spherosomes function as the primary structures of lipid storage in cotyledonary tissues of Gossypium hirsutum L. seeds. After osmium fixation, the author observed that vacuolar areas (spherosomes) appeared electron-opaque, caused by the high content of unsaturated fatty acids in the cottonseed oil, indicating that these areas were the sites of oil storage. Furthermore, after permanganate fixation the vacuolar areas were electron-translucent due to the extraction of lipid since permanganate is not miscible with lipids; thus further confirming that the material occupying

the vacuoles was probably lipid. Jacks et al. (1967), in a study of isolation and characterization of peanut spherosomes, concluded that the spherosomes were the principal sites of lipid storage in the cells, but not of lipid degradation. Similarly, Horner and Arnott (1966) reported that oil bodies, the equivalent of spherosomes, functioned merely as storage sites of oil in Yucca seeds. The large amount of triglycerides in isolated lipid bodies from shoot apices of young corn seedlings is a further indication of the storage role for lipid bodies in plant cells (Trelease, 1969). Observations of wheat aleurone cells by Jelsema et al. (1977) suggest that spherosomes are lipid storage organelles unique to plants. Their findings were based upon the observation that spherosomes disappeared from cells during periods of increased metabolism (germination) paralleling the concomitant utilization of storage material within the seed. Allen et al. (1971) reported that the primary function of simple and composite lipid vesicles in bush bean and pea cotyledons was unknown, but the sequence of transformation within the germinating tissue suggested that both simple and composite vesicles served as triglyceride storage deposits. However, they felt that composite vesicles might have another metabolic function.

Guilliermond (1941) considered lipid granules as products of metabolism and/or degradation products of lipo-protein compounds. The abundance and motility of spherosomes in the parietal layers of cytoplasm lead to the suggestion that they might function in the transportation of substances throughout the cell (Sorokin, 1958). Oxidase and dehydrogenase enzymes have been reported

localized on the spherosomes. Perner (1952) reported the occurrence of the Nadi reaction (synthesis of indophenol blue by oxidation of alpha-naphthol and dimethyl-p-phenylenediamine) on the spherosomes. These observations led to the idea that the spherosomes were special cell organelles involved in oxidation-reduction reactions. However, this was disproved by Drawert (1953) who showed that mitochondria were responsible for such reactions and that the reaction product (indophenol blue) was a lipid dye and readily accumulated in the spherosomes.

The occurrence of a number of hydrolytic enzymes associated with spherosomes and spherosome-like organelles has been reported by many authors. Semadeni (1967) found a number of hydrolytic enzymes associated with plant spherosomes (including protease, RNase, beta-amylase, alpha-glucosidase, phosphatase, esterase, arylsulphatase-C, and NADH-diaphorase. Because of the hydrolytic enzymes, some authors have drawn an analogy between spherosomes of plant cells and lysosomes of animal cells. Matile and Spichiger (1968) found that isolated spherosomes from tobacco endosperm contained five different acid hydrolases: two proteases, assayed by their attack upon hemoglobin (pH 3.5) and casein (pH 6.0), p-nitrophenylphosphatase, α-naphtylacetate-esterase, RNase and DNase. However, electron micrographs of isolated particles were not presented, leaving open the possibility that their preparations were contaminated with other organelles. Similarly, investigations by Walek-Czernecka (1962, 1963, 1965), reported the presence of acid phosphatase and a nonspecific

esterase in the epidermis of onion scales. The activity of these enzymes was specifically in the spherosomes; the supposition was expressed that spherosomes played a role in intracellular hydrolytic processes and were, therefore, compared with lysosomes in animal cells.

Many authors have concluded that spherosomes are lysosomes on the basis of cytochemical demonstration of acid phosphatase (Avers and King, 1960; Holcomb et al., 1967; Matile et al., 1965; Matile and Spichiger, 1968; Semadeni, 1967; Sorokin and Sorokin, 1968; Wilson et al., 1970; and Wilson, 1973). Earlier reports on the localization of acid phosphatase activity in cytoplasmic particles of plants have been reviewed by Jensen (1962). Later, Poux (1963) identified the phosphatase reactive particulates as dictyosomes, while Flinn and Smith (1967) pointed out that they were larger than mitochondria and may have resulted from aggregation of smaller bodies. Walek-Czernecka (1962, 1965) found that acid phosphatase activity was localized in spherosomes and had no association with mitochondria or plastids in epidermal cells from the scale leaves of onion. Furthermore, Matile et al. (1965) working with density gradient-centrifuged extracts of corn and tobacco seedlings, isolated two fractions both containing phosphatase. In one fraction, small spherosomes were the principal structures while larger spherosomes predominated in the other. Spichiger (1969) found that isolated spherosomes accounted for 70% of the hydrolase activity associated with tobacco endosperm tissue, including phosphatase, protease, RNase and DNase and proposed that spherosomes have a double function; storage

and mobilization of lipid. Lipase appeared to be attached to or part of the spherosomal membrane in castor beans (Ory et al., 1968). The results did not indicate, however, whether lipase was present within the organelle. Ory and coworkers (1968), concluded that spherosomes in the endosperm of mature resting castor beans have a dual function both as fat-storage organs and as sites of fat breakdown; therefore, they suggested that these intracellular particles were equivalent to lysosomes.

Matile et al. (1965), indicated that spherosomes in tissues of higher plants morphologically resembled lysosomes of animal cells. They observed that both organelles appeared as spherical bodies, delimited by single membranes and possessed a more or less homogeneous fine granular stroma. However, Jelsema et al. (1977) expressed a lack of homology between spherosomes and animal lysosomes. They reported that the delimiting membrane of lysosomes was of the unit-type (with a clear dark-light-dark pattern in osmium fixed preparations); spherosomes did not show this characteristic pattern. In another report, Matile et al. (1965) found major differences in the density of spherosomes and lysosomes. The density of lysosomes was 1.22 gcm^{-3}, while that of spherosomes was considerably lower at 1.105 gcm^{-3}. A clearer distinction between lipid inclusions in the cell and lysosomes is needed, and the role of lysosomes in lipid synthesis needs further study.

Other investigators have reported little or no activity of hydrolytic enzymes associated with spherosomes. Jelsema et al. (1977) studied the lysosomal nature

of spherosomes in wheat aleurone layers based upon incubation of the tissue according to the Gomori (1952) procedure for acid phosphatase. In previous studies, electron-dense deposits of lead phosphate were reported to accumulate within spherosomes. However, after incubation of wheat spherosomes in the reaction mixture, the interiors were relatively free of lead deposits verifying the absence of acid phosphatases. Heavy deposits of lead phosphate were found, however, associated with protein body membranes. Protein bodies have frequently been reported to have acid phosphatase activity (Ashford and Jacobsen, 1974; Matile, 1975; Matile and Spichiger, 1968; Poux, 1963; Yatsu and Jacks, 1968). Jelsema and coworkers (1977) also reported that spherosomes of wheat aleurone cells did not contain phospholipase D nor acid lipase and that these lipid hydrolases were associated with protein bodies. Jacks et al. (1967) found little lipase activity associated with spherosomes from peanut cotyledons, whereas lipase activity was associated with the mitochondrial fraction.

Some researchers have reported that spherosomes act as sites of reserve protein storage in plant cells (Srivastava and Paulson, 1968), however, this view is not generally accepted.

Cellular origin

Although the structure and composition of spherosomes or oil bodies have been the subject of many published papers, their origin and development are less well-understood.

Frey-Wyssling, Grieshaber and Muhlethaler (1963), attempted to trace the origin and development of spherosomes in the coleorhiza of corn seedlings. They proposed that spherosomes start as minute vesicles originating from enlarged fragments of the endoplasmic reticulum. Before abcission of such vesicles, they noted an apparent accumulation of granular material in the end of the strand, and then a neck was eventually established by constriction, forming numerous juvenile spherosomes (immature spherosomes). Gradually these bodies increased in size and evolved first into spherosomes and then into a transitional stage. In the final stages of development, they claimed that the center cleared as the granulated stroma became more and more restricted to the periphery of the spherosome and eventually it appeared as a clear body of reserve oil. Frey-Wyssling and coworkers also paid special attention to whether juvenile spherosomes could also be produced by the Golgi apparatus, but in their preparations there was no association.

Working with Ricinus communis, Schwarzenbach (1971) agreed with Frey-Wyssling et al. (1963) that spherosomes are formed from the endoplasmic reticulum, but changed the terminology referring to spherosomes as prospherosomes, a term used earlier by Matile et al. (1965), and to oil bodies as spherosomes. However, Harwood et al. (1971), proposed that oil bodies ("which cannot be equated with spherosomes"), in maturing Ricinus communis, originate around a cluster of enzymes in the cytoplasm of the cell, which involves fatty acid biosynthesis and triglyceride formation. Similarly, Smith (1974) reported that

spherosomes and oil bodies were separate entities with different sites of origin in developing cotyledons of Crambe abyssinica. Smith claimed that spherosomes developed as small terminal vesicles formed on the endoplasmic reticulum which subsequently increased in size and became filled with osmiophilic material, similar to that reported by Frey-Wyssling et al. (1963) and Simola (1969). On the other hand, he found that oil bodies originated in the cytoplasm from small areas of electron-dense particulate material, which by nature of its staining with uranyl acetate appeared to be proteinaceous.

Mollenhauer and Totten (1971a,b) reported that composite lipid vesicles (i.e. lipids interassociated into lipid vesicle sheets) in bush bean and pea cotyledons appeared to be synthesized in association with, but external to, the endoplasmic reticulum. There were no instances where lipid droplets were continuous with, or appeared to be budding from, the endoplasmic reticulum. Therefore, they concluded that lipid synthesis occurred external to, or on the surface of, the endoplasmic reticulum. In contrast, Mann and Engleman (1972) reported that no special association of spherosomes with the endoplasmic reticulum was noticed in developing embryos of Lepidium virginicun L. (pepper grass).

Although the morphological origin of spherosomes was not investigated in the work of Spichiger (1969), he biochemically detected the localization of two oxidoreductases (NADH cytochrone C reductase and NADH dichlorophenol

indophenol oxidoreductase) in isolated spherosomes from tobacco endosperm, and suggested that these were indicators of endoplasmic reticulum derivation.

Germination and Developmental Studies of Soybean Cotyledons

Microscopic examinations of cellular constituents of soybean seeds during germination and developmental stages, have been scanty. By far, most studies of germinating and developing soybean seeds have dealt with changes in the chemical composition of cotyledons during either germination or maturation periods (Privett et al., 1973; Roehm and Privett, 1970; Rubel et al., 1972; Wilson and Rinne, 1974; Yazdi-Samadi et al., 1977). Furthermore, no reports have appeared concerned with changes in chemical composition complemented with microscopic observations from a single soybean cultivar over the entire process.

Germination

Germination of a seed may be regarded as those consecutive steps that cause a quiescent seed with a low water content to show a rise in general metabolic activity and to initiate the formation of a seedling from the embryo (Mayer and Poljakoff-Mayber, 1975). During the process of germination, seed reserve materials are utilized for growth and development. In soybean seeds, there are two major types of reserve materials stored in their cotyledons, protein and oil, in contrast to many other seeds which store large quantities of starch.

In a histochemical study of soybean cotyledons during germination, Suryanarayana (1976), observed that during early stages of germination, neutral lipid, stained with Nile blue chloride, appeared as a faintly stained viscous mass covering the protein bodies. From day 1 to day 5 there was little qualitative change in the oil globules in most cells, but subsequently from day 5 to day 14 there was a noticeable progressive decrease in oil globule content. Holman (1948) found that the oil content of soybean cotyledons decreased during germination and after 15 days was only about 2 percent. Kahn (1959), reported an accelerated loss of oil following a peak in lipase activity which was reached on the fifth day. Other authors have reported similar depletion of oil in soybean cotyledons following peak lipase activity (Holman, 1948; Bils and Howell, 1963). However, the rate of oil loss during germination in other tissues seems to vary. Spichiger (1969) reported that 20% of reserve lipid in tobacco seedlings was depleted after the first 24 hours of germination. Decomposition of lipid then stopped and continued again at the beginning of the 3rd day, and by 96 hours post-germination only 10% of the original lipid content remained. Jacks et al. (1967) found that only one-half of the lipid content per peanut cotyledon was utilized.

Microscopically, Tombs (1967) observed that germination of soybean cotyledons seemed to be accompanied by early structural changes in protein bodies. First, the protein bodies became more granular in appearance and apparently lost their limiting membranes. Then, eventually the bodies became

irregularly shaped and sometimes appeared to be coalesced into a large single mass. These observations of protein bodies are similar to those reported for other tissues, such as peanut (Bagley et al., 1963), pea (Bain and Mercer, 1966), Yucca (Horner and Arnott, 1965) and Phaseolus (Opik, 1966). Suryanarayana (1976), suggests that protein bodies in soybean cotyledons undergo progressive fragmentation into smaller particles and finally disappear from most cotyledon cells by 9 days after germination. In a study by McAlister and Krober (1951), protein decreased at about the same rate as oil for the first two weeks, but protein depletion was much slower thereafter. By the fifth week after planting each cotyledon contained about 2 mg of protein.

A study of the proteins of soybeans during germination, as followed by disc electrophoresis and disc immunoelectrophoresis, revealed differences in rates of breakdown for the various proteins. The 11 S protein, the major component of the protein bodies, was detectable up to 16 days after germination. One of the 7 S components, however, decreased to low levels after only 9 days of germination (Catsimpoolas et al., 1968).

Tombs (1967) found that disruption and coalescence of protein bodies generally occurred in the epidermis layer and around the vascular bundles, particularly near the embryonic end. Similarly, Suryanarayana (1976) reported that the size and stainability of all soybean reserve materials decreased in cells bordering vascular strands compared to those away from them. He suggested that this was partly due to the enzyme stimulating effect of hormones that moved into

these cells from the phloem and partly due to the sustained enzyme activity in the cells created by the continual removal of end products of degradation by the phloem transport mechanism. The disruption of protein bodies, which appeared to be present around vascular bundles in soybean cotyledons, however, does not seem to be the case in other species (Opik, 1966).

Tombs (1967) noticed that lipid granules decreased and immature plastids together with what appeared to be starch grains proliferated during germination. In day 0 and day 1 germinated soybean cotyledons, Suryanarayana (1976) found that most cells were devoid of starch grains. By day 2 to day 5, however, almost all cells contained them. They appeared as clusters of spherical to oblong shaped grains, which upon subsequent days of germination steadily increased in size and number and eventually joined to form large, polygonal compound grains. Beyond day 5, there was a progressive loosening of the compound grains accompanied by a decrease in their size and stainability until finally only traces of them were seen in the palisade cells by day 11. McAlister and Krober (1951) showed that carbohydrates in soybean cotyledons were depleted much more rapidly than the fat-fraction approaching zero by the 3rd day of germination.

The conversion of fat to carbohydrate via β-oxidation of fatty acids and the glyoxylate shunt is well authenticated in oil-rich seeds during their germination (Beevers, 1961; Breidenbach et al., 1968; Gruber et al., 1970; Kornberg and Beevers, 1957; Richardson, 1974). Furthermore, the formation and activity of

microbodies (i.e. glyoxysomes) during germination of oil containing seeds has been reported (Gruber et al., 1970 and Schnarrenberger et al., 1971). However, the presence of enzymes associated with the glyoxylate shunt and the formation of glyoxysomes have not been investigated in soybeans.

Maturation

Bils and Howell (1963) reported that the prominent structures of the mature soybean cotyledon, lipid granules and protein bodies, were absent during early stages of development. At 15 days after flowering (DAF), little more than, nuclei, ribonucleoprotein particles and endoplasmic reticulum were observed within the cotyledon cells. A few days later, when the fresh weight of the seed was almost 80 mg, they noted that many mitochondria and immature chloroplasts with associated starch grains were seen in the cells. During this period a relatively high respiration was observed which probably produced the energy for the subsequent appearance and rapid synthesis of lipid and protein. By 26 DAF, starch, lipid granules and protein bodies were present in the cytosol of soybean cotyledons.

Rubel et al. (1972) have shown that developing soybean seeds contained 5% oil at 25 DAF. The oil percentage increased to around 20% by 40 DAF and essentially remained constant during the remainder of seed development. Hsu (1970) observed essentially the same pattern with oil in Amsoy and Harosoy cultivars. Yazdi-Samadi et al. (1977) reported oil content at 10 DAF was only

3.5% in Harosoy 63 and 4.1% in Steele; then from 20 to 40 DAF in both cultivars the greatest amount of oil was synthesized. Similarly, Bils and Howell (1963) determined that rapid lipid synthesis occurred in seeds with fresh weights of 150 to 280 mg, corresponding to a period of 26 to 36 DAF. They found the lipid granules to be extremely small, measuring only 0.1 to 0.2 μm in diameter.

Synthesis of protein in the form of protein bodies followed a pattern similar to that reported for lipid. Twenty-six to 36 DAF, protein bodies rapidly increased in size and numbers in soybean cotyledons (Bils and Howell, 1963). Most of the protein bodies were about 5.0 μm in diameter. However, soybean seeds analyzed for total protein, only showed a gradual increase in this reserve material throughout development. They proposed that since the number and size of protein bodies increased rapidly during this period, other cell protein must decrease. Therefore, they suggested that total protein should be separated into active or metabolic protein and inactive or storage protein. Krober and Gibbons (1962) showed that during the development of the soybean seed the percent nonprotein nitrogen decreased with a concomitant rise in the percent protein nitrogen. Rubel et al. (1972) observed similar results as did Hsu (1970). Yazdi-Samadi et al. (1977) reported that percent protein did not fluctuate much from 20 to 40 DAF, however, the largest amount of protein accumulated during a period from 20 to 40 DAF.

During this time of increased lipid and protein accumulation, mitochondria and chloroplasts began to disappear from the rapidly enlarging cells (Bils and Howell, 1963). When the fresh weight of the developing seed reached a maximum, they observed that the cells were filled with lipid granules, protein bodies and numerous starch grains (5.0 μm in diameter). They claimed that the starch bodies disappeared during dehydration of the seed, just prior to seed maturation. Similarly, Tada and Kawamura (1963) found that starch decreased to almost zero at maturity, whereas immature soybeans contained 4 to 5%. In Harosoy 63 cultivar, starch content rapidly increased from 10 to 40 DAF followed by a rapid decrease to 70 DAF, while in the Steele cultivar, starch sharply increased up to 30 DAF followed by a decrease to 50 DAF, after which there was a slight decrease until 60 DAF (Yazdi-Samadi et al., 1977). At maturity, however, Harosoy and Steele contained 1.1% and 0.8% starch respectively.

Relationship of Processing and Soybean Ultrastructure

Conversion of soybeans to several food products

The relationship of soybean ultrastructure to processing is sketchy and little of the known information has been applied to developing new processing methods. A better understanding of the original soybean structure at the cellular and subcellular level and how it is modified by present practices may provide insights for new approaches to processing.

Saio and Watanabe (1968) investigated structural changes that occur during conversion of soybeans into several Japanese foods. After soaking soybeans in water overnight, they found no changes in the distribution of protein bodies and spherosomes in the swollen cells, however, on steaming at 115°C for 30 minutes, cells appeared shrunken and the distance between them increased considerably. Also, the outer distinct unit-membrane of the protein bodies was no longer observed and the reserve protein within them seemed to be curdled. Steaming also disrupted spherosomes, releasing their oil throughout the cytoplasm, which appeared as large droplets 1-3 μm in diameter. In the Japanese fermented food natto, prepared by soaking, steaming and inoculating soybeans with Bacillus natto, cell walls were partially destroyed and some of the oil-droplets appeared meshlike in structure. Fresh tofu (bean curd) prepared by soaking beans in water, grinding, heating, filtering to yield soybean milk and addition of calcium salts to coagulate the protein-oil complex was also examined. Electron microscopy of this revealed a meshy network of coagulated protein particles (0.1 to 0.2 μm in diameter) interspersed with oil-droplets measuring up to 1.0 μm in diameter. On the other hand, Kori-tofu (dried or frozen tofu) exhibited a denser network of coagulated protein than ordinary tofu. Saio and Watanabe observed that upon freezing the protein-oil network became more compact and on thawing and drying a fibrous structure developed.

In a study on soymilk preparation, Johnson and Snyder (1978), found that

typical protein bodies (2 to 10 μm in diameter) were present only in heated soy slurries, whereas unheated preparations appeared to contain much smaller particles. Johnson claimed that protein bodies appeared to be "fixed" in some manner by the heat treatment prior to grinding and suggested that they were possibly more resistant to disruption upon grinding than protein bodies in unheated preparations.

Wolf (1970) studied the effects of grinding, defatting and drying on the integrity of soybean protein bodies using scanning electron microscopy. Examination of vacuum-dried isolated protein bodies revealed numerous spherical or nearly spherical particles 1.0 to 3.0 μm in diameter plus some adhering amorphous material. Freeze-dried preparations, however, contained more of the amorphous substance and a greater number of smaller particles (1.0 μm or less in diameter). These particles were considerably smaller than those protein bodies (2.0 to 20.0 μm in diameter) observed in soybean flour. Wolf believed that most of the protein bodies probably did not survive the isolation process; nevertheless, he found some aggregates of particles, presumably clumps of protein bodies which apparently survived initial grinding of the soybeans, as well as homogenization during their isolation.

In a scanning electron microscopy study of soybeans, soy flours, protein concentrates and isolates, Wolf and Baker (1975) reported that processing practices used in the preparation of these products altered the ultrastructural appearance of some soybean components. Protein bodies and spherosomes

characteristic of the native cellular structure were clearly discerned in freeze-fractured surfaces of whole soybean cotyledons, as well as in full-fat flour. Defatted soybean flour also contained protein bodies, but more of them were present in an unheated flour as compared to a toasted flour. They observed relatively intact protein bodies in protein concentrates prepared by alcohol leaching, whereas an acid leaching preparation consisted of partially collapsed spheres. In the isoelectric preparation of protein isolates, particles appeared rough in surface texture, while the proteinate forms of isolates were predominantly smooth, apparently as a result of differences in solubility during spray drying.

Cumming et al. (1972) in a study on textural properties and ultrastructure of texturized soy protein, found that texturization of a defatted soybean meal appeared to reform protein bodies into continuous fiber-like structures. Many fissures, and holes were observed in and around the fibers, which they claimed were caused by the abrupt release of pressure which occurs during and just prior to extrusion. Similar results were reported by other researchers (deMan, 1976; Stanley et al., 1972).

Recently, ultrastructural changes in defatted soy flour induced by extrusion and nonextrusion texturization were studied (Taranto et al., 1978; Taranto and Rhee, 1978). The work has shown that the texture created by nonextrusion texturization caused a number of ultrastructural changes: extensive fusion of cellular fragments, a well-formed protein-carbohydrate matrix and fibrillation

of the protein matrix. Some areas of incomplete protein body fusion could still be found, but most of the protein bodies were severely deformed. In extruded texturized soy flour, no fibroid structures were detected; however, a protein matrix in which insoluble carbohydrates were dispersed throughout was observed.

Processing of soybeans to desolventized-toasted flakes

In the United States the major form of processing soybeans is an integrated operation consisting of extracting the oil and then desolventizing-toasting the defatted flakes for use as animal feeds (Wolf, 1972).

After harvesting, soybeans are cleaned and dried before storing. Proper moisture content is needed for successful storage of soybeans, therefore they are dried to a moisture content of approximately 12%. From storage the soybeans are cleaned of iron, steel and other magnetically susceptible objects by passing through a magnetic separator and screening operation. Next the cleaned beans flow to the cracking rolls where the beans are cracked into six to eight pieces, and the hulls are loosened. The hulls are eventually removed by aspiration of the cracked beans. The cracked beans are conditioned to 10 to 11% moisture at 63 to 74°C, and then flaked to a thickness of 0.01 to 0.015 inches by passing through smooth rolls. Flaking ruptures the cells in the soybean and reduces the distance the oil and solvent must diffuse, thereby facilitating extraction with organic solvent (Wolf and Cowan, 1971). However, cell disruption during flaking of soybeans does not appear to have been investigated

(Kamofsky, 1949a). Moreover, there is as yet no agreement whether flaking of other oilseeds disrupts a large fraction of the oil-bearing cells (Norris, 1964). Studies on the effects of flaking and grinding processes on the ultrastructure of soybeans might provide the information needed for the successful development of an air classification process for increasing protein content of soy flours (Wolf, 1972). Next, the soybean flakes flow by conveyor to the extractor where hexane (boiling point 66 to 69°C) is widely used as a solvent. Extractors employ a variety of different ways to contact the flakes, including a presoaking period to remove some of the oil, and stationary baskets with the pumping of solvent and miscella in a progressive stepwise counter-current flow of flakes and solvent.

For feed use the hexane-laden flakes are passed through a desolventizer-toaster which recovers the hexane and simultaneously toasts the flakes to obtain optimum nutritive value. Steam is introduced to vaporize the hexane and condenses on the flakes to increase their moisture content. As the flakes descend through the unit, temperature is gradually increased to 110°C to lower the moisture to 13 to 15% at the end of the cooking cycle. The effect of desolventizing-toasting on cellular structure of hexane-defatted soybean flakes has been observed under the light microscope (Sipos and Witte, 1961). They found an unusual destruction of the cellulosic cell walls and agglomeration of protein bodies present within the individual cells into large protein masses. Sipos

and Witte claimed that this almost total destruction of cell walls was due to the explosive vaporization of solvent in the desolventizing zone and is characteristic of the desolventizer-toaster process. Finally, after desolventizing and toasting the flakes are dried and cooled and then ground into meal.

Solvent extraction of lipids

When whole or half soybeans were extracted with hexane for a week, respective percentages of the original oil extracted were only 0.08 and 0.19% (Othmer and Agarwal, 1955). It has long been known that to extract oil, soybeans and other oilseeds must be decreased in particle size so that at least one dimension is about 0.010 inches (Karnofsky, 1949b). Such results demonstrated that to successfully extract oil from the soybean, the seed structure needed to be disrupted; however, the results do not answer the question whether the oil is unextractable due to impermeability of cell walls to hexane or impermeability of spherosomes (Wolf, 1972).

To date, the literature contains no information on the effects of lipid extraction utilizing various organic solvents on the ultrastructure of soybean tissue. Moreover, studies of this nature seem to be lacking for most oilseeds. Hensarling et al. (1970), however, microscopically investigated ultrastructural effects of lipid extraction in cottonseed following extraction of the tissue with chloroform-methanol-water (CMW), hexane-acetone-water (HAW), chloroform-methanol (CM), hexane-acetone (HA), hexane (H) and acetone (A). The

cellular structure in tissues extracted with CM, HA, H or A appeared similar to untreated samples, except that spherosomes were empty of oil and were electron-translucent after fixation with osmium tetroxide. However, extraction of tissue with CMW or HAW resulted in cells that had disorganized cytoplasm and extensive disruption of intracellular structures without apparent cellular rupture. From this evidence, the authors concluded that disruption of intracellular cytoplasm occurred after extraction of tissue with water-containing solvents.

MATERIALS AND METHODS

Soybeans

Soybean seeds of the Amsoy 71 variety were used for all experiments (except) for those samples obtained from commercial processors). The soybeans were sorted by hand to remove broken or discolored beans as well as any debris and were stored at 4° C prior to use to minimize changes in moisture content. For most experiments soybean seeds were imbibed in water (overnight, 4° C), unless otherwise specified.

Processed Soybean Samples

Seven soybean samples — field beans, dried beans, cracked and conditioned beans, full-fat flakes, defatted flakes, desolventized-toasted flakes and dried meal — were obtained from a commercial soybean processing plant in Iowa. Samples were stored in polyethylene self-sealing bags at 4° C and under nitrogen to prevent loss of moisture.

Proximate Analysis

Moisture determination

Approximately 3.0 g duplicate samples were weighed into tared aluminum weighing dishes, which were previously cleaned and dried. The samples were dried in a vacuum oven (85° C, 635 mm Hg) for 14 hr and cooled in a $CaCl_2$ desiccator. Moisture content was determined gravimetrically.

Protein determination

A modification of the micro-Kjeldahl method (38.012 AOAC, 1970) was used to determine total nitrogen in all samples. The total nitrogen was converted to

protein using a conversion factor of 6.25.

Duplicate dried samples, 0.1 to 0.2 g, were digested with 2 ml of concentrated sulfuric acid on a Lab. Con. Co. digestion apparatus. Cupric selenite, 0.2 g, was used as a catalyst during digestion along with 0.3 g of potassium sulfate to raise the boiling point of acid. Gentle heating was applied for 15 min, the flask neck was rinsed with additional acid, and the temperature was elevated and digested for an additional 45 min. The Kjeldahl flask was rotated periodically while on the digestion apparatus.

After cooling the digested samples to room temperature, about 10 ml of distilled deionized water was added, then the mixture was quantitatively transferred to a Lab. Con. Co. distillation apparatus. Excess sodium hydroxide solution 40% (w/v) was added to release the ammonia which was trapped in a 4.0% boric acid solution. The ammonia in the solution was titrated with standard HCl prepared according to AACC (1969) method 70-20. Tashiro's indicator, prepared by dissolving 0.25 g methylene blue and 0.375 g methyl red in 300 ml of 95% ethyl alcohol gave a light blue to greyish endpoint.

Crude lipid

Crude lipid was determined by skelly B extraction (unless specified) using a Goldfisch extraction apparatus according to the procedure of AACC (1969) method 30-20. Duplicate dried samples, 1.5-2.0 g were weighed into previously dried and tared extraction thimbles. The thimbles were placed on the Goldfisch

apparatus and extracted 16 to 18 hr at a setting adjusted for high heat. After the extraction period, thimbles were dried in a vacuum oven (85°C, 635 mm Hg) for 14 hr and then placed into a $CaCl_2$ desiccator to cool. The percent lipid was determined gravimetrically as weight loss due to extraction.

Some samples of soybean cotyledon meal were extracted with hexane (H), chloroform:methanol (CM) 2:1 (v/v), chloroform (C), methanol (M) and acetone (A) in a process identical to the skelly B extraction of crude lipids.

Ash determination

A modification of AOAC (1970) method 15.016 was used for the determination of ash. Duplicate 2.0 g samples were weighed into tared porcelain crucibles, previously cleaned and fired to a red glow. The 2.0 g samples were charred using a Bunsen burner, being careful not to allow the samples to burn with an open flame, then ignited in a muffle furnace at 500°C for 12 to 16 hr to get carbon-free ash. Samples were cooled in a $CaCl_2$ desiccator, and the percentage ash was determined gravimetrically.

Carbohydrate

Carbohydrate was determined by difference; therefore, it includes all material not included as protein, lipid, ash or moisture.

Protein Dispersibility Index (PDI)

A modification of the procedure described in Smith and Circle (1972) was used to determine the PDI for the seven processed soybean samples. Samples

(ground to pass through a 20 mesh screen) weighing 20 ± 0.1 g, were placed into 600 ml beakers. For each sample, a 250 ml volumetric flask was filled to the mark with distilled water and approximately 50 ml of this was poured into a Waring blender jar. The sample was added to the blender jar containing the water and stirred with a spatula to disperse the particles, forming a slurry; the additional 200 ml of distilled water was used to rinse the beaker, spatula and blender walls of adhering material. The slurry was homogenized at high speed for 10 min and poured back into the 600 ml beaker where it was allowed to stand at room temperature until it separated into two distinct layers (about 30 min). Twenty-five ml of the upper layer was pipetted into a 50 ml centrifuge tube and centrifuged for 10 min at 955 x g. After centrifugation, duplicate 2.0 ml samples of the supernatant liquid were quantitatively transferred to 100 ml micro-Kjeldahl flasks and protein was determined by a modification of the micro-Kjeldahl method.

$$\% \, H_2O \text{ dispersible protein} = \frac{\text{ml HCl} \times 0.1\text{N} \times 0.014 \times 100 \times 6.25}{\frac{\text{weight of sample}}{250}} \times 2.0$$

$$\text{Protein dispersibility index (PDI)} = \frac{\% \, H_2O \text{ dispersible protein}}{\% \text{ Total protein}} \times 100$$

Microscopy Studies

Light microscopy (LM)

Paraffin embedding Whole soybean cotyledons or pieces were fixed in 3.0% glutaraldehyde-1.5% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.2 for 12 to 24 hr at 4°C. The material was then passed through three 20-min buffer rinses, dehydrated through an ethanol series, infiltrated with xylene and slowly infiltrated with paraffin. Sections (8 to 10 μm) were made on a rotary microtome. The sections were mounted on glass slides and stained in the following ways using general and histochemical techniques to localize several major groups of chemical components: proteins — mercuric-bromphenol blue (Mazia et al., 1953) coupled with trypsin extraction (Horner, 1976); carbohydrates — periodic acid-Schiffs reaction (PAS) (Jensen, 1960, 1962); starch — iodine-potassium iodide (Johansen, 1940) and PAS technique (McManus, 1948) coupled with alpha-amylase extraction (Casselman, 1959); lipids — Sudan III, Sudan IV and Sudan black (Baker, 1947; Gomori, 1952); general staining — Toluidine blue O.

Resin embedding Small pieces of soybean cotyledons, processed soybean samples or agar embedded lipid bodies were fixed in 3.0% glutaraldehyde-1.5% paraformaldehyde in sodium phosphate buffer (0.1 M, pH 7.2) for 12 to 24 hr at 4°C. The pieces were then passed through three 20-min buffer rinses and postfixed for 2 hr in 2.0% osmium tetroxide (same buffer and pH) at room temperature. Fixation was followed by three 20-min buffer rinses, dehydration

through an acetone series, infiltration with propylene oxide and final infiltration and embedding in Spurr's embedding resin (Spurr, 1969). Sections were cut at 1 to 2 μm using an LKB Ultratome III and glass knives. Sections were stained with warmed 1% Toluidine blue O.

Transmission electron microscopy (TEM)

Samples were prepared as described for resin embedded material for light microscopy. Sections having silver to gold interference colors were cut with glass knives using an LKB Ultratome III. Sections were picked up on copper grids and stained with methanolic uranyl acetate (Stempak and Ward, 1964) and lead citrate (Reynolds, 1963). Grids were examined on a Hitachi HU-11C transmission electron microscope operated at an accelerating voltage of 50 KV.

Scanning electron microscopy (SEM)

Pieces of material were fixed in 3.0% glutaraldehyde-1.5% paraformaldehyde in sodium phosphate buffer (0.1 M pH 7.2) for 12 to 24 hr at 4°C. The material was given three 20-min buffer rinses and postfixed in 2.0% osmium tetroxide (same buffer and pH) at room temperature. Fixation was followed by three 20-min buffer rinses and dehydration through an acetone series. After the third absolute acetone change, the pieces were frozen in Parafilm pillows filled with absolute acetone and cryofractured in liquid nitrogen using a razor blade held in a hemostat. Fractured pieces were dropped into liquid 100% acetone to thaw, infiltrated with Freon TF (113) and critical point dried using liquid carbon dioxide. Specimens were

mounted on brass discs with silver conductive paint, lightly coated with carbon and about 10 nm of gold in a Varian VE-30 vacuum evaporator.

Fresh unfixed specimens (calcium oxalate crystals) were concentrated from cut ends of soybean cotyledons in drops of double-distilled water on brass discs, dried and coated with carbon and gold as just described.

Energy dispersive X-ray (EDX) analysis

Small pieces of soybean cotyledons and agar embedded lipid bodies were prepared as described for resin embedded material for light microscopy except for the use of cacodylate buffer (0.1 M pH 7.2) in place of phosphate buffer. Sections were cut at 0.5 to 1 μm using an LKB Ultratome III and glass knives. Sections were collected on formvar coated 200 mesh copper grids and lightly coated with carbon in a Varian VE-30 vacuum evaporator. The coated grids were each attached over a hollow graphite rod mounted on top of a hollow brass stub, using silver conductive paint. Sections were examined using a transmitted electron detection system (TED) and analyzed with an energy dispersive X-ray (EDX) microanalyzer. The EDX system was also used for elemental analysis of the unfixed calcium oxalate crystals.

The chemically fixed and fresh preparations were examined on a JEOL JSM-35 scanning electron microscope fitted with a Kevex Model 5000 A energy dispersive X-ray microanalyzer. The scanning electron microscope was operated at 15, 20 or 25 KV at beam currents between 80 and 140 μA.

Photography: (LM), (TEM) and (SEM)

Photographs of paraffin and plastic sections were taken on a Leitz Ortholux microscope fitted with bright field, phase contrast, and polarization optics. Kodak plus-x and pan-x film were used in the attached Leitz Orthomat automatic microscope camera.

TEM photographs were taken on 3 1/4 x 4 inch Dupont Cronar Ortho S Litho sheet film using Kodak D-19 developer. SEM photographs were taken on 3 1/4 x 4 1/4 Polaroid type 665 positive/negative land film. Positive prints made from all negatives were printed on Kodak polycontrast paper using dektol as the developer.

Germination and Maturation Studies

Germination

The germination towel method [number 8 as described by Burris and Fehr (1971)] was used for soybean germination. Seeds were planted directly on two layers of moistened paper towel without surface sterilization (with micropyle oriented toward the top of the paper) and covered with a third layer of towel. The towels were then rolled, placed in a one-liter plastic carton with 100 ml distilled water and covered with a plastic bag secured with a rubber band.

The containers with the soybeans were placed into a Percival Model E54-U growth chamber at an alternating temperature sequence of 18 hr at 30 ± 1°C and 6 hr at 22 ± 1°C. The 18 hr period had illumination of 300 foot-candles and

the 6 hr period was dark. After 5 days, the plants were transferred and planted into large (30 cm diameter) pots containing soil. Twelve to 13 soybean plants were placed into each pot. The soil and plants were sufficiently watered and placed into a percival growth chamber operating at a constant temperature of 30° C with 12 hr of illumination and 12 hr of darkness. The relative humidity of the chamber fluctuated from 76 to 86% during the 24 hr period.

Soybean cotyledons were collected at 24 hr intervals from day 1 to day 17 postgermination. Following each collection period, seed coats and hypocotyl-radicle axes were removed and fresh weights of the cotyledons were recorded. Some of the cotyledons were immediately sectioned in fresh fixative and processed as described for resin embedded material for (LM) and (TEM), while other cotyledons were minced (to facilitate drying) and placed overnight in a vacuum oven (85° C, 635 mm Hg). The dried cotyledonary tissue was ground in a Wiley mill to pass a 20 mesh screen and analyzed for total lipid, protein and ash.

Cytochemical studies

Acid phosphatase localization Localization of acid phosphatase in ungerminated (3 hr water soak), 2, 4, 7, and 9 day germinated cotyledons of both cotyledonary tissue and lipid body isolates (as described previously) was determined utilizing a modification of the procedure of Barka and Anderson (1962). Aldehyde-fixed tissues were placed in 5 to 10 ml of the reaction mixture (adjusted to pH 5.5 prior to use) and incubated at 37° C for different periods of time (30, 60, 90, and 120 min). The following controls were used: (1) tissues

incubated in reaction medium minus the substrate (sodium beta-glycerol phosphate), (2) tissues incubated in complete reaction medium containing 0.01 M sodium fluoride, (3) tissues carried through the entire procedure after having been boiled for 15 min in water. After incubation, the tissues were well rinsed in buffer and postfixed in 2.0% phosphate-buffered osmium tetroxide (2 hr, room temperature Tissues were further processed as described for resin embedded material for (LM) and (TEM).

Catalase localization Histochemical investigations have shown that glyoxysomes specifically stained for catalase activity (Breidenbach et al., 1968; Richardson, 1974). The method of incubating tissue in 3,3'-diamino-benzidine (DAB) described by Mollenhauer and Totten (1970) was used to detect catalase cytochemically in soybean cotyledons. Ungerminated (3 hr water soak) and germinated (1, 2, 3 through 17 day) cotyledons were used in this experiment. Small pieces of cotyledonary tissue were prefixed in a mixture of 3.0% glutaraldehyde-1.5% paraformaldehyde in a 0.1 M sodium phosphate buffer, pH 7.2 for 2 hr at 4°C. After prefixation, the tissues were rinsed in three 20-min changes of buffer and incubated in one of the described media. Following incubation, the tissues were rinsed in three 20-min changes of buffer and then postfixed in phosphate buffered 2.0% osmium tetroxide for 2 hr at room temperature. After postfixation, the tissues were rinsed in several changes of buffer and further processed as described for resin embedded material for (LM) and (TEM).

Lipase localization Histochemical localization of soybean lipase was tested by a modification of the lipase localization procedure described by Ory (1969). Pieces of soybean cotyledon as well as agar embedded lipid body isolates (isolated according to Jacks et al., 1976), were obtained from ungerminated (3 hr water soak), 1, 3, 5, and 7 day germinated cotyledons. In addition to the acetate buffers (pH 4.5 and 5.5), a tris-HCl buffer was used to attain a pH of 7.0. Samples were incubated in the lead containing reaction mixture at each respective pH for different periods of time (8, 30, 60, and 300 min) at 45° C. Controls consisting of complete reaction mixture without lead were run along with the experimental tissue. After incubation, tissues were fixed and processed as described for resin embedded material for (LM) and (TEM).

Maturation

Developing cotyledons were collected from soybean plants at various time intervals as determined by days after plant flowering (DAF), and by cotyledon fresh weight. Following each collection period, seed coats and hypocotyl-radicle axes were removed and fresh weights of the cotyledons were recorded. Some of the cotyledons were immediately sectioned in fresh fixative and processed according to the procedure described for resin embedded material for (LM) and (TEM), while other cotyledons were minced (to facilitate drying) and dried overnight in a vacuum oven (85° C, 635 mm Hg). The dried cotyledonary

tissue was ground in a Wiley mill to pass a 20 mesh screen and analyzed for total lipid, protein and ash.

Processing Methods

Solvent extraction of soybean cotyledons

A modification of Hensarling et al. (1970) was employed for lipid extraction of cotyledonary tissue. Pieces of dry soybean cotyledons, cut from the mid-section, were placed over water in a Petri plate for 1 hr; then smaller pieces, about 1 mm^3 were excised from this pliable tissue. Pieces were dried over $CaCl_2$ in vacuo and placed into vials containing: (CM), (C), (M), (H) and (A). The capped vials, each containing approximately 8 ml of solvent and 10 pieces of tissue, were placed on a rotating wheel to ensure continuous mixing. After 2 changes of respective solvents during a 24 hr period, the tissues were fixed for microscopic observation. A method was developed to fix the tissue in the extracting solvent. Tissues were fixed in 2.0% osmium tetroxide (2 hr, room temperature), which appeared both soluble and stable in the solvents. The fixed tissues were then thoroughly rinsed with the respective solvents, dehydrated through an acetone series and further processed as described for resin embedded material for (LM) and (TEM).

Physical treatments

Whole soybean cotyledons, imbibed overnight in water at 4° C, were subjected to treatments described below. The cotyledons were sectioned

(mid-sections) into small pieces (approximately 1 mm^3) and processed as described for resin embedded material for (LM) and (TEM).

Heat treatment (boiling) Imbibed soybean cotyledons were placed into boiling water for 15 min.

Freezing-thawing treatment Imbibed soybean cotyledons were frozen at -20° C for 12 hr prior to microscopical processing.

Sonication treatment Imbibed soybean cotyledons were placed into a small beaker of water, and sonicated at a constant temperature of 22° C for a period of 10 min in an L & R Ultrasonic Model 320 Sonicator. (L & R Manufacturing Company, Kearny, New Jersey).

Enzyme treatment (trypsin) Small pieces of imbibed cotyledons, both unheated and heated, as well as agar embedded lipid bodies, were placed into vials containing 5 ml of a trypsin solution (prepared according to Horner, 1976) and incubated at 37° C for 12 hr. Samples were processed as described for resin embedded material for (LM) and (TEM).

Lipid Body Isolation

Isolation of lipid bodies from soybean cotyledons was done using three methods, as described by: Jacks et al. (1967), Kahn et al. (1960), and Yatsu and Jacks (1972). The methods are based upon sequential centrifugation of a tissue homogenate in an appropriate medium and collecting the resultant floating layer "fat pad" for microscopical examination and chemical analysis.

Floating layers from each method were dialyzed 2 days in distilled water (4° C) before analysis. A portion of the isolated lipid bodies was embedded in an agar medium to facilitate manipulation through the fixation procedures. The lipid body fraction was warmed to 43° C and mixed with a 2% (w/v) solution of agar at the same temperature, and centrifuged at 2600 x g until the agar gelled. One mm^3 cubes of the gel which contained the lipid bodies were removed from the centrifuge tube and taken through the fixation, dehydration and embedding process as described for resin embedded material (LM). Thin sections of the material for (TEM) and freeze-fractured samples for (SEM) were used for microscopical examination.

The remaining portion of the dialyzed sample was placed into a VirTis freeze-dryer and dried (approximately 2 days) before chemical analysis. For comparative studies, other centrifugal fractions of soybeans (pellet and supernatant) were similarly processed.

Density Gradient Centrifugation

Isolated lipid bodies prepared according to the procedure of Yatsu and Jacks (1972) were placed under continuous and discontinuous sucrose density gradients. A small portion of the lipid isolate was resuspended in 2.0 ml distilled water and carefully layered over the gradients. The discontinuous gradient consisted of zones containing 0.05 M, 0.1 M, 0.2 M, 0.4 M and 0.6 M sucrose whereas

the continuous gradient was prepared with a range of 0.1 M to 0.6 M sucrose concentrations. Sample and gradients were held at 4° C during preparation and ultracentrifugation. The sucrose gradients were centrifuged for 7 hr at 100,000 x g in a Beckman Model L3-50 ultracentrifuge with a SW 41 rotor.

RESULTS AND DISCUSSION

Soybean Cytology with Emphasis on Protein Bodies

Protein bodies constitute the most abundant organelle of soybean cotyledon cells. They are oval or irregularly oval in shape and range in size from 1.0 μm to 20.0 μm; however, most appear to be slightly over 5.0 μm in diameter (Fig. 4). The protein bodies readily stain deep blue after treatment with mercuric bromphenol blue (Fig. 2), and literally fill the cotyledonary tissue as well as some of the epidermal cells. Fig. 3 shows cotyledonary tissue as it appears following trypsin treatment and subsequent mercuric bromphenol blue staining. Protein bodies undergo dissolution and are not evident as discrete bodies in the treated tissue; however, a lightly stained mass and darkened cell walls contrast with the structures in Fig. 2. Probably the remaining stained mass is proteinaceous and is indicative of partially extracted cells.

Protein bodies are interspersed within a cytoplasmic network consisting of small (0.1 to 1.0 μm in diameter) spherical lipid bodies, sometimes referred to as spherosomes (Fig. 5). The freeze-fractured tissue reveals the small lipid bodies, some protein bodies and numerous surface depressions probably caused by the loss of the protein bodies adhering to the complemental fractured surface. More protein bodies are retained in the fractured cells shown in Figs. 6-8. These protein bodies are predominantly spherical, smooth surfaced, 5.0 μm in diameter and are randomly dispersed throughout the cytoplasmic network which contains

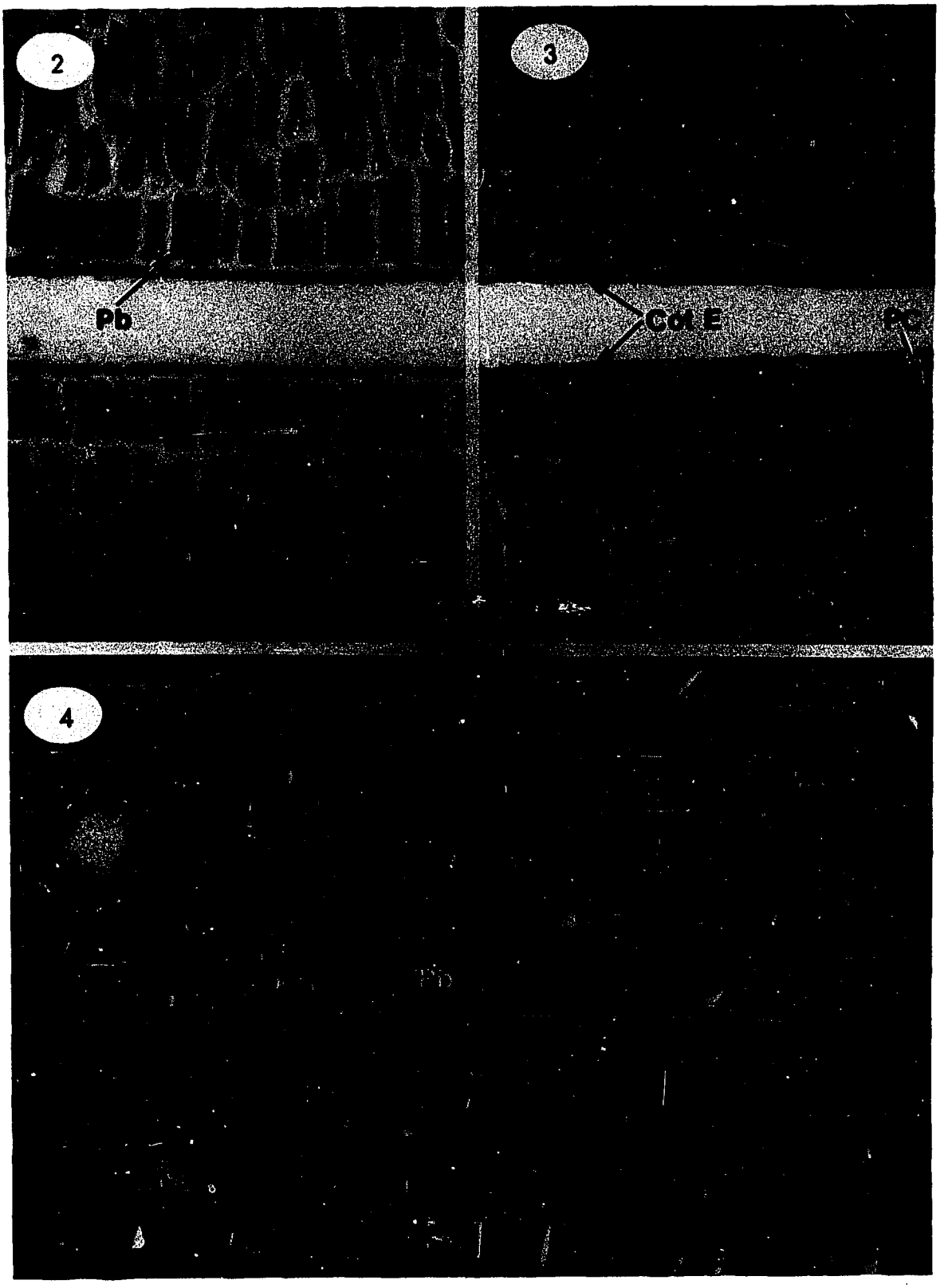
Figs. 2-4. Paraffin sections of soybean cotyledons

Fig. 2. Protein bodies stained with mercuric bromphenol blue 380X

Fig. 3. Trypsin treatment of protein bodies followed by mercuric bromphenol blue staining 520X

Fig. 4. Stained with toludine blue O 1,400X

Explanation of figure abbreviations appear in Fig. 162 found on page 252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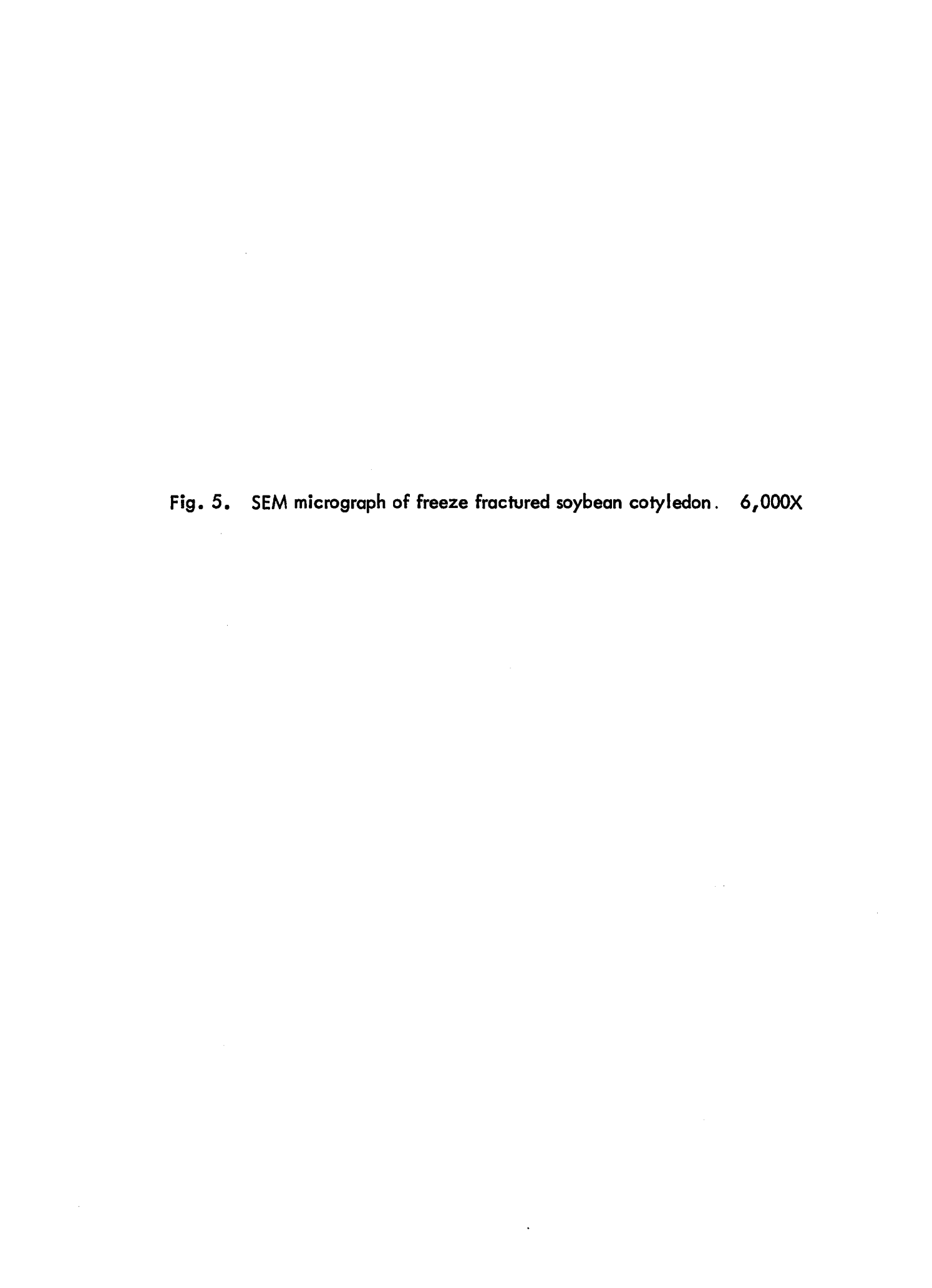
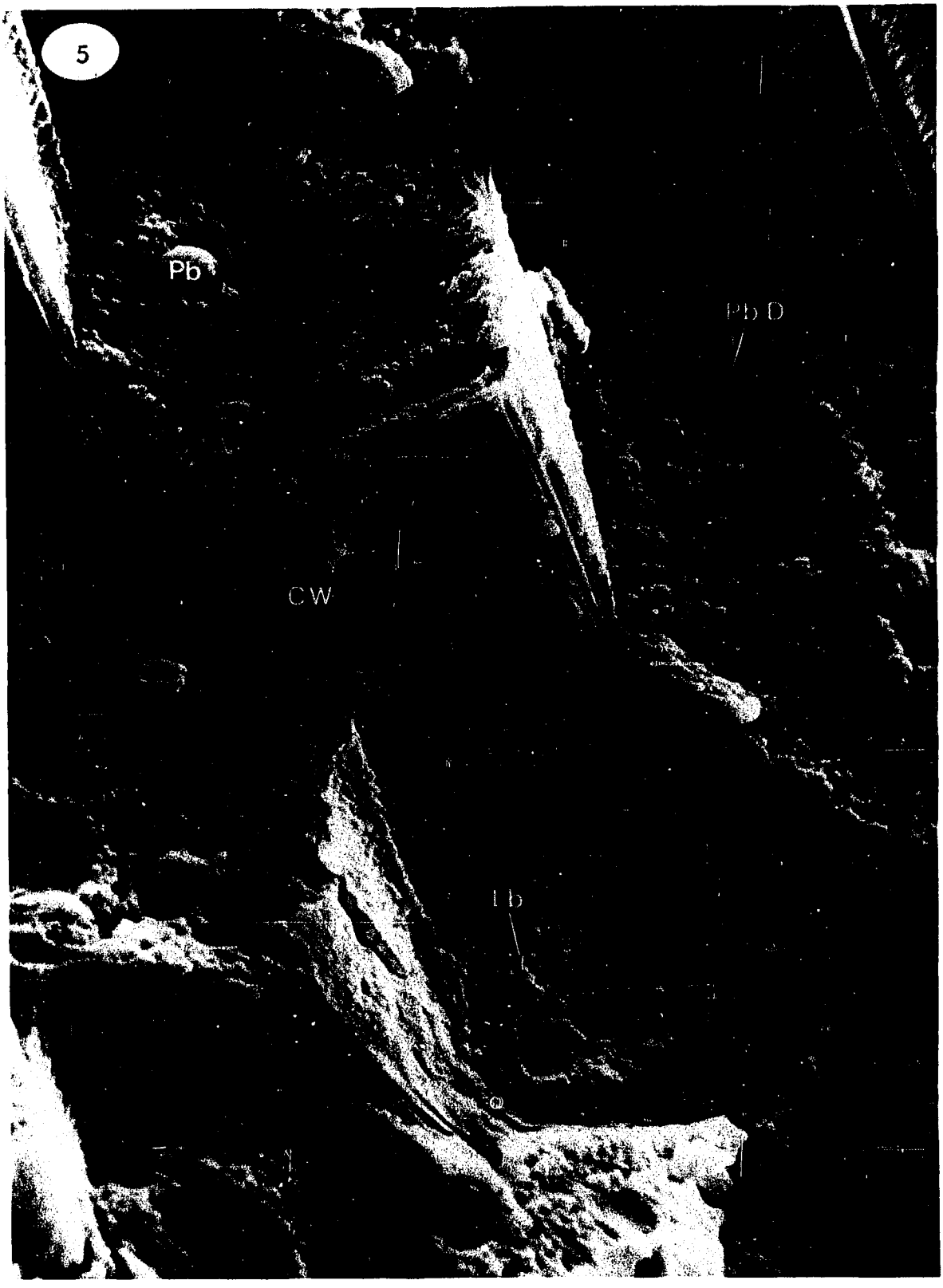


Fig. 5. SEM micrograph of freeze fractured soybean cotyledon. 6,000X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Pb
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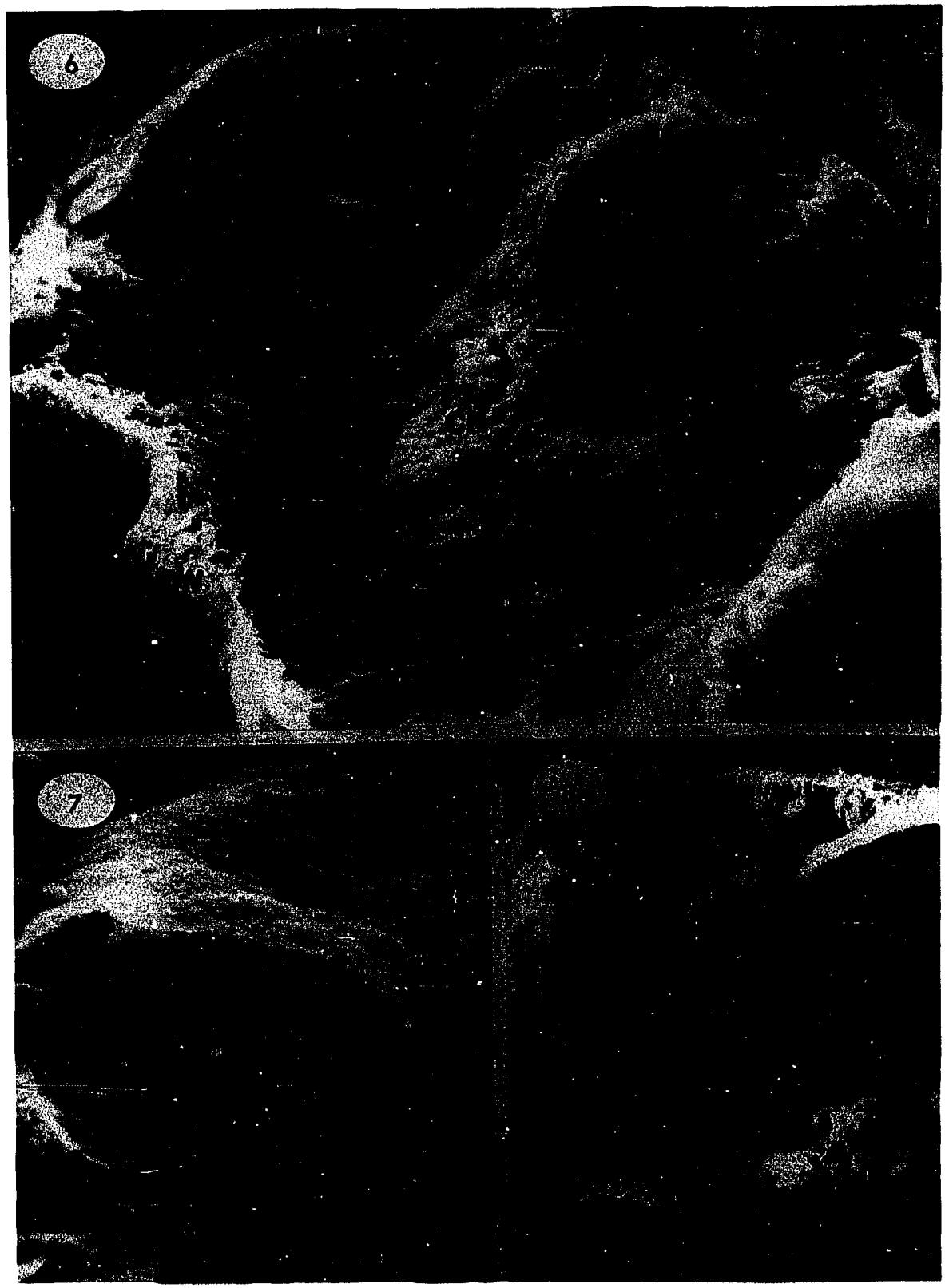
Figs. 6-8. SEM of freeze fractured soybean cotyledons

Fig. 6. Fractured cotyledon cell 5,700X

Fig. 7. Fractured cotyledon cell 4,600X

Fig. 8. Fractured cotyledon cell 4,200X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the lipid bodies and other cellular constituents. One protein body in Fig. 6 is partially exposed through the sheet-like cytoplasmic network (arrow).

Much can be learned of soybean cellular organization by TEM. In Fig. 9 the protein bodies appear irregular in shape and show a close association of lipid bodies to their surface. This association is evident in cells where there are fewer lipid bodies (compare Figs. 9 and 10). Protein bodies are delimited by a membrane (Fig. 11), which appears as a dark-light-dark line, typical of the well-known tripartite unit-membrane and is easily detected in tissues where lipid bodies are less concentrated. This membrane is similar to that reported by Tombs (1967) and Webster and Leopold (1977) for soybean protein bodies.

Cotyledon cell walls range in size from 1.0 to 8.0 μm in thickness (Fig. 9) and appear to have a rough or corrugated surface texture (Fig. 5). The surface texture is most likely caused by the close association of lipid bodies with the cell wall. In some freeze-fractured preparations, the cell walls are composed of fibrous material (Figs. 6-8).

Most researchers dealing with soybean protein bodies have described the interior matrix of the body as being homogenous and uniform, with no apparent structural entities contained within them (Tombs, 1967; Saio and Watanabe, 1966), similar to those shown in Figs. 9-11. I have frequently found, however, that some soybean protein bodies accommodate small circular inclusions (Fig. 12). At first glance, these spherical objects might appear as artifacts due to fixation

Figs. 9–11. TEM of soybean cotyledon cells

Fig. 9. Irregular shaped protein bodies and other cellular organelles 8,280X

Fig. 10. Cotyledon cell 21,996X

Fig. 11. Protein body membrane 25,790X


9

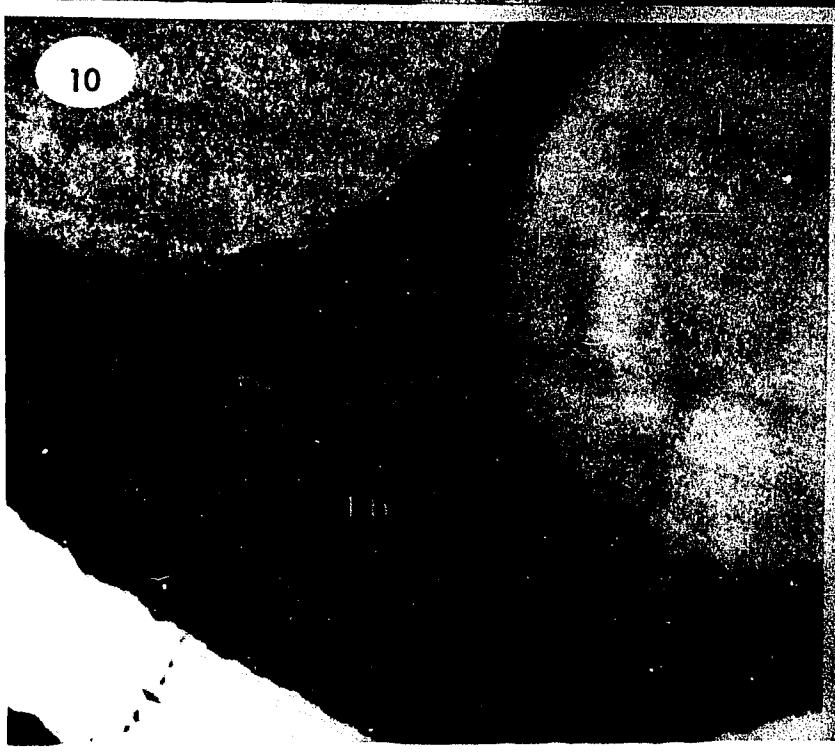
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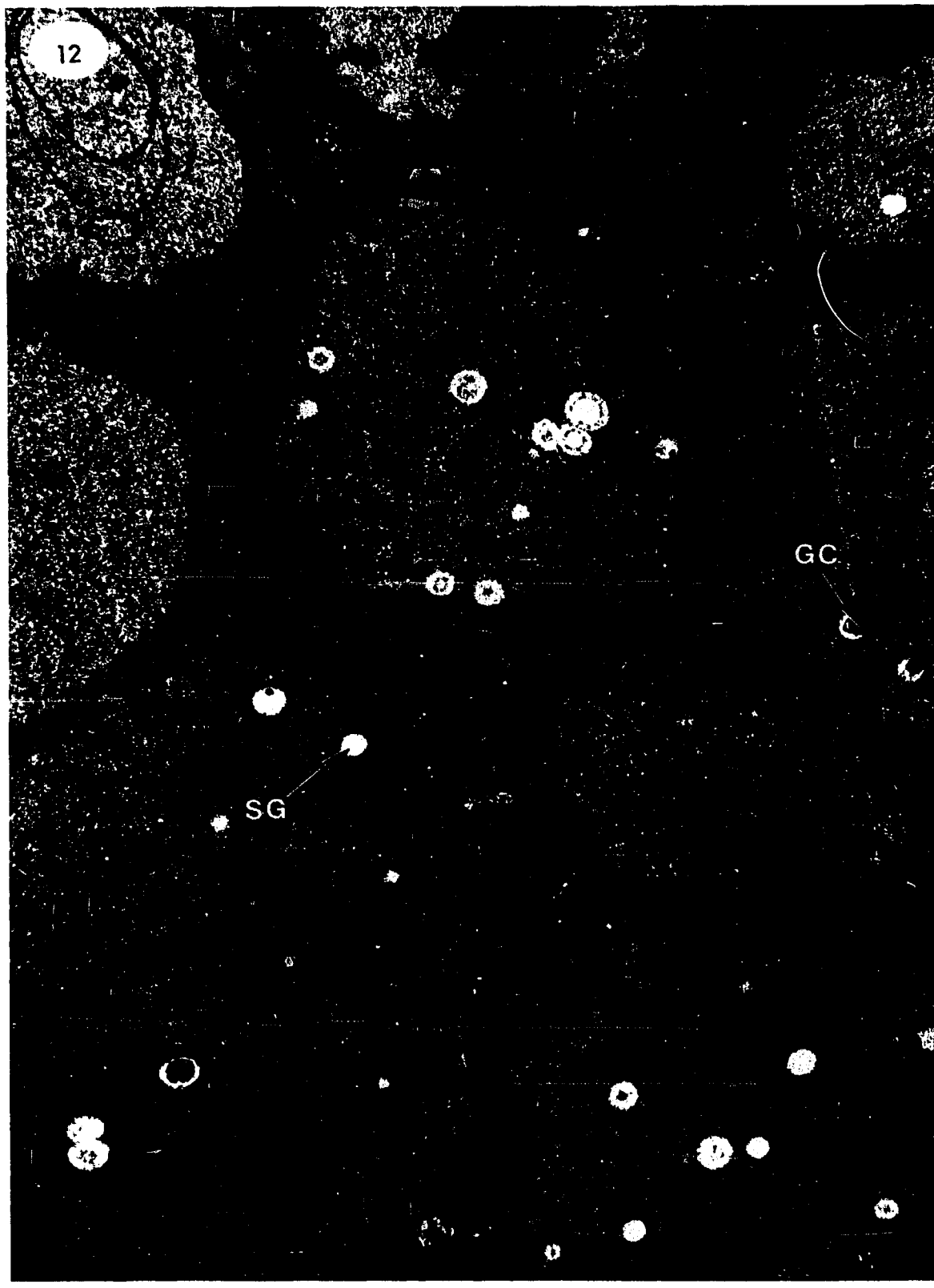
Fig. 12. TEM of soybean cotyledon showing distribution and types of globoid inclusions found in protein bodies 10,350X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GC
SG

and preparation of tissue for microscopical examination. However, protein bodies have been found in many plant seeds which contain inclusions similar to those reported here (Jacobsen et al., 1971; Rost, 1972; Sharp, 1934).

The inclusions in soybean protein bodies vary in size, distribution and electron-density. Most of the inclusions appear spherical to slightly ellipsodial in shape and are 0.5 μm in diameter, but a range in size from 0.1 μm to 3.0 μm has been observed. There appears to be a cell-to-cell (or tissue-to-tissue) variation in the distribution of these inclusions. Often, I observed inclusion-containing protein bodies in one cell, while a neighboring cell would have protein bodies completely devoid of inclusions. Most of the inclusions appear electron-translucent, but some contain electron-dense or scattered electron-dense material (Figs. 12-17). In this study, the terminology of Lott et al. (1971) was followed. Spherical inclusions in a protein body are termed globoids. An electron-dense portion of a globoid is called globoid crystal, and an electron-translucent portion is termed soft globoid.

The electron-dense globoid crystals (Figs. 13 and 14) are about 0.2 μm in diameter and are rarely found in the globoid. Globoid crystals in many plant seeds contain high levels of phosphorus and are thought to be rich in phytin (Lott, 1975; Lui and Altschul, 1967; Ogawa et al., 1975). Considering that soybean protein bodies contain approximately 0.5% to 0.8% phosphorus on a dry weight basis (Saio and Watanabe, 1966; Tombs, 1967; Lolas et al., 1976),

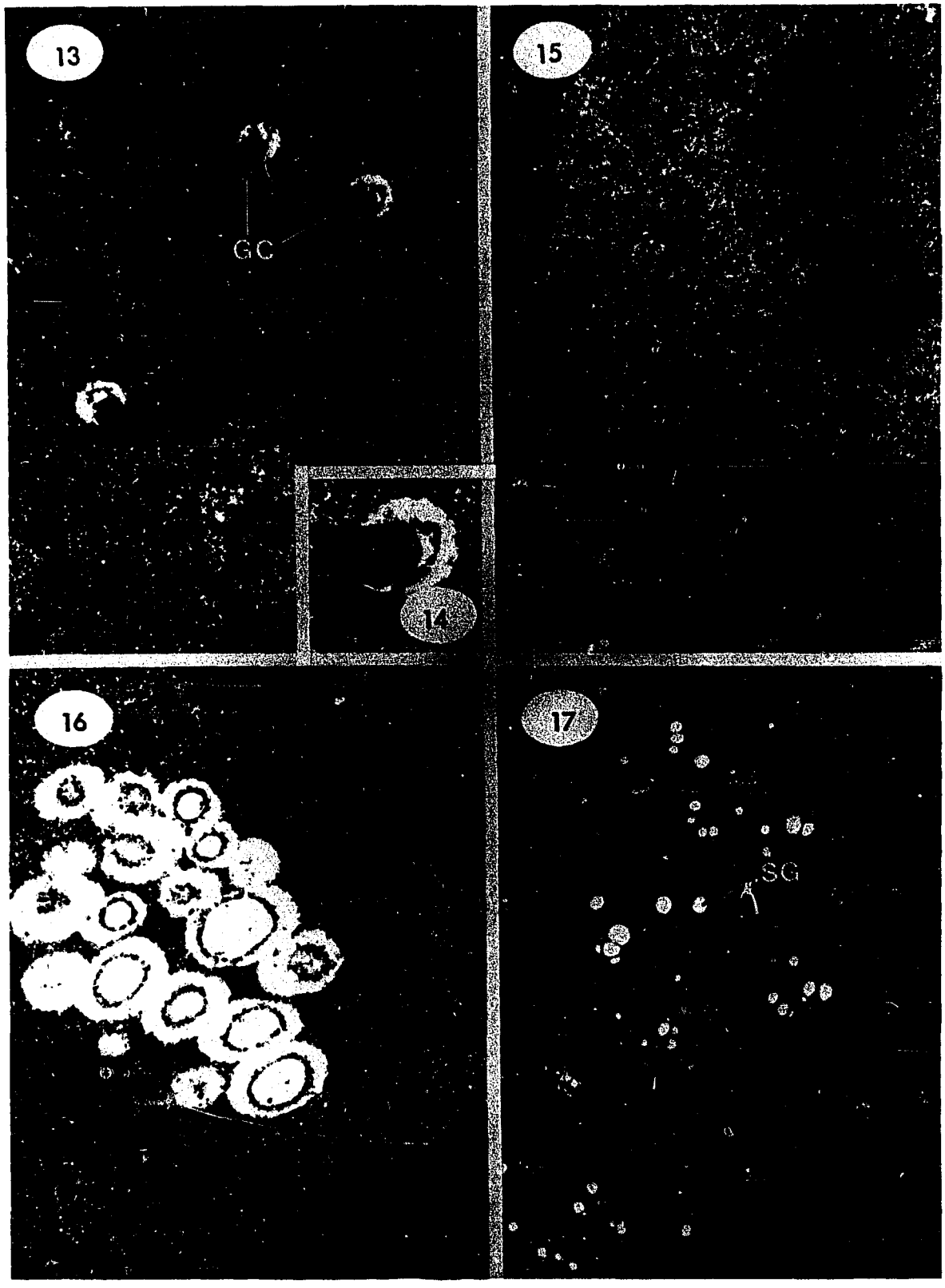
Figs. 13-17. TEM of soybean cotyledons showing globoid containing protein bodies

Fig. 14. Higher magnification of globoid crystal 37,740X

Fig. 15. Lipid containing globoids 11,728X

Fig. 16. Partially extracted globoid crystals 23,000X

Fig. 17. Soft globoid inclusions 11,750X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and that phytin accounts for over 50% of the total phosphorus of the seeds (Smith and Rackis, 1957), there is the possibility that these globoid crystals in soybean protein bodies might be rich in phytin. However, Tombs (1967) writes, "Phytic acid interacts strongly with glycinin and is presumably protein bound in the body, but there is no evidence for its localization, unlike wheat where it appears to be present in specific areas of the protein bodies."

Globoid crystals from thick-sectioned soybean tissue, similar to those shown in Fig. 13, were subjected to energy dispersive X-ray (EDX) analysis. EDX analysis of the electron-dense crystals generally revealed the presence of the elements P, K and Cu (Fig. 18). The copper peak is probably an artifact from the use of copper grids, and thus will be ignored. In the sectioned material, the highest peak was phosphorus, with a smaller one corresponding to potassium. EDX analysis of sections of electron-translucent globoids (soft globoids) did not reveal the presence of any detectable elements. Since elements differ in the number of X-rays they will produce when under the electron beam of a microscope, one must not use peak heights as a strict measure of the concentration of different elements in a sample.

Bils and Howell (1963), Saio and Watanabe (1966) and Tombs (1967) studied protein bodies in soybean seed tissue by TEM, but never recognized the presence of globoid inclusions. Although the structural preservation they obtained is not good by the standards of today, many of their electron micrographs contain protein bodies having small electron-dense and electron-translucent regions. The protein body in

Fig. 18. Energy dispersive X-ray (EDX) analysis of an electron-dense globoid crystal in a soybean protein body

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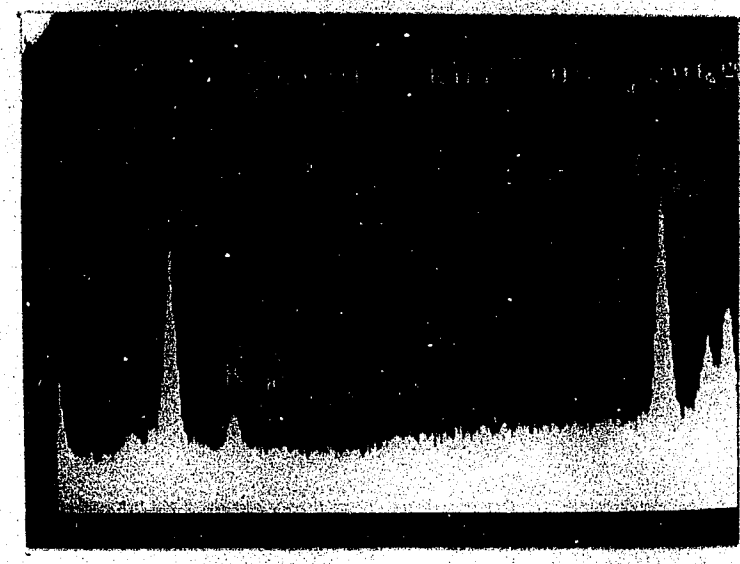


Fig. 1n of Tombs's paper has numerous electron-translucent areas which may represent actual inclusions.

Recently, Lott and Buttrose (1978) reported that soybean protein bodies contained globoid inclusions. They found that EDX analysis of globoids in thick-sectioned and freeze-fractured protein bodies revealed the presence of the elements P, K and Mg, with the highest reading for phosphorus, similar to the results obtained in this study for EDX analysis of thick-sectioned globoid crystals. Lott and Buttrose concluded that globoid crystals in soybean protein bodies were rich in phytin and that this may be the predominant storage form of phosphorus in the soybean seed.

Some of the soybean globoids appear to be lipid. Fig. 15 shows two globoid inclusions in a protein body which appear similar in electron-density to the lipid bodies in the surrounding cytoplasm. Jacobsen et al. (1971), demonstrated that protein, phosphate (phytin) and lipid were present in the globoid of protein bodies from the aleurone layer of barley. However, Lott and Buttrose (1978) found no evidence of lipid containing globoids in their preparations of soybean tissue.

The globoids shown in Fig. 16 contain electron-dense rings. These structures may result from partial extraction of the globoid crystals during the fixation of the tissue. Lolas and Markakis (1975), in a study of phytin in bean proteins, reported that phytin is almost completely water soluble, therefore extraction of globoid crystals is possible early in the fixation process.

From the evidence presented in this study coupled with the results of Lott and Buttrose (1978), I am convinced that soybean protein bodies do contain globoid inclusions. The globoids not only vary in size and distribution, but also in electron-density. The electron-dense globoid crystals appear to be high in phosphorous. Based upon reports that over 50% of the soybean phosphorus is in the form of phytin and the widespread occurrence of phytin containing electron-dense crystals in protein body globoids from other tissues, I strongly suggest that the globoid crystals in soybean protein bodies are storage forms of phosphorous.

Starch Granules

Mature soybean cotyledons were microscopically observed to contain small starch granules measuring from 0.5 μm to 3.0 μm in length (Fig. 21). In sections, the cell walls and numerous spherical particles stain intensely when treated with the PAS procedure (Fig. 19). The cytoplasm appears as a faintly stained mass whereas no other cell components react with the stain. Alpha-amylase extraction and subsequent PAS staining of the tissue reveals densely stained cell wall; however, the small spherical particles (presumably starch granules) no longer appear within the tissue (Fig. 20).

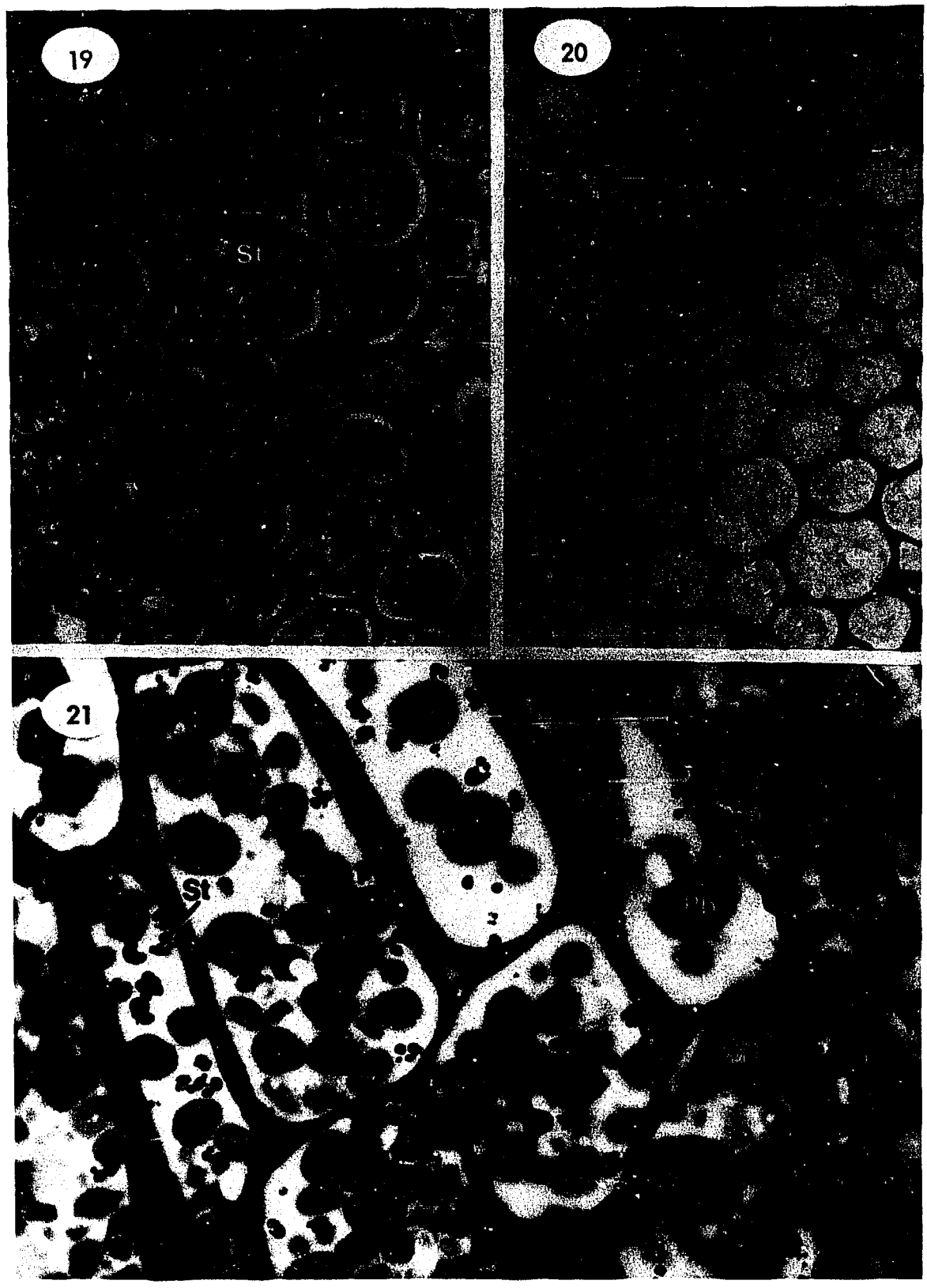
Treatment of cotyledonary tissue with iodine-potassium iodide readily stains small clusters of starch granules dark blue (Fig. 21). Cell walls and protein bodies are stained due to subsequent staining with toludine blue O. Most starch granules appear to be grouped in clusters of two or more granules, although some

Figs. 19-21. Paraffin sections of soybean cotyledons

Fig. 19. Cross section treated with PAS procedure. Dark staining bodies are starch 950X

Fig. 20. Cross section following alpha-amylase treatment and subsequent PAS staining. Note absence of dark staining bodies 950X

Fig. 21. Longitudinal section stained with iodine-potassium iodide poststained with toludine blue O 2,240X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do occur singly within the tissue.

Starch is a birefringent compound which is able to rotate the plane of polaried light and is observed when soybean cotyledonary tissues are examined under polarized optics. The typical birefringence pattern (centric polarization crosses) is shown for soybean starch in Fig. 23 (compare Figs. 22 and 23).

The distribution of starch granules in the Amsoy 71 variety is restricted towards the midline of the cotyledons, similar to that reported by Wilson et al. (1978). It is suggested that if microscopic examinations are confined to sections near the periphery of the cotyledon, then the failure to find starch is understandable. Possibly this occurred when MacMasters et al. (1941) were unable to identify starch in soybean cotyledons by microscopic examination. However, varietal differences in starch content have been reported (Piper and Morse, 1923; Wilson et al., 1978).

Soybean starch exists as compound granules in double-membrane bound amyloplast (Figs. 24 and 25). The granules in Fig. 24 appear to be spherical to irregular in shape, whereas the starch granules shown in Fig. 25 are elongated with one surface being concave. Scanning electron microscopy of isolated starch from Amsoy 71 (Wilson et al., 1978) revealed many starch granules which were round to ovoid, while some were observed to have one concave surface.

The results of this study indicate that mature soybeans contain starch, contrary to that reported by other researchers (Bils and Howell, 1963; Blondell,

Figs. 22-23. Paraffin section of soybean cotyledon

Fig. 22. Viewed under phase contrast 1,136X

Fig. 23. Viewed with polarized optics. Shows typical birefringence pattern (centric polarized crosses) of soybean starch 1,136X

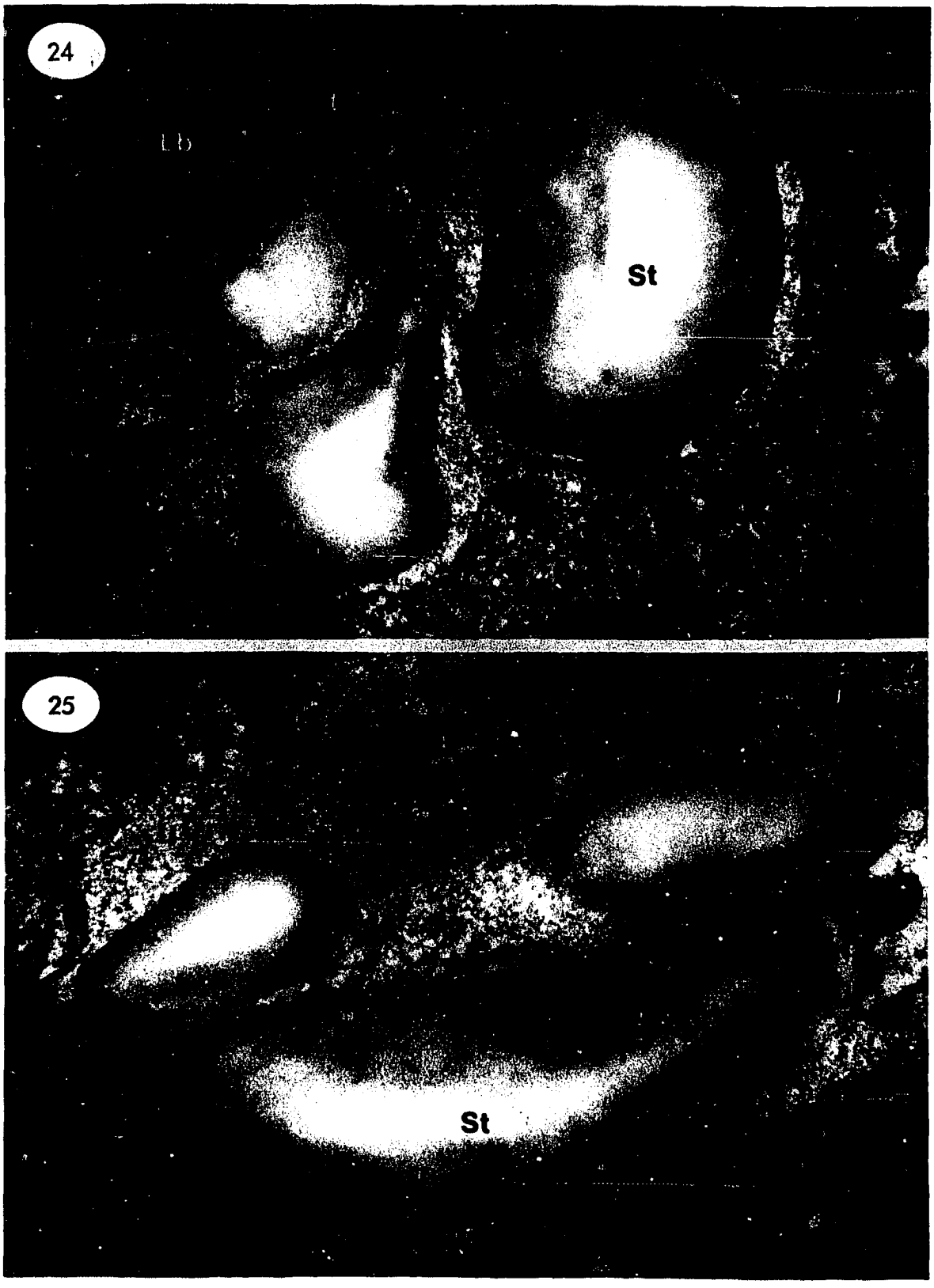

22


23

Figs. 24-25. TEM of soybean cotyledons containing starch granules

Fig. 24. Amyloplast containing three starch granules 33,488X

Fig. 25. Elongated starch granules 51,856X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1888; MacMasters et al., 1941; Meissl and Böcker, 1883; Prinsen, 1896; Tada and Kawamura, 1963; Wallis, 1913).

Calcium Oxalate Crystals

When sectioned soybean cotyledons are viewed under polarized optics, numerous birefringent particles appear (Figs. 26 and 27). Some of the particles are considerably larger than soybean starch granules and do not possess the characteristic centric polarized cross pattern typical of starch. These polarizing particles have been described by Wallis (1913) as crystals of calcium oxalate; however, their distribution, shape and polarizing characteristics have never been photographed.

Distribution of the crystals is limited to the soybean cotyledons; crystals do not appear in the hypocotyl tissue of the seed (compare Figs. 26 and 27). These highly refringent crystals can be viewed in sectioned soybean seeds excised from any region within the cotyledons.

Wallis (1913) described the calcium oxalate crystals as small prismatic crystals arranged in pairs end to end, measuring 24.5 μm long and 4.0 to 5.0 μm wide. However, most of the crystals I found were only 8.0 to 10.0 μm long and 2.0 to 3.0 μm wide, considerably smaller than those described by Wallis. Many of the crystals do appear to be paired end to end (Figs. 30 and 34), similar to those found by Wallis, but other shapes and crystal arrangements have been observed in my preparations (Figs. 29, 31-33). A single prismatic

Figs. 26-27. Paraffin section of soybean cotyledon and hypocotyl tissue

Fig. 26. Phase contrast of tissue stained with toludine blue O 105X

Fig. 27. Same tissue as it appears with polarized optics. Note absence of crystals in hypocotyl region 105X

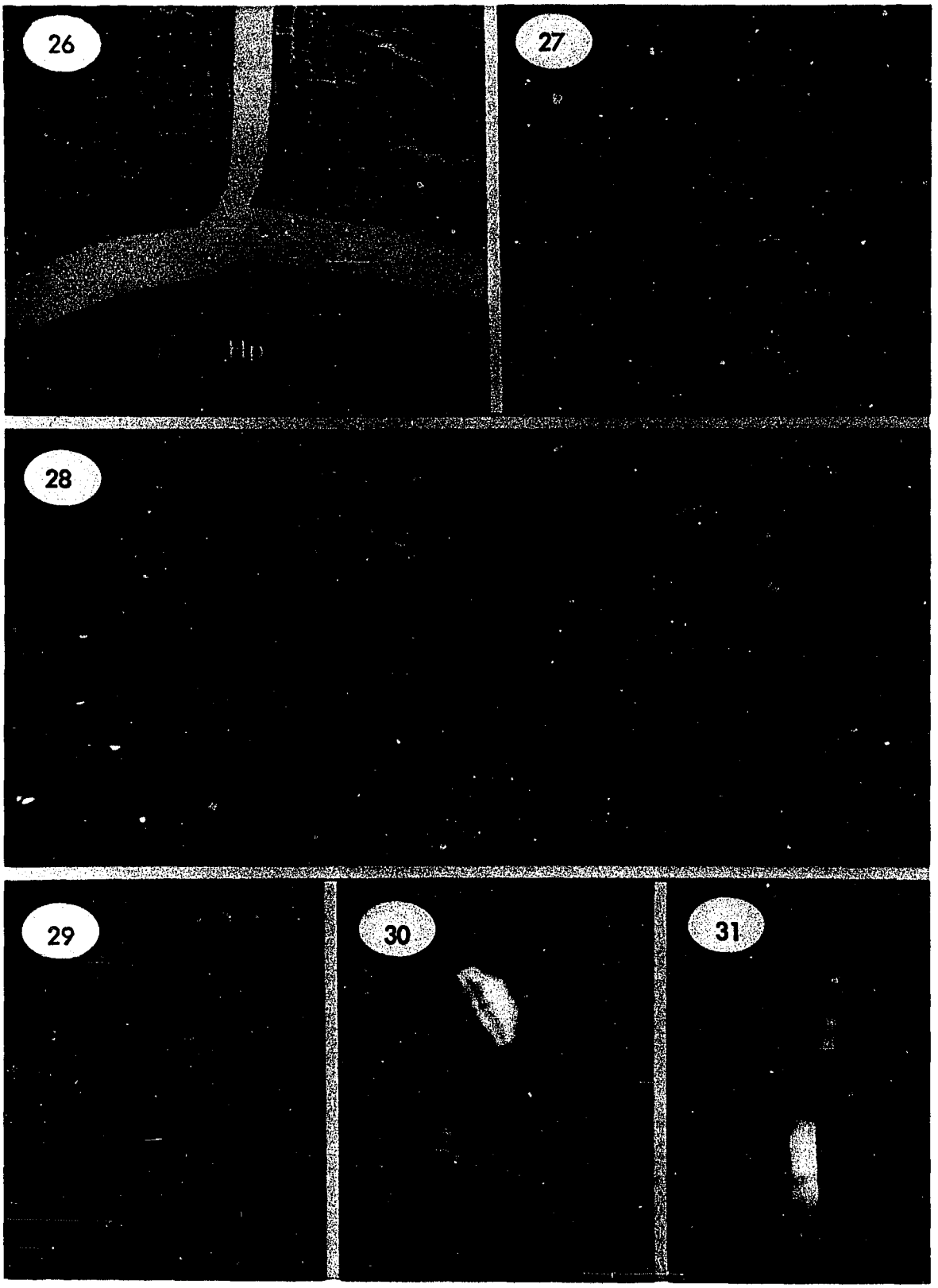
Figs. 28-31. Paraffin sections of soybean cotyledons showing distribution and shapes of calcium oxalate crystals viewed with polarized optics, stained with toludine blue O

Fig. 28. Cotyledon tip showing crystal distribution 150X

Fig. 29. Prismatic calcium oxalate crystal 1,720X

Fig. 30. Double crystals 1,720X

Fig. 31. Cylindrical crystals 1,720X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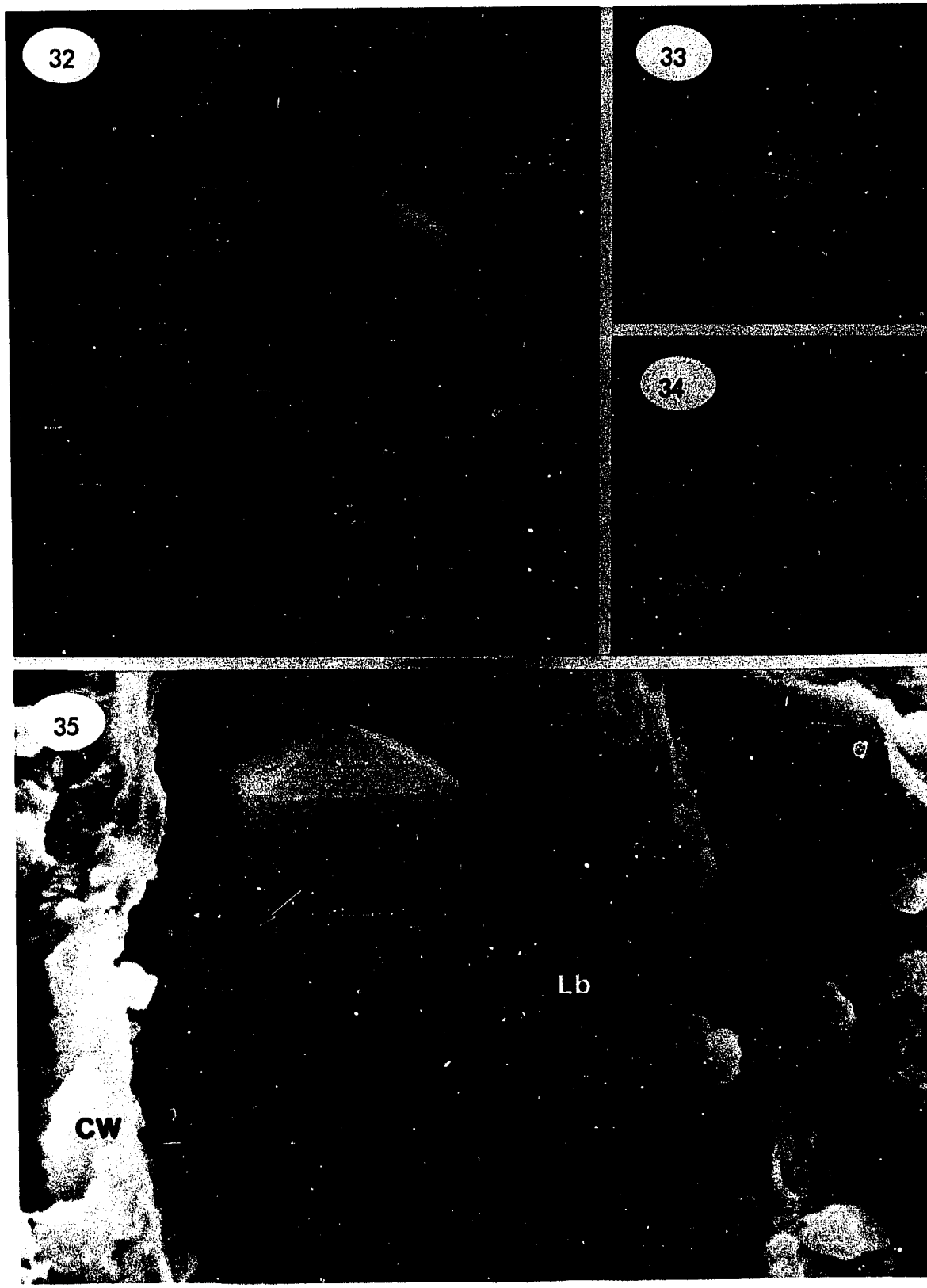
Figs. 32-34. Paraffin sections of soybean cotyledons viewed with polarized optics showing shapes of calcium oxalate crystals

Fig. 32. Star-shaped and cylindrical-shaped crystals 1,100X

Fig. 33. Higher magnification of star-shaped crystal 2,720X

Fig. 34. Double crystals of calcium oxalate crystals 2,720X

Fig. 35. SEM of freeze fractured soybean cotyledon showing a prismatic calcium oxalate crystal. Arrow indicates bump on crystal surface 12,000X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crystal similar to that shown in Fig. 29 was found in a freeze-fractured preparation of cotyledonary tissue (Fig. 35). The pyramid-shaped ends are typical of the prismatic crystals and are clearly displayed in preparations of crystals isolated from unfixed soybean tissues (Figs. 36 and 37). The small pyramid-shaped crystal in Fig. 37 may represent the fractured end of one of the larger crystals. Fig. 32 and 33 show crystals which appear star or cross-shaped possibly created by sectioning horizontally through the tip of a prismatic crystal, however, druse-shaped (star-shaped) crystals have been reported as anatomical features of a number of oil seeds (Vaughan, 1970).

The scanning electron micrographs reveal that the surface of the crystals are textured (Figs. 35-37). The crystal in Fig. 37 appears to have a rough or porous surface while some crystals have small surface bumps (Fig. 35, arrow).

EDX analysis of the isolated crystals in Figs. 36 and 37, reveals the presence of the element calcium; however, only preliminary studies on their elemental composition have been achieved. Cartter and Hopper (1942) reported that calcium in 10 varieties of soybeans ranged from 0.163% to 0.470% with a mean of 0.275%.

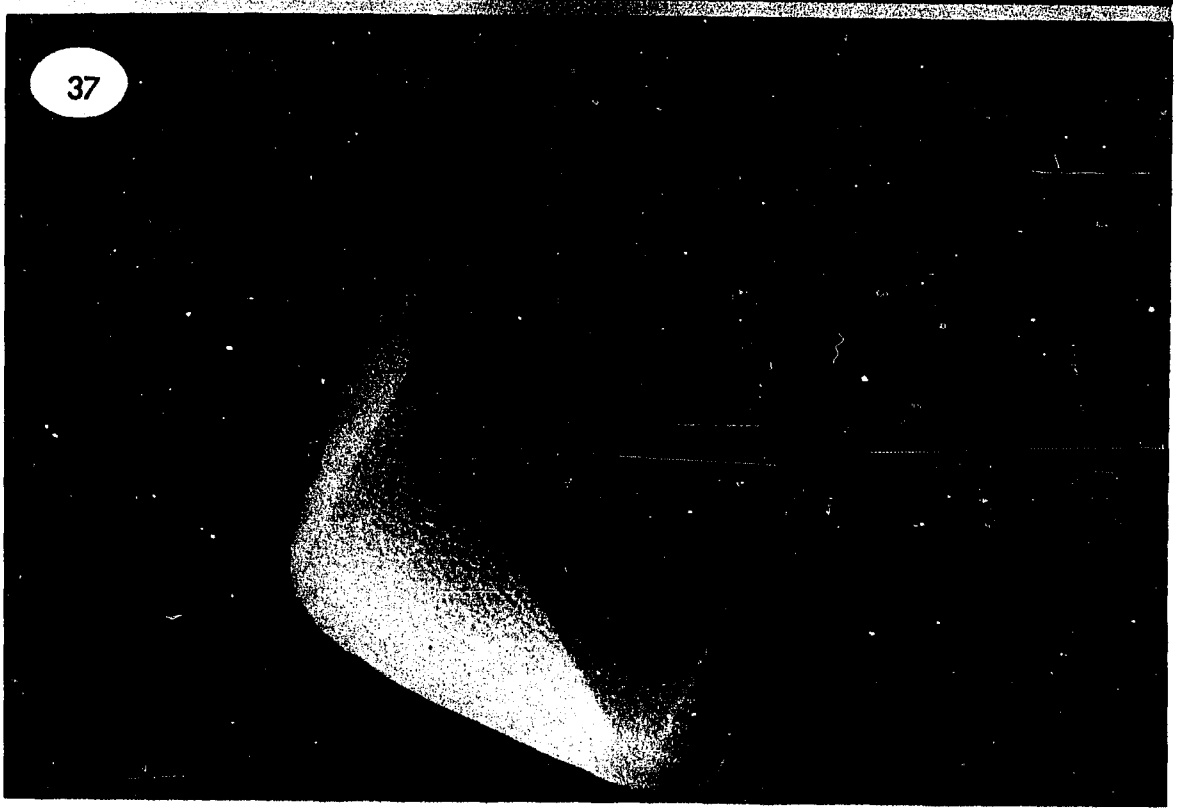
Although concern has been expressed for ingestion of foods high in oxalates (rhubarb, spinach and beets) and for their effect on calcium absorption, no epidemiological cases of oxalate toxicity have been reported from consuming soybeans. However, since there are no reports of the oxalate content in soybeans,

Figs. 36-37. SEM of calcium oxalate crystals isolated from unfixed soybean cotyledons

Fig. 36. Three calcium oxalate crystals 6,480X

Fig. 37. Possibly a fractured end of a prismatic calcium oxalate crystal. Note textured surface 7,200X


36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I feel that more research is needed to establish its amount and its nutritional significance.

Soybean Lipid Bodies

Appearance and distribution

Light microscopy Upon breaking the cotyledonary cells of a hydrated soybean seed, a fluid mass flows freely from the interior of the cells to the surrounding medium. When observed under the light microscope the fluid mass appears to be composed of particulate matter consisting of very small spherical bodies. However, the bodies are sometimes difficult to distinguish as separate entities since their size approaches the limit of the microscopes resolution power.

The minute particles are characteristically prone to Brownian motion and are highly refractile when observed through phase contrast optics. They stain with various known fat dyes, such as, Sudan black, Sudan III, Sudan IV, Nile blue and oil red O. Some nonspecific staining occurs after treatment of the material with aniline blue black, a dye used to localize proteins; however, it appears to be faint and concentrated in the amorphous material surrounding the refractile bodies.

Similar particles have been described in a variety of tissues by other researchers (Sorokin, 1967; Sorokin and Sorokin, 1966; Yatsu et al., 1971), and have been referred to variously as lipid bodies, oil droplets, oleosomes or

spherosomes. On the basis of their appearance and staining characteristics I prefer to use the term lipid body.

Some cotyledon cells containing lipid bodies are shown in Fig. 38. It must be noted, however, that the appearance of these cells are atypical of most cotyledon sections, since very few protein bodies can be seen. Nevertheless, the lipid body mass shows up nicely within the cotyledon cells.

The use of a light microscope to study soybean lipid bodies is limiting since their size approaches the resolution power of the microscope. However, the use of electron microscopy, both TEM and SEM provides the magnification needed to adequately study their characteristics.

Electron microscopy Fig. 39 is an electron micrograph of soybean cotyledon cells showing lightly-stained, irregularly-shaped protein bodies and numerous electron-dense lipid bodies. Lipid bodies appear extremely electron-dense primarily due to the reactivity of osmium tetroxide with unsaturated fatty acids. Soybean lipid is reported to contain 88% unsaturated fatty acids on a dry weight basis (Daubert, 1950).

Lipid bodies are spherical and considerably smaller than protein bodies. They range in size from 0.1 to 1.0 μm in diameter; however, some as large as 2.0 μm have been observed.

Most cotyledon cells appear to be filled with protein and lipid and show few other recognizable cellular organelles (Figs. 39 and 40). In some cells,

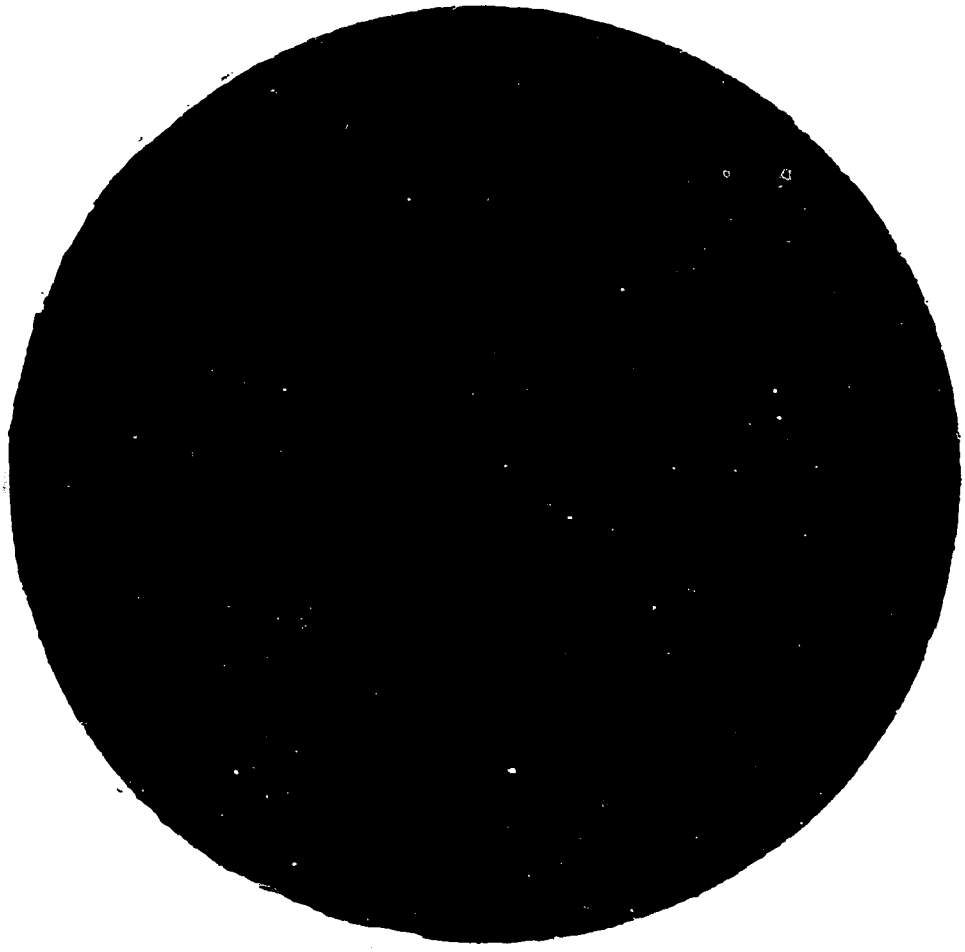


Fig. 38. Plastic section of soybean cotyledon stained with toludine blue O. Cells are atypical of soybean cotyledons, however, show the particulate lipid body mass and few protein bodies. 1,800X

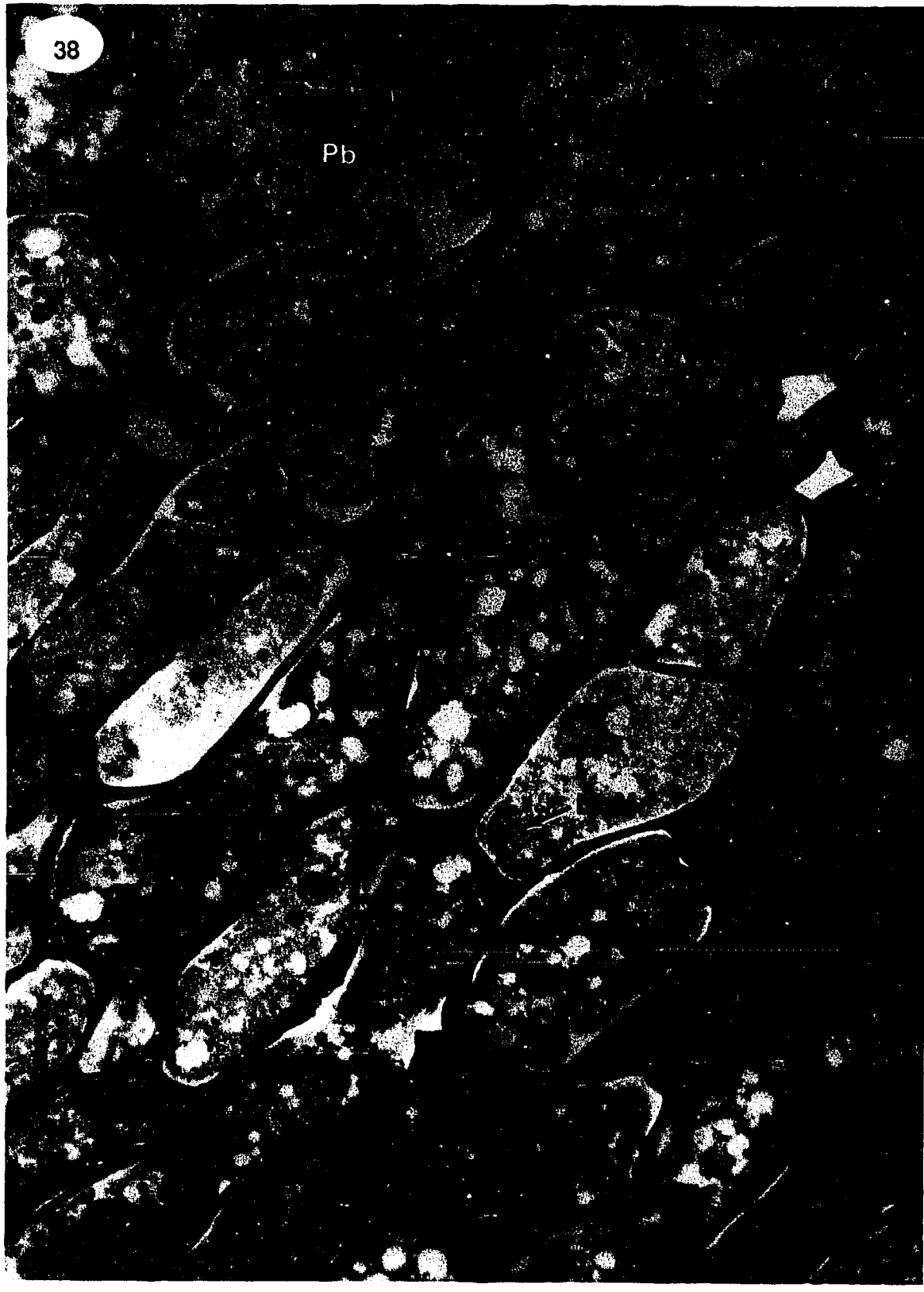
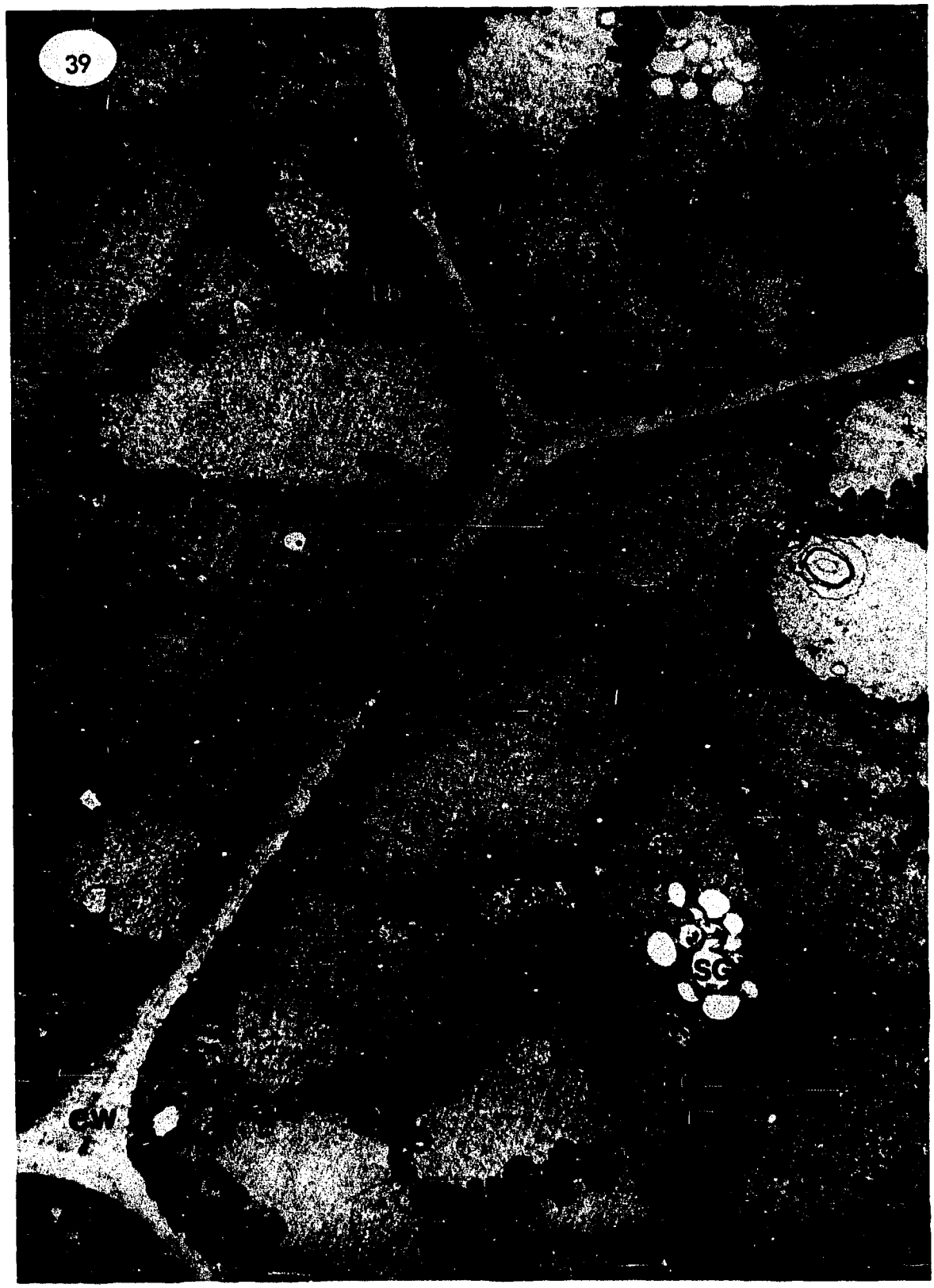

38
Pb

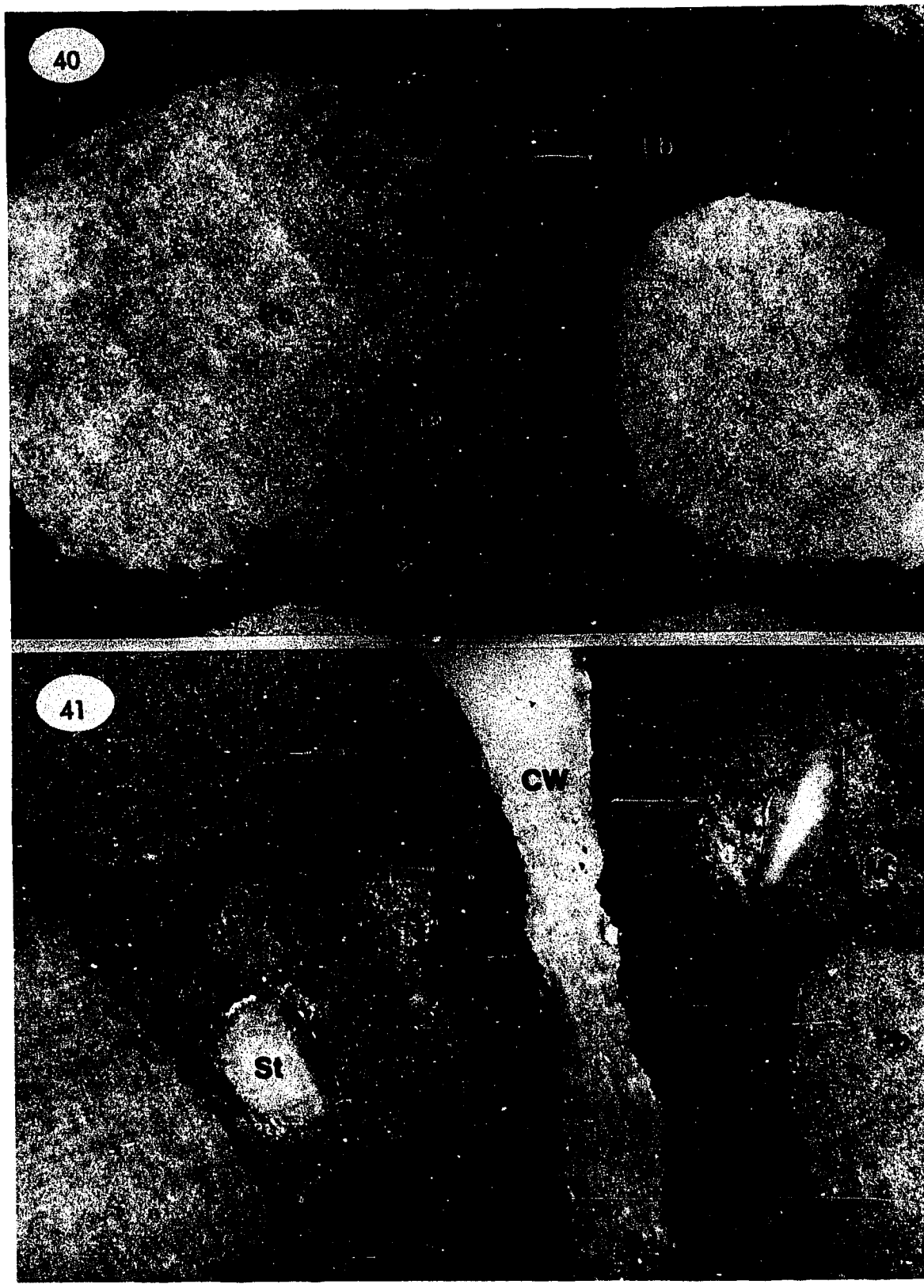
Fig. 39. TEM of sectioned soybean cotyledon, showing appearance and distribution of lipid bodies and protein bodies 5,985X


39
SG
CW

Figs. 40-41. TEM of soybean cotyledons showing distribution of lipid bodies

Fig. 40. Cell filled with protein bodies and lipid bodies 20,750X

Fig. 41. Cells having less concentration of lipid bodies 14,553X


40
41
CW
St

however, where lipid is less concentrated, other cellular constituents can be observed (Figs. 41-45). Fig. 41 shows portions of two adjacent cells where lipid bodies are less concentrated, revealing two double-membrane bound amyloplasts with their characteristic starch granules and some small mitochondria scattered throughout the cytoplasm. In cotyledon cells where the lipid is more dispersed, some lipid bodies appear to adhere or align themselves along various cellular structures. In Fig. 41, lipid bodies preferentially line-up along the surface of protein bodies and cell wall; however, they do not appear around the amyloplast. Similarly, Webster and Leopold (1977) found that soybean lipid bodies were distributed typically in a single layer around protein bodies and at the margins of the cytoplasm. Furthermore, Tombs (1967) reported that lipid attached itself tenaciously to protein in homogenates of whole soybeans, thus suggesting a strong lipid body-protein interaction.

In addition to the close association of lipid bodies with protein bodies and cell walls, I often observed lipid bodies surrounding various other cellular organelles and structures (Figs. 42-45). Some unidentified light-colored, spherical structures in Fig. 43 also have adhering lipid bodies. Other unidentified structures shown in Fig. 44 also demonstrate an arrangement of lipid bodies at their surface; however, mitochondria and nuclei do not appear to have any associated lipid bodies. Rough endoplasmic reticulum is another subcellular constituent which typically show an associated concentration of

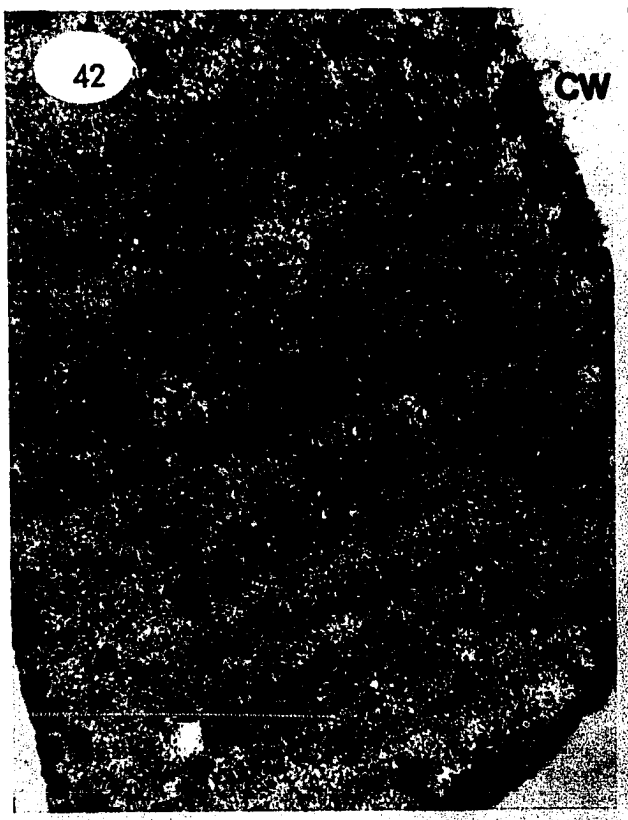
Figs. 42-45. TEM of soybean cotyledons showing distribution and association of lipid bodies with various cellular components

Fig. 42. Association with cell wall 17,600X

Fig. 43. Association with cell wall and unidentified cellular bodies 12,504X

Fig. 44. Association of lipid bodies with rough endoplasmic reticulum and unidentified cellular bodies 14,560X

Fig. 45. Association with rough endoplasmic reticulum 27,200X


42
CW


43


44


45

lipid bodies. For comparison, lipid bodies were examined in sectioned soybean hypocotyl tissue (Fig. 46). Again, lipid bodies were found to be associated with certain cellular components. Unidentified structures similar in appearance to those observed in cotyledonary tissue were also prevalent throughout the tissue.

The cause of the affinity or association of lipid bodies with certain cellular structures is presently unclear; however, it might suggest an interrelationship between the two components. It is interesting to point out that lipid bodies have been observed adhering or aligning protein bodies, cell wall, rough endoplasmic reticulum and various unidentified structures which are characteristically bound by unit-membranes. However, there appears to be little lipid body association with amyloplasts, mitochondria and cell nuclei; organelles which are double-membrane-bound.

As already mentioned, the distribution of lipid bodies appears to be less concentrated in some cotyledon cells than in others. Distinct differences in their distribution, however, were not recognized when cotyledon material excised from inner, outer and middle portions of soybeans were microscopically examined. Corresponding sections were analyzed for total lipid (skelly B extraction) with no significant difference in their lipid content.

Cells in close proximity to vascular tissue (i.e. mid-vein, lateral-vein and vascular bundles), appear to contain less lipid and protein reserves. This was generally observed by the decreased intensity of staining (with various dyes) in

Fig. 46. TEM of sectioned soybean hypocotyl showing lipid body distribution and association with various cellular components 14,715X


46

cells bordering the vascular strands than in those away from them. Suryanarayana (1976) reported similar observations in soybean cotyledons during germination. He suggested that this dissolution of reserve materials near vascular strands may be partly due to the enzyme stimulating effect of hormones that may have moved into these cells from the phloem. Also, the dissolution may be due to the sustained enzyme activity in these cells resulting from the continued removal of end products of degradation by the phloem transport mechanism.

Isolation and chemical characterization of lipid bodies

Studies of soybean lipid bodies are limited when research is based solely upon observations made in intact tissue. Most studies of subcellular components involve an isolation step that is designed to separate the desired components from the tissue to facilitate further characterization. Methodology for lipid body or spherosome isolation from intact tissue involves: homogenizing the tissue of interest in an isolation medium (usually a sucrose or saline solution) followed by sequential centrifugation of the homogenate to produce a floating film or fat pad which appears as a creamy layer concentrated at the surface of the supernatant.

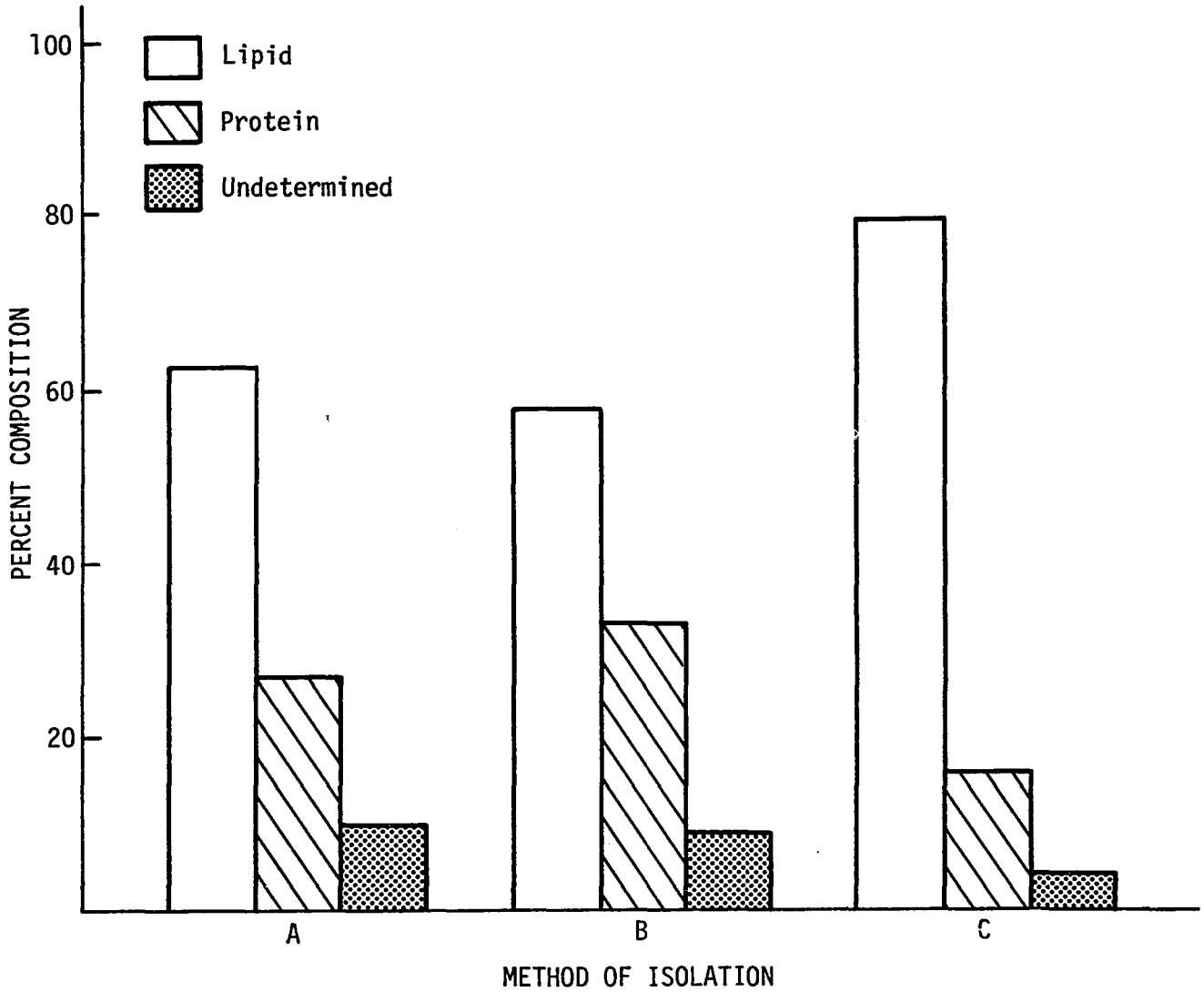
Isolation of lipid bodies Fat pad yield (on a dry weight basis) varied among the three methods used for lipid body isolation from soybeans. Method A (isolated in 0.25 M sucrose) yielded a fat pad weighing 6.3 gm; method B (isolated in 0.5 M sucrose) yielded 7.2 gm while method C (isolated in 0.5 M saline) resulted with a fat pad weighing 5.0 gm. Intact soybean cotyledons

contain about 23% lipid, and considering that the starting weight of cotyledons for each isolation was 100 gm, the resultant fat pads only accounted for about 25% of the total cotyledon lipid. A considerable amount of lipid either is retained in the tissue following homogenization due to insufficient cellular disruption and/or is lost in the pellet or supernatant fractions during subsequent centrifugations.

Chemical composition The effects of the three methods on the lipid and protein composition of the soybean fat pad are shown in Fig. 47. Method C, using the 0.5 M saline solution, resulted in the highest percentage of lipid, 81% as compared with method A and B having only 63% and 58% respectively. All preparations contained a considerable amount of protein which ranged from a low of 15% for method C, up to 32% for method B. The fraction designated as undetermined may be mostly carbohydrate; however, oligosaccharides should have been removed during dialysis of the fat pad.

The amount of protein I found associated with the lipid in the fat pad layer of soybean cotyledons appears to be considerably higher than that found in fat pads isolated from other tissues. Jacks et al. (1967) reported that isolated spherosomes from peanut cotyledons were composed of 98.1% lipid and 1.27% protein by dry weight. Light and heavy spherosomes isolated from aleurone layers of wheat were found to contain 1.8% and 11.7% protein respectively (Jelsema et al., 1977). The source of the variation in lipid to protein ratios

Fig. 47. Effect of lipid body isolation method on percent composition of the floating fat pad (dry weight basis). A represents Jacks et al. (1967) procedure using 0.25 M sucrose. B represents Kahn et al. (1960) procedure using 0.5 M sucrose. C represents Yatsu and Jacks (1972) procedure utilizing a 0.5 M saline solution


100
80
60
40
20
Lipid
Protein
Undetermined
PERCENT COMPOSITION
A
B
C
METHOD OF ISOLATION

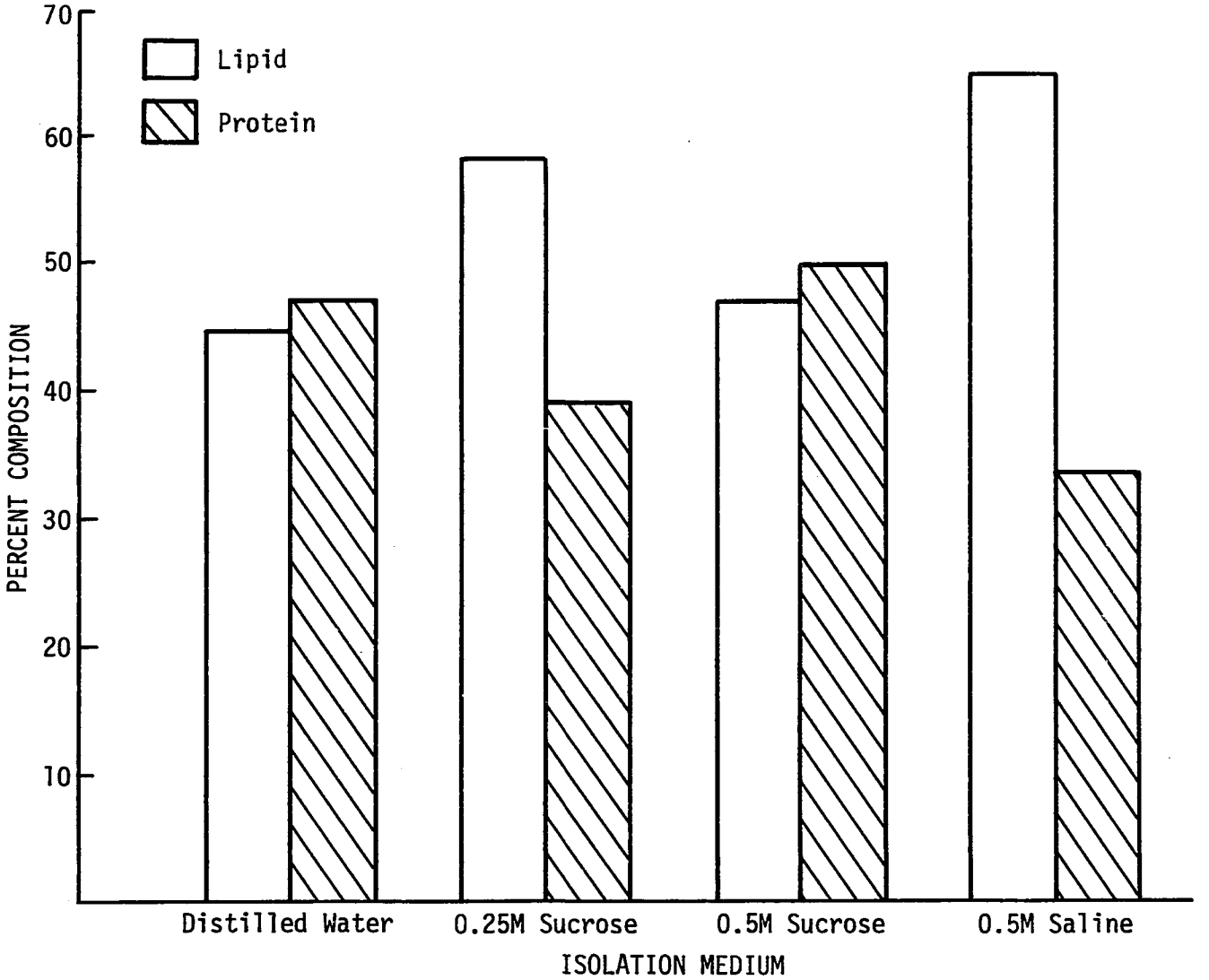
is unclear, but may be due to different types of tissues. However, the variation in lipid and protein content of fat pads obtained from the three methods (A, B and C) show that isolation technique is an important variable.

Effect of isolation medium To study the effect of isolation medium on the lipid and protein composition of the fat pad, method A was performed on 100 gm lots of soybean cotyledons using four different isolation media (Fig. 48). Isolation with 0.5 M saline gave a fat pad containing 63% lipid while the fat pad from the distilled water isolation had only 44%. Fat pad isolations using 0.5 M sucrose and distilled water resulted in higher percentages of protein than lipid. The decrease in the protein content of the A isolate shown in Fig. 47 compared to that in Fig. 48 (0.25 M sucrose) may be partially accounted for by the absence of an undetermined fraction (Fig. 48) due to sufficient dialysis of the fat pads. I might also mention that lipid and protein percentages vary 3 to 4% for any one procedure when subsequent isolations are performed.

From the results presented in Fig. 48, it appears that the isolation medium used for soybean lipid body isolation influences the chemical composition of the resultant fat pads. However, Mollenhauer and Totten (1971a,b) reported that isolation media were not critical for isolation of lipid vesicles from bush bean and pea cotyledons.

Effect of soybean cotyledon:isolation medium ratio Preliminary research has shown that the ratio of lipid to protein and the amount of floating fat layer can be influenced by the initial soybean cotyledon:isolation medium

Fig. 48. Effect of isolation medium on the percent composition of the floating fat pad utilizing the lipid body isolation method of Jacks et al. (1967)


70
60
50
40
30
20
10
Lipid
Protein
PERCENT COMPOSITION
Distilled Water
0.25M Sucrose
0.5M Sucrose
0.5M Saline
ISOLATION MEDIUM

ratio. By increasing the initial concentration of soybean cotyledons to isolation medium, the percentage of lipid and the yield of the floating fat layer is increased. The isolation procedures for method A, B and C use cotyledon:isolation medium ratios of 1:3 and 1:4, and always result with a sizeable floating fat pad layer. However, when an initial 1:10 (cotyledon: isolation medium) ratio was tried the amount of the floating fat pad was considerably less, having most of its creamy material sedimented. Isolation of lipid bodies using distilled water and the same 1:10 ratio, resulted in no floating layer, but rather all of the creamy substance pelleted to the bottom of the centrifuge tube. More research is needed to clarify the effect and significance of bean to isolation medium ratio.

Effect of centrifugation temperature Since the three methods of isolation varied in centrifugation temperature (Method A, room temperature; Method B, 4° C, Method C, 0° C), two entire sets of isolation preparations were examined at room temperature and 4° C to study the effect of temperature on fat pad yield and lipid composition. No differences were noted between the two temperatures in yield or composition of fat pads within identical preparations.

Effect of heating Soybean cotyledons, boiled in distilled water 15 minutes prior to lipid body isolation, gave floating fat pads which were considerably larger and more yellow in color than those isolated from unheated cotyledons. Lipid content of the dried material often exceeded 90% with the

remaining amount as protein. One might speculate that boiling disrupts the lipid bodies causing coalescence which facilitates the extraction of oil from the cotyledonary tissue.

Microscopic examination of isolated lipid bodies

Isolated lipid bodies from mature soybean cotyledons appear to be relatively sturdy and easily survive homogenization and centrifugation. They exhibit the same structure and size as those of unfractionated tissue (Figs. 49-53). Microscopical examination indicates that the isolation medium is not critical for their isolation. Lipid bodies isolated by the three methods are spheroidal and have an average diameter of 0.5 μm with a range from 0.05 μm up to 1.2 μm. Some larger bodies (3.0 μm to 4.0 μm) have been observed; however, they appear to be aggregates of coalesced lipid bodies.

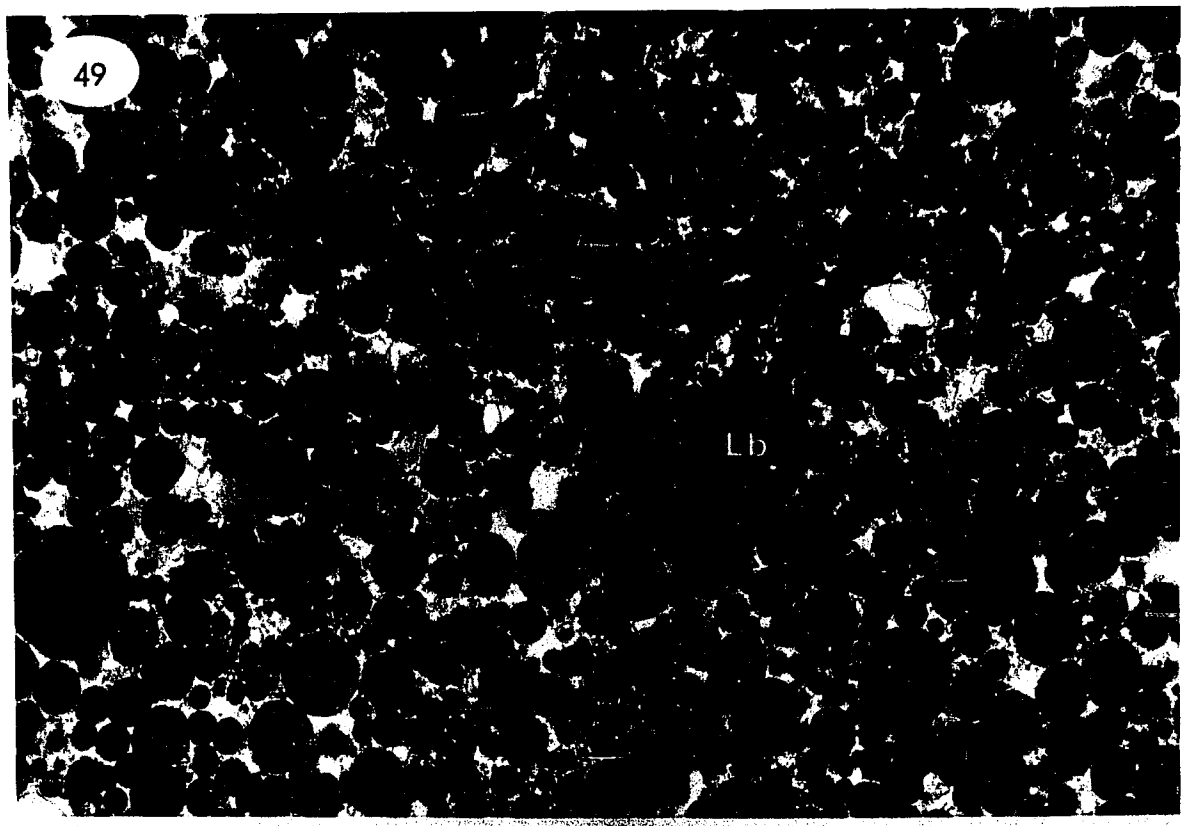
In most preparations, a small amount of contaminating material was found associated with the isolated lipid bodies (Figs. 49-52), but was not morphologically recognizable. However, based upon chemical analysis of the fat pad and its staining characteristics, there is good evidence that this material is proteinaceous. Lipid bodies isolated by method C contained less of this amorphous background material (Fig. 53), and correspondingly had the least amount of protein associated with the fat pad.

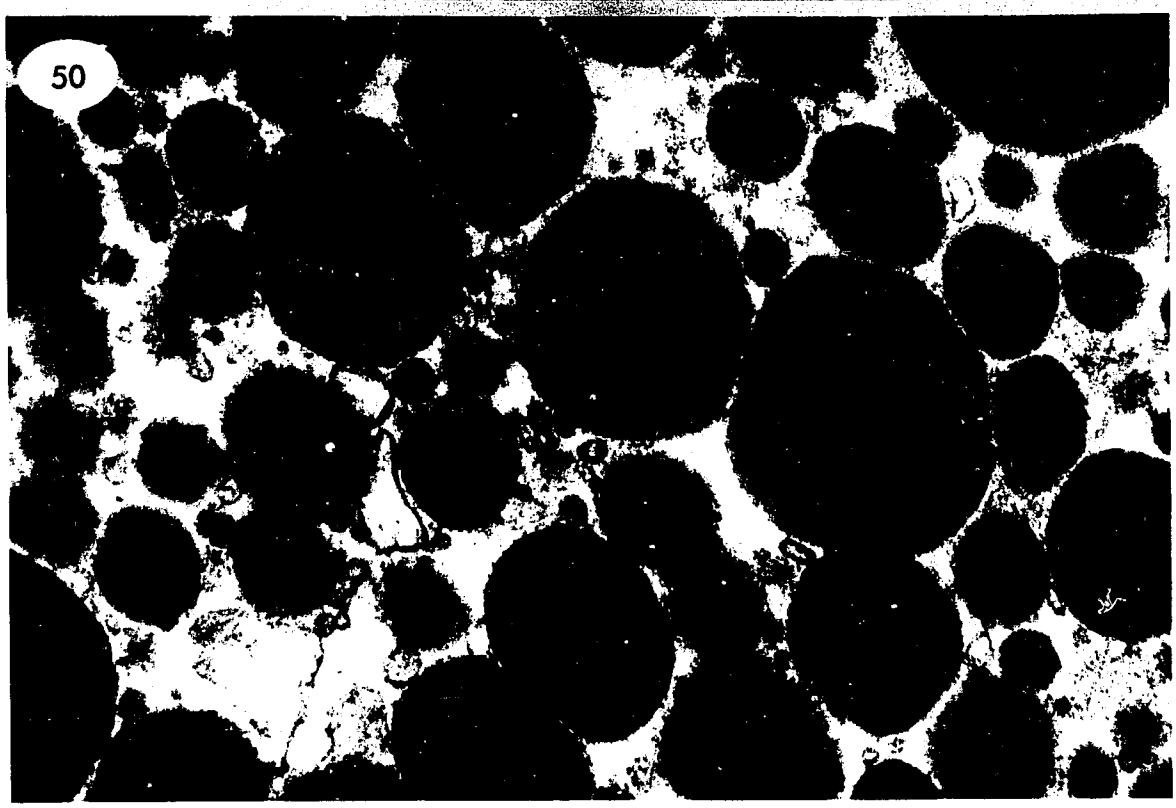
Staining characteristics of isolated lipid bodies are similar to those found

Figs. 49-50. TEM of soybean lipid body isolates, prepared according to the procedure of Jacks et al. (1967)

Fig. 49. Isolated lipid bodies 14,225X

Fig. 50. Isolated lipid bodies 55,622X


49
Lb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Figs. 51-52. TEM of soybean lipid body isolates prepared by the procedure of Kahn et al. (1960)

Fig. 51. Isolated lipid bodies 55,622X

Fig. 52. Isolated lipid bodies 53,708X


51
Lb

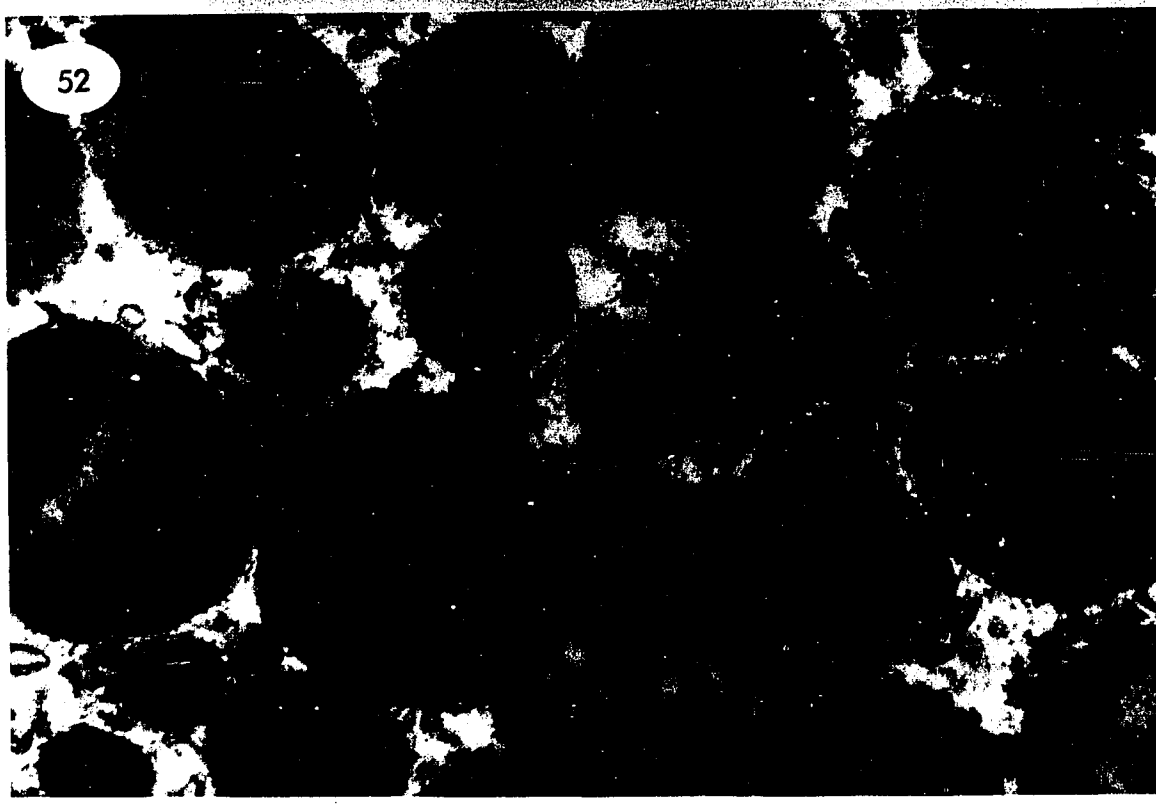
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Fig. 53. TEM of soybean lipid body isolate prepared according to the procedure of Yatsu and Jacks (1972). Flattened or concave surface of lipid body (arrow) 20,535X


53
Lb

in intact soybean cotyledon tissue (compare Figs. 49-50 and Figs. 39-45). Isolations prepared by method A (Figs. 49-50) and method B (Figs. 51-52) have lipid bodies which appear relatively electron-dense. Differences in staining characteristics sometimes encountered with different preparations are shown in Figs. 51 and 52. Many of the lipid bodies in Fig. 51 appear more electron-dense at their outer edge and more electron-permeable towards their center. It is possible that this is an artifact created during chemical fixation. Their appearance could arise from insufficient fixation with osmic acid or from partial extraction of the lipid following dehydration. However, Sorokin and Sorokin (1966) observed spherosomes in doubly-fixed (glutaraldehyde-osmium tetroxide) tissue of Campanula persicifolia L. as containing an electron-permeable medullary zone occupying three-fourths of the central region of the spherosome surrounded by a cortex of osmiophilic material. The wave-like or striped pattern shown in Fig. 52 is probably not inherent to the lipid bodies, but is likely caused by complications incurred during thin-sectioning of the specimen block.

Lipid bodies isolated according to method C, have a uniform staining matrix of lower electron density than those prepared by the other two methods. Nevertheless, their size and shape are indicative of those observed in situ, and they contain the highest lipid content.

While most of the lipid bodies appear spherical in shape, occasionally a

body with a flattened or concave surface is found in isolated preparations (Fig. 53, arrow). SEM of a freeze-fractured lipid-agar block (Fig. 56) reveals two lipid bodies with concave depressions on their surface. These depressions probably are caused by two closely appressed lipid bodies, an occurrence often observed in the intact cotyledon tissue. In another freeze-fractured preparation (Fig. 57), an isolated lipid body appears to have a textured surface (Fig. 58). The nature of the rough and irregular surface is unknown.

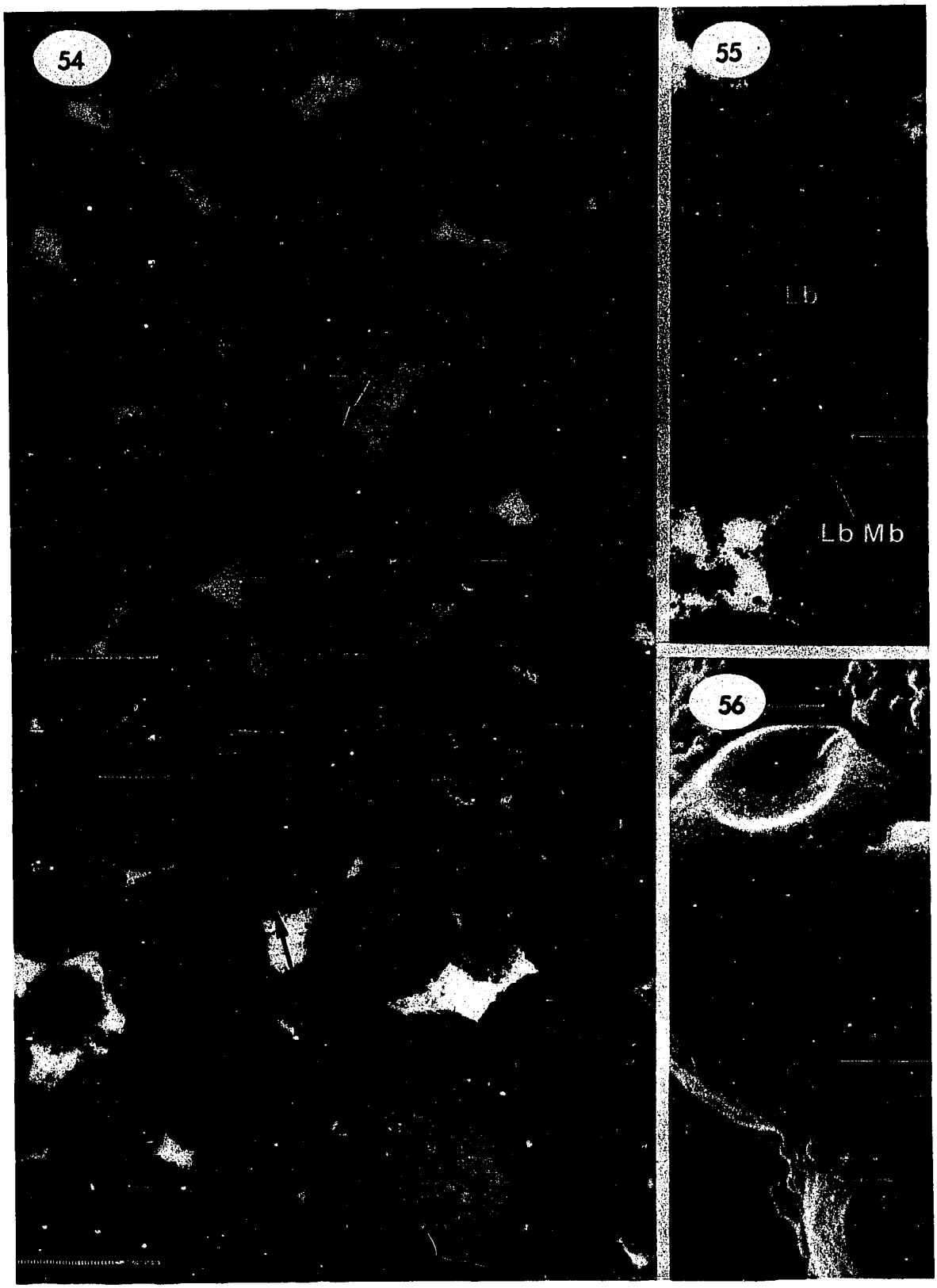
Membrane Soybean lipid bodies appear to be delimited by a membrane; however, there is some question as to whether it is a unit or single-line-membrane. Most soybean lipid bodies appear to be bounded by unusual single-line "membranes" (Figs. 49-52 and 55) which measure 2.5 to 4.0 nm in width, contrasted to the well-known tripartite unit-membranes, which measure 6 to 8.5 nm in overall thickness. Morphologically these membranes are similar to those found by Yatsu and Jacks (1972) for peanut spherosomes which were described as half-unit-membranes whose polar surfaces face the hyaloplasm and whose lipoidal nonpolar surfaces contact internal storage lipid. In some preparations, soybean lipid bodies appear to be delimited by thicker (9.0 nm to 10.0 nm), more electron-dense boundaries (Figs. 53 and 54). Fig. 59 is an electron micrograph of lipid body "ghosts" doubly-fixed following treatment of the lipid body isolate with hexane. They appear as electron-transparent bodies surrounded by electron-dense membranes which measure 9.0 nm to 10.0 nm in width. Lipid bodies possessing the thicker

Figs. 54-55. TEM of soybean lipid body isolates prepared according to the procedure of Yatsu and Jacks (1972)

Fig. 54. Some lipid bodies appear to have halos (indicated by arrow) 48,704X

Fig. 55. Single-line-membrane surrounding lipid bodies 98,000X

Fig. 56. SEM of freeze fractured lipid body isolate prepared by the procedure of Yatsu and Jacks (1972), showing surface depressions of some lipid bodies 28,500X


54
55
Lb
Lb Mb
56

Figs. 57-58. SEM of freeze fractured lipid body isolates prepared by the procedure of Yatsu and Jacks (1972)

Fig. 57. Spherical appearance of lipid bodies 12,760X

Fig. 58. Higher magnification showing surface of isolated lipid body 34,500X

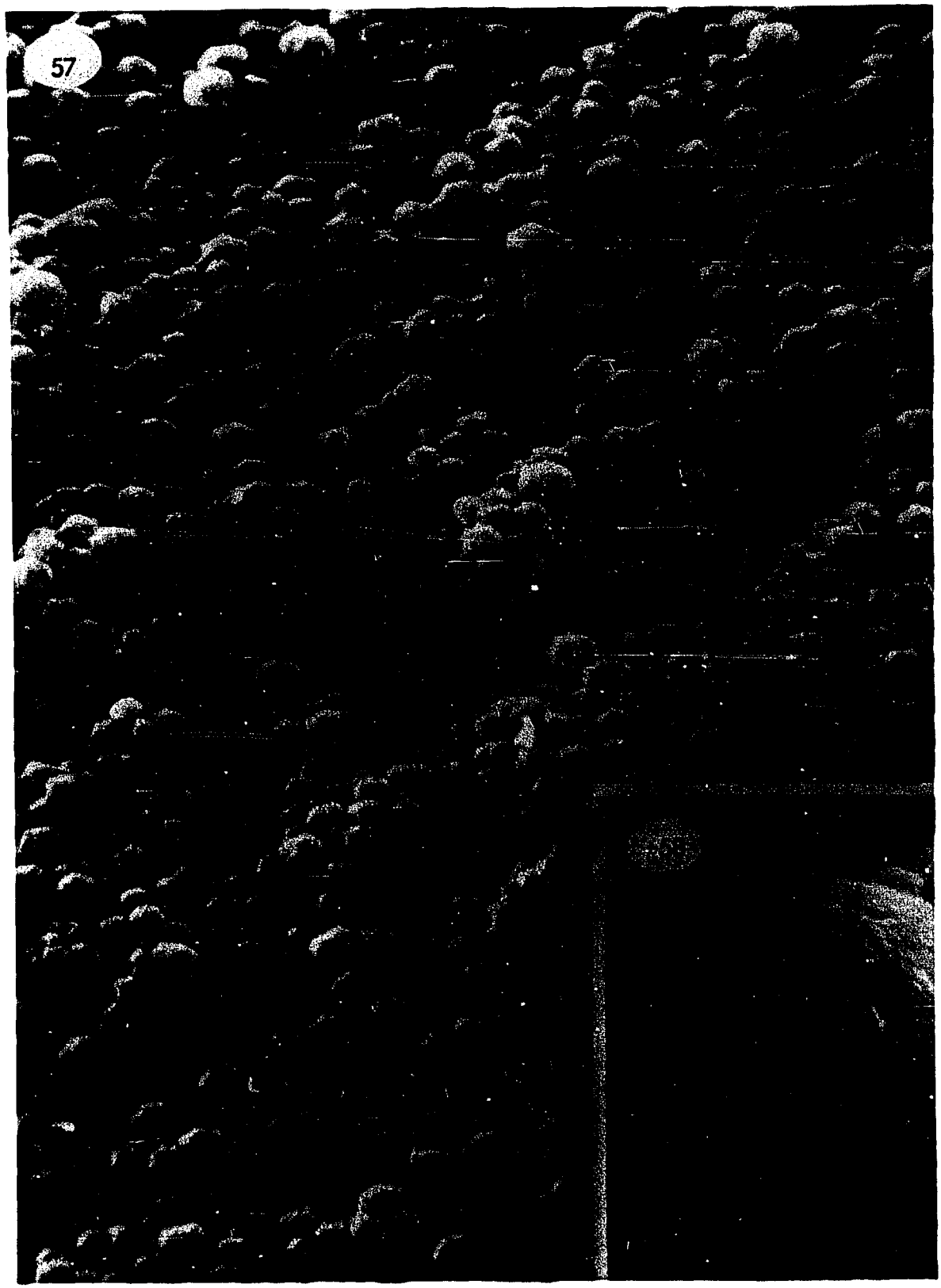

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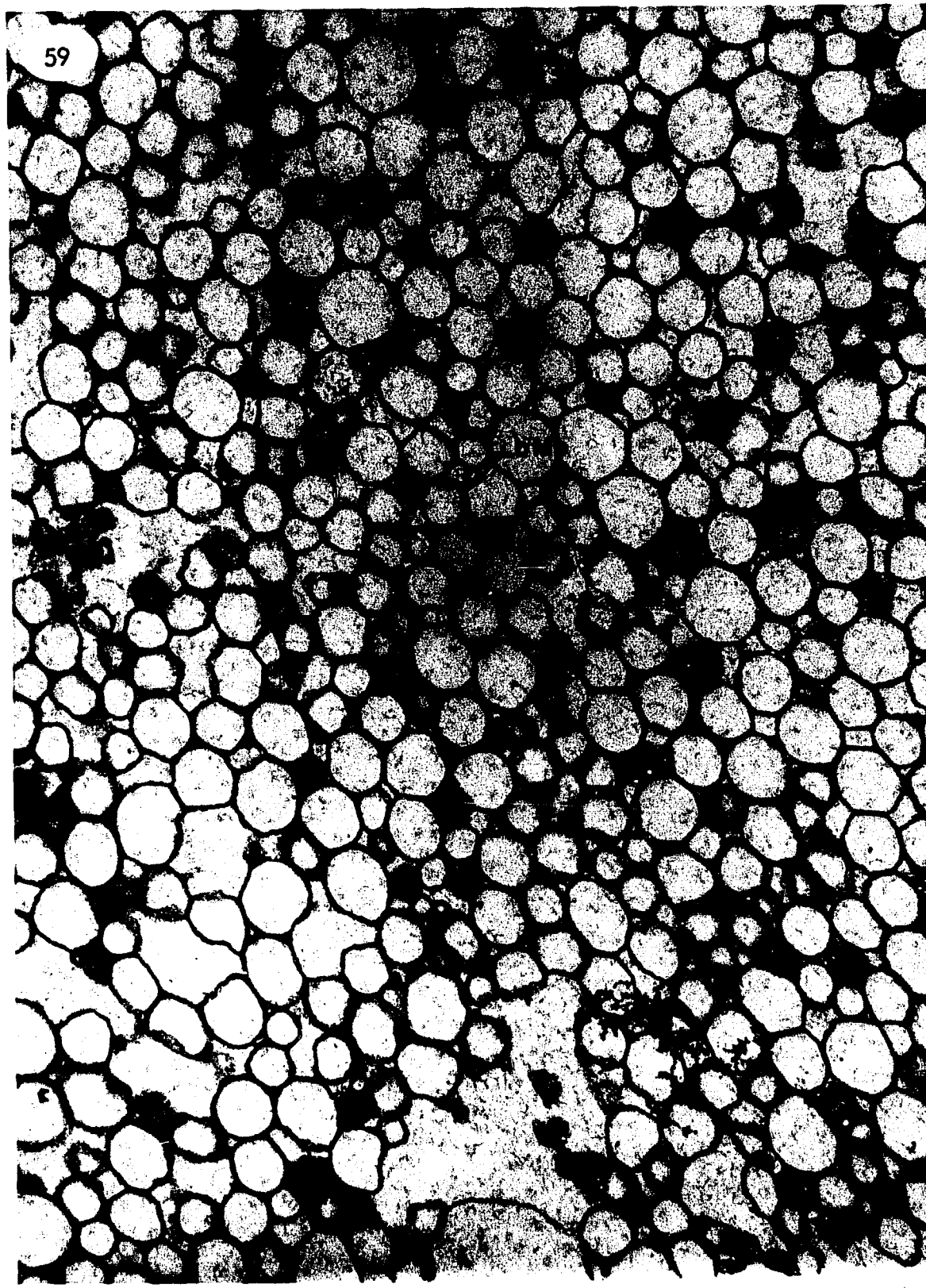
Fig. 59. TEM of a lipid body isolate prepared by the procedure of Yatsu and Jacks (1972), which was extracted with hexane prior to chemical fixation. Shows electron-permeable lipid body "ghosts " 31,620X


59

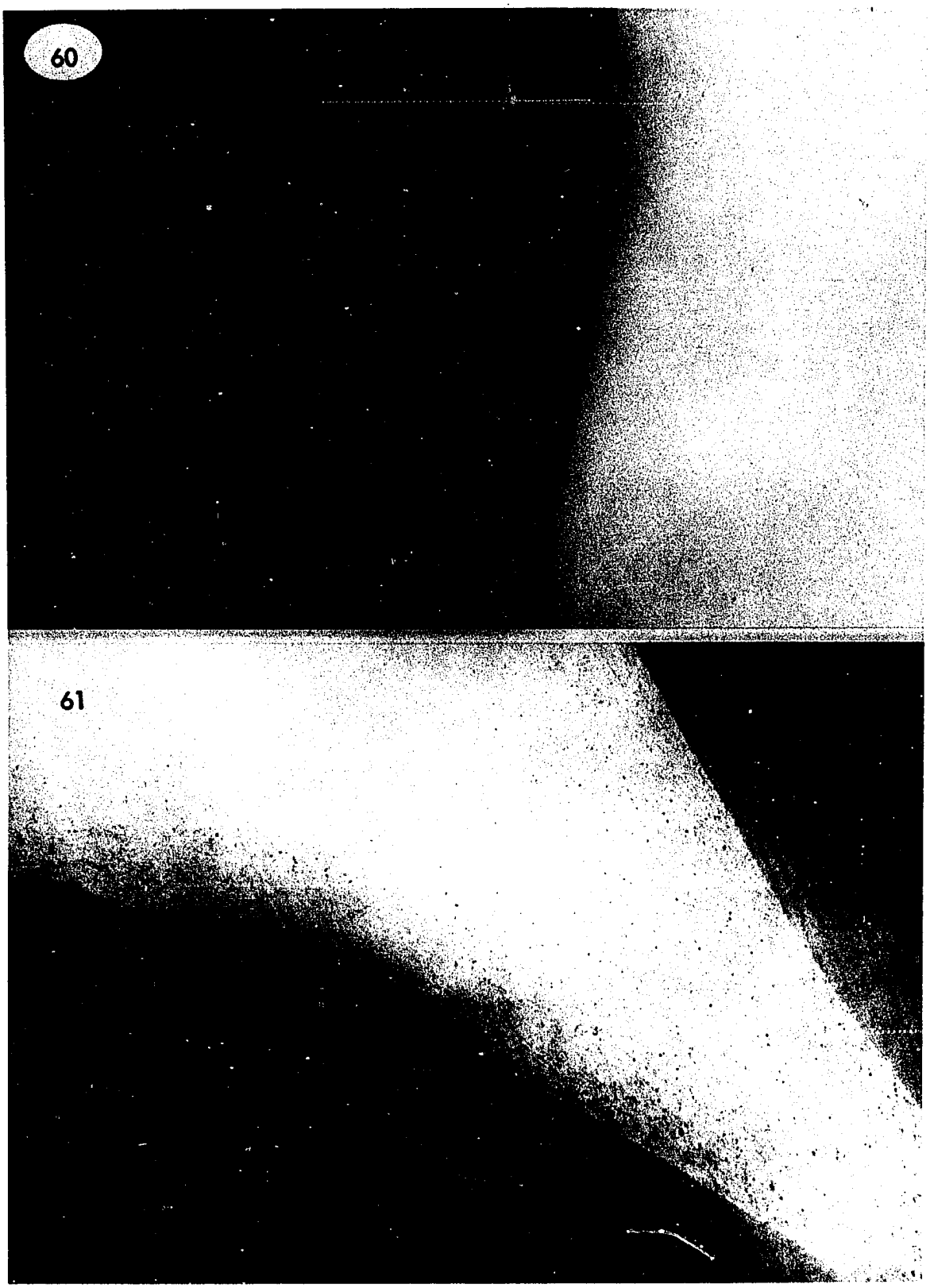
membranes were found in isolates prepared by method C, while those having the thinner single-line-membranes were obtained from methods A and B. However, some of the lipid bodies appear to be surrounded by halos (Fig. 54, indicated by arrows). This may be solubilized protein, due to the disruption of protein bodies, covering or adhering to the surface of the lipid bodies. Furthermore, since a close association of lipid bodies and protein bodies was observed in the intact tissue, this might represent a similar phenomenon. Observations similar to this are found in Figs. 49, 50, 51, 53 and 59.

Commercial vegetable oil (Hy-Vee vegetable oil, specially processed soybean oil) and hexane extracted oil from 20 mesh full-fat soybean flour were embedded in agar and processed identically to that of the lipid body isolates (Figs. 60 and 61). Upon microscopical examination, the oil appeared as large droplets with no visible surface boundaries. Therefore, I suggest that the structure observed around isolated lipid bodies is not merely a phase boundary artificially created during fixation or subsequent processing for microscopic examination. Also, closely appressed lipid bodies, observed in situ and in lipid isolates do not appear to coalesce which further substantiates that a limiting boundary is present surrounding the lipid body.

Effect of trypsin treatment Soybean lipid bodies appear to be susceptible to enzyme attack. Trypsin treatment of lipid body isolates causes disruption of delimiting membranes and allows the bodies to coalesce into large

Fig. 60. TEM of soybean oil, prepared by extracting 20 mesh soybean flour with hexane 10,791X

Fig. 61. TEM of commercial vegetable oil 15,059X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61

irregular-shaped oil masses (Fig. 62). The dark particulate material observed surrounding the lipid mass might be remnants of lipid body membranes. When lipid isolates are hexane extracted prior to trypsin treatment, the lipid bodies appear as elongated saccules containing smaller unidentified spherical objects (Figs. 63 and 64). The effect of trypsin on soybean lipid bodies suggests that the membraneous material surrounding the lipid bodies is proteinaceous.

Examination of centrifugation pellet Chemical analysis of centrifugation pellets obtained during lipid body isolations revealed that a considerable amount of lipid was being sedimented. Since the density of lipid is obviously less than that for water or the isolation media used, it seemed odd that lipid would appear in the pellet. Therefore, a centrifugation pellet from the final step of lipid body isolation prepared by method C, was processed for TEM. Microscopical examination revealed numerous electron-dense particles, recognized as intact lipid bodies, embedded in a gray mass consisting of various subcellular organelles, membranes and amorphous material (Figs. 65-67). Lipid bodies appear smaller (0.05 μm to 0.1 μm in diameter) in the pellet fraction than those normally found in the floating fat pad layer. Possibly the smaller lipid bodies, having a greater surface area than larger particles, are dense enough when surrounded by solubilized protein to allow their sedimentation during centrifugation.

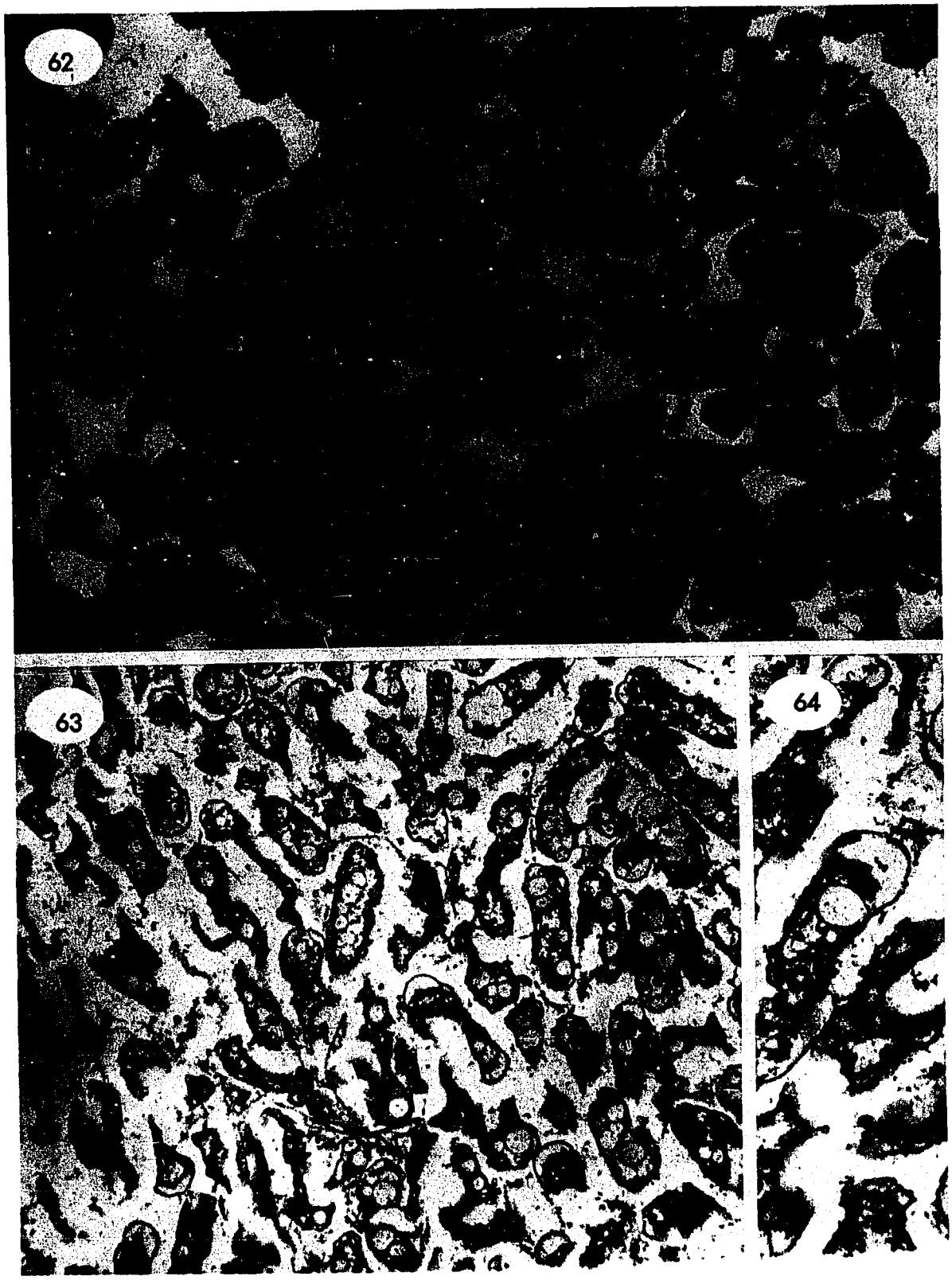
In some electron micrographs, lipid bodies often appear to be closely associated with or encircling vacuolated areas (Fig. 66, arrow). These areas

Figs. 62-64. TEM of lipid body isolates prepared by the procedure of Yatsu and Jacks (1972), and treated with trypsin

Fig. 62. Treated with trypsin 13,992X

Fig. 63. Hexane extraction followed by trypsin treatment 17,193X

Fig. 64. Higher magnification of Fig. 63 35,784X

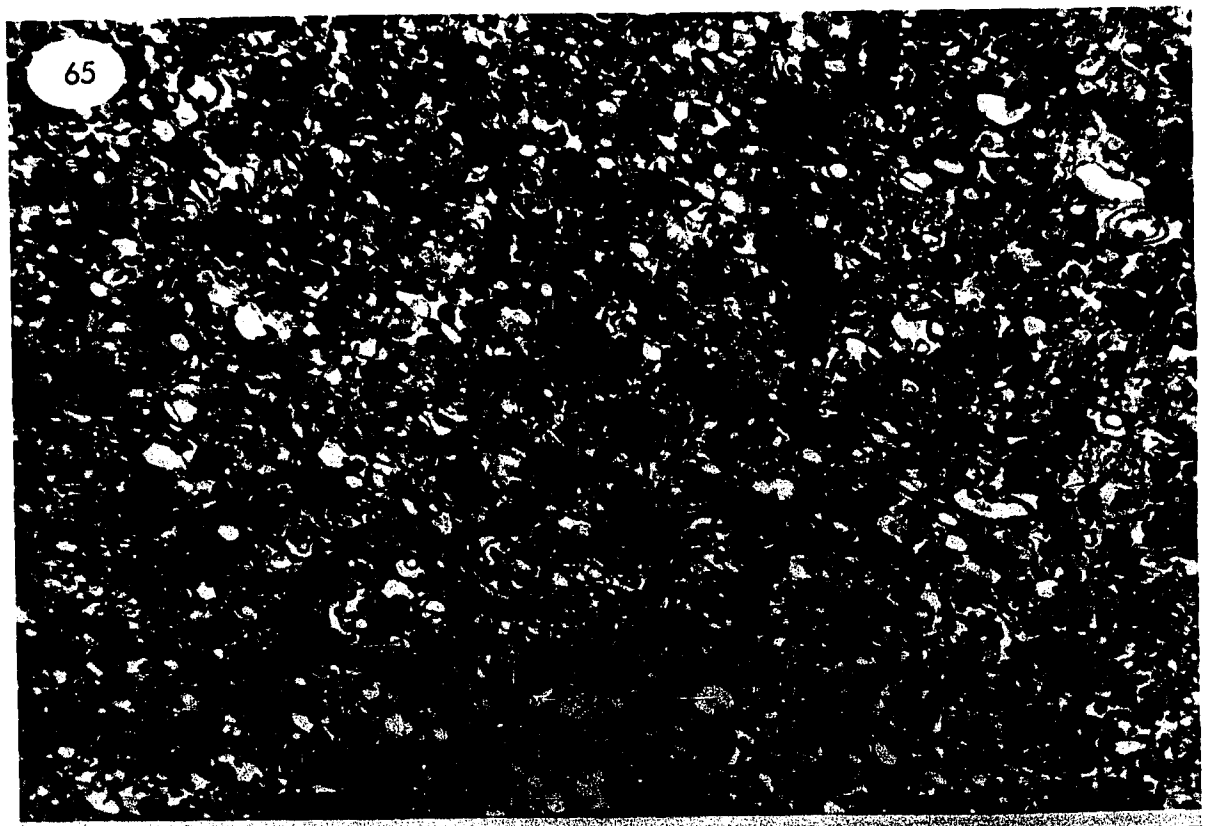

62
63
64

Figs. 65-67. TEM of the final centrifugation pellet obtained from a lipid body isolation prepared by the method of Yatsu and Jacks (1972)

Fig. 65. Lipid bodies interspersed in proteinaceous substance 12,397X

Fig. 66. Higher magnification showing some lipid body association with vacuolated areas 18,500X

Fig. 67. Higher magnification of Fig. 66 22,250X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66


67

might represent remnants of protein bodies, indicating a strong association of lipid bodies to the surface of protein bodies, as that observed in intact cotyledonary tissue. However, in most cases lipid body distribution was nonspecific.

Problems associated with microscopical preparation of isolated lipid bodies

Problems with fixation and embedding of the lipid-agar blocks were frequently encountered during early stages of the research project. Insufficient polymerization of the lipid-agar blocks resulted when a mixture of araldite-epon resin was used for specimen embedding. Following incubation, the resin appeared rigid and possessed good trimming and cutting qualities; however, the embedded lipid-agar block remained pliable and did not facilitate sectioning. The high lipid nature of the isolate may have prevented infiltration of the viscous araldite-epon resin. Spurr (1969) described a low viscosity resin that adequately infiltrated and polymerized the specimen block and possessed good sectioning qualities.

An attempt to fix the lipid isolate in permanganate fixative for comparative studies, resulted in poorly fixed material inadequate for microscopical examination.

Density gradient centrifugation of lipid bodies

When lipid body isolates were centrifuged on linear sucrose gradients, an evenly distributed pattern was observed throughout the gradients. After centrifugation on discontinuous sucrose gradients, lipid bodies collected at different interfaces of the gradient as well as in a floating lipid layer. On the

basis of their position in the discontinuous gradient and their dispersed appearance in the linear gradient, it appears that the floating fat pad (lipid bodies) is made-up of a broad range of particles having different densities.

Germination and Developmental Studies of Soybean Cotyledons

Germination

Fig. 68 shows the fresh weight of soybean cotyledons from day 0 to day 17 after germination. The cotyledons elongated three-fold and reached a maximum fresh weight of 750 mg about a week after germination. During the early stages of seedling growth the cotyledons appear green and carry out some photosynthesis in addition to supplying stored minerals and reserve food to the seedling until the leaves and roots are well-established. The cotyledons then lose a considerable amount of water, so that they have fresh weights of 425 to 400 mg at 17 days postgermination. Ultimately, the cotyledons turn yellow and fall from the plant.

Soybean cotyledons were morphologically and chemically examined during the 17 day period. In day "0" (ungerminated cotyledons (Figs. 69 and 70), the palisade cells appear to be filled with spherical to irregular-shaped protein bodies, with the remaining space occupied by numerous lipid bodies. The small electron-translucent area found in Fig. 69 appears to be the site of a calcium oxalate crystal. Also a few soft globoids are present within one of the protein bodies shown in Fig. 70.

By day 2, the protein bodies are the first of the reserve food substances to

Fig. 68. Fresh weights of soybean cotyledons from 0 to 17 days after germination

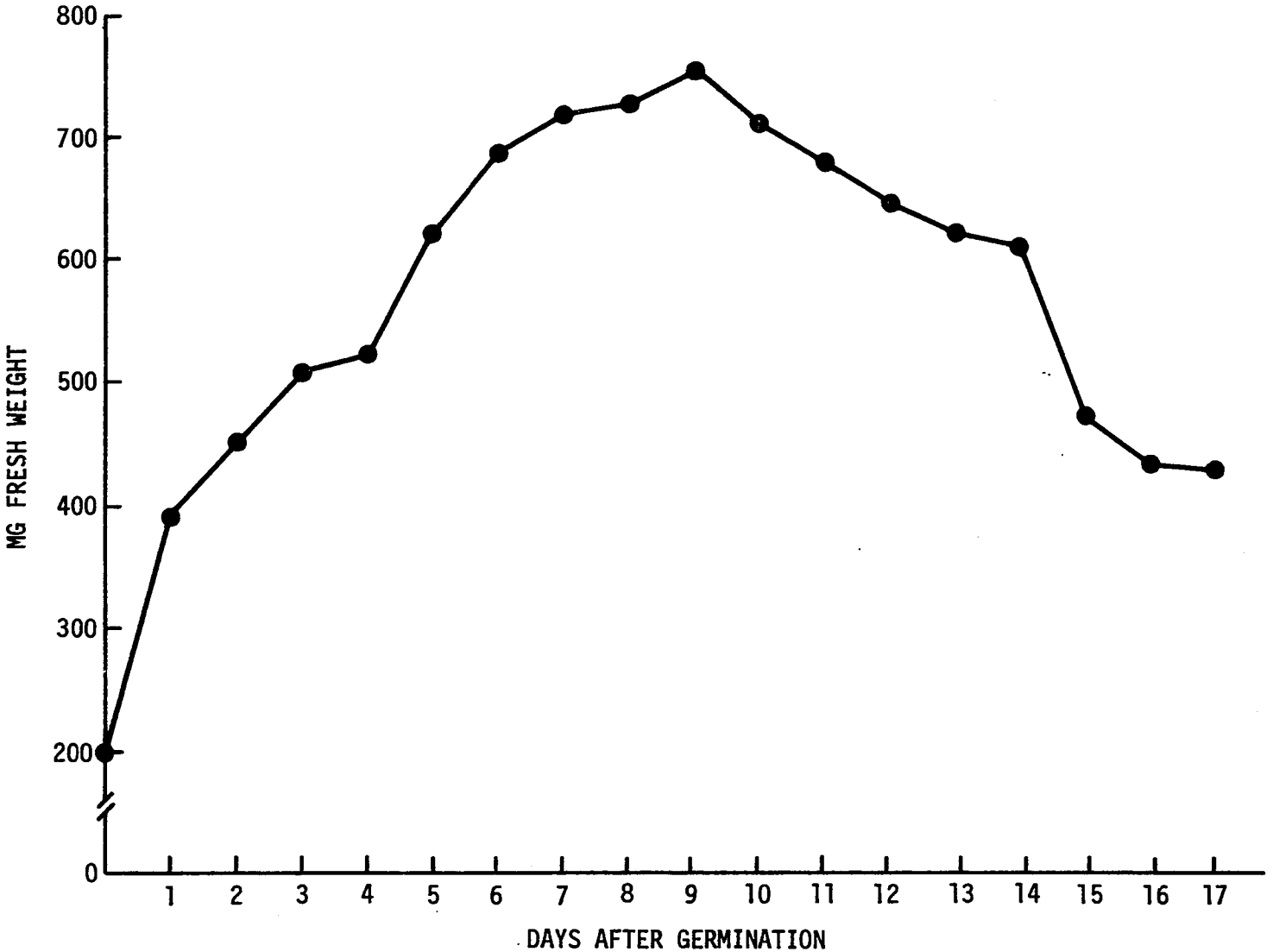
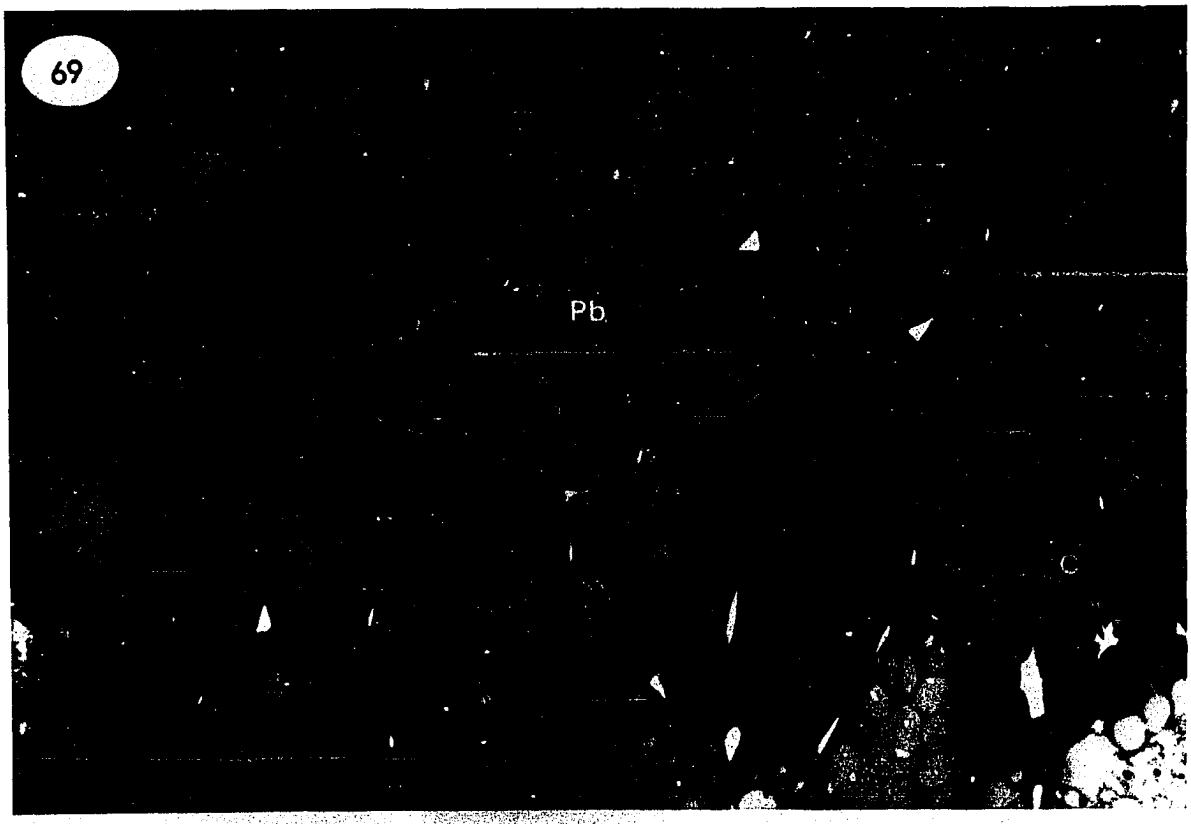

800
700
600
500
400
300
200
0
MG FRESH WEIGHT
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
DAYS AFTER GERMINATION

Fig. 69. Plastic section of mature (day 0) soybean cotyledon stained with toludine blue O. Acalcium oxalate crystal is shown. 1,275X

Fig. 70. TEM of mature (day 0) soybean cotyledon. 5,704X


69
Pb
C


70
Lb
CW

exhibit change. Most of the protein bodies become granular in appearance and more irregular in shape and begin to coalesce to form larger protein masses (Figs. 71-75). Tombs (1967) and Suryanarayana (1976) reported similar observations in germinating soybean cotyledons. Such a breakdown pathway is strikingly similar to that shown for peanut protein bodies (Bagley et al., 1963) and for barley (Paleg and Hyde, 1964). Protein bodies apparently lose their limiting membranes upon coalescence (compare Figs. 73 and 74 with Fig. 75). Little change is seen in the protein content of soybean cotyledons during this period (Fig. 76); however, there is a surprising gradual increase in cotyledonary protein over the next 6 days of germination.

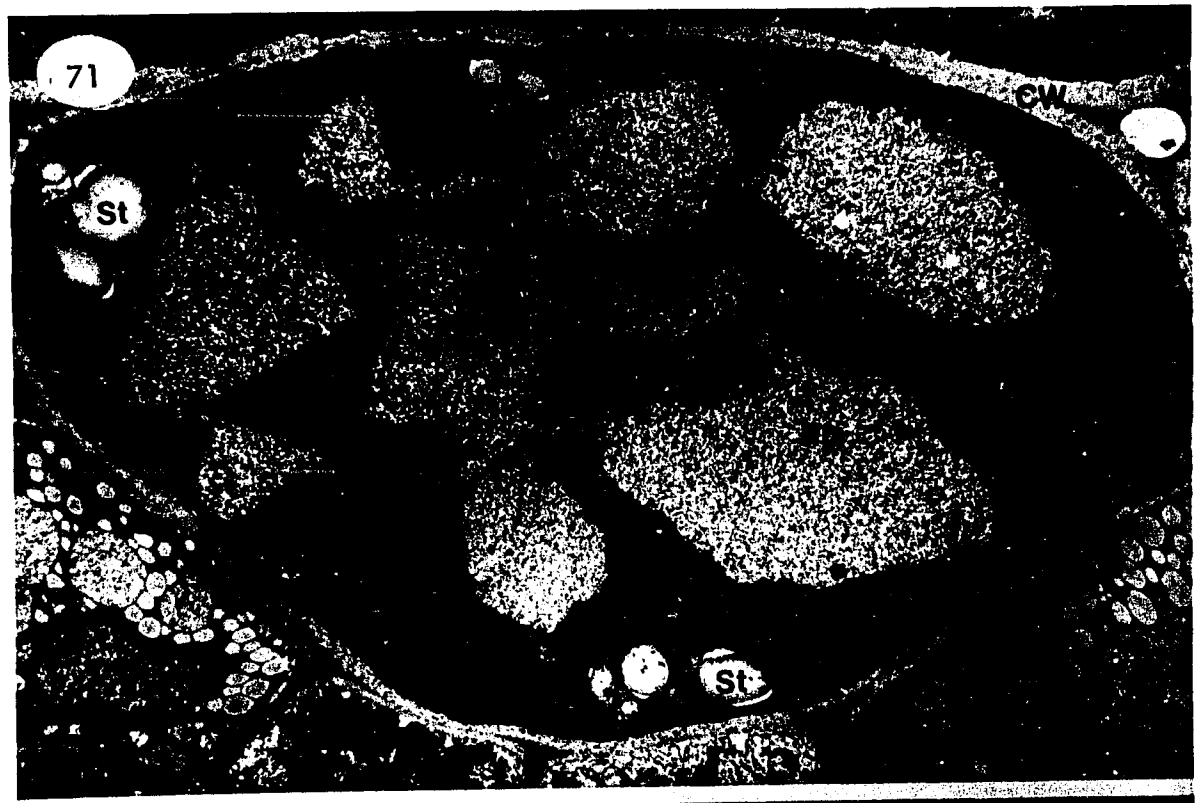
The occurrence and proliferation of starch granules is also apparent in 2 day cotyledons (Figs. 71 and 75). Suryanarayana (1976) found that by day 2 to day 5, almost all soybean cotyledon cells contained starch granules. From day 1 to day 5 there was little qualitative change in the lipid bodies of most cells.

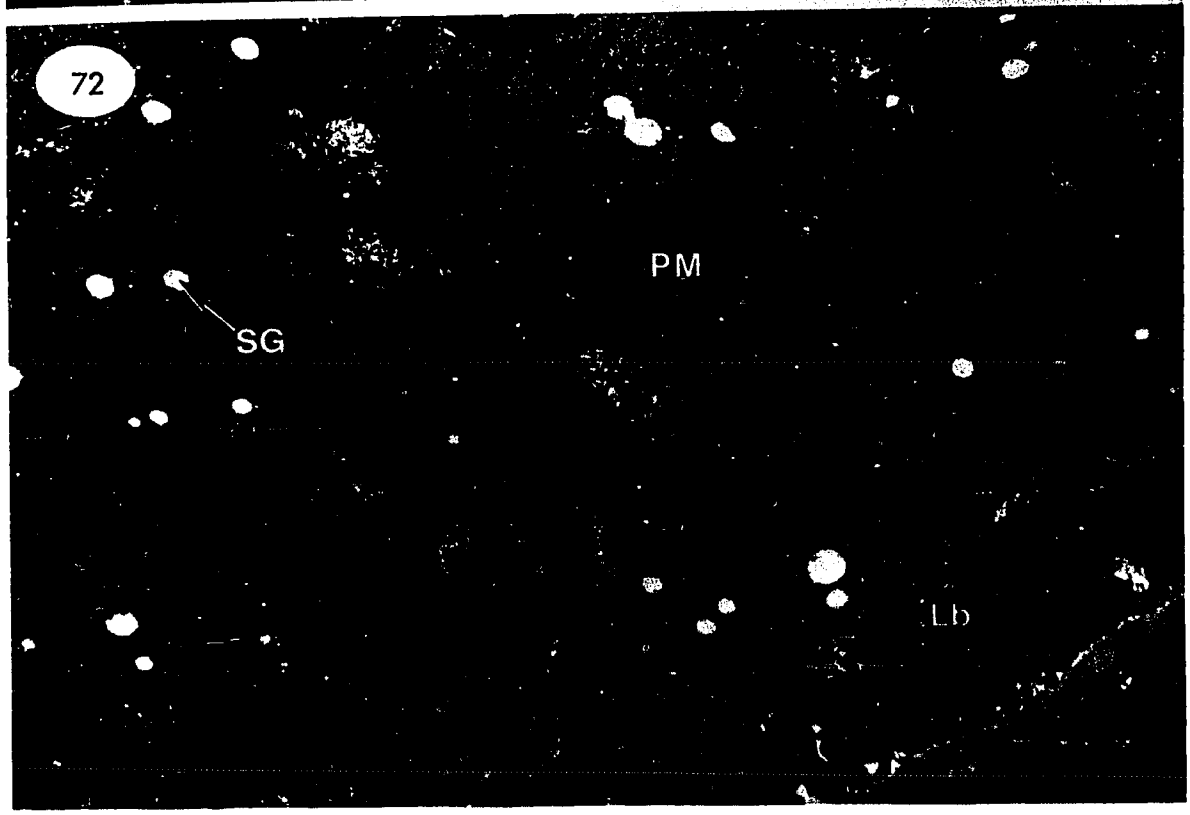
Cotyledon cells of 4 day germinated soybeans appear to have large vacuolated regions occupying their central portion (Fig. 77). Upon closer examination (Fig. 78), the vacuoles appear to be filled with a uniform low electron-dense material, presumably protein. Some remnants of protein body fusion can still be seen in some of the cotyledon cells (Fig. 77, arrow). During this stage of germination, the number of lipid bodies observed in the

Figs. 71-72. TEM of cotyledonary tissue from 2 day germinated soybeans showing protein body fusion

Fig. 71. 5,456X

Fig. 72. 7,590X


71
St
CW
St


72
PM
SG
Lb

Figs. 73-75. TEM of cotyledonary tissue from 2 day germinated soybeans showing protein body fusion

Fig. 73. Fusion of two protein bodies 13,992X

Fig. 74. Higher magnification of Fig. 73 33,484X

Fig. 75. Protein body fusion with complete disappearance of membrane 18,325X

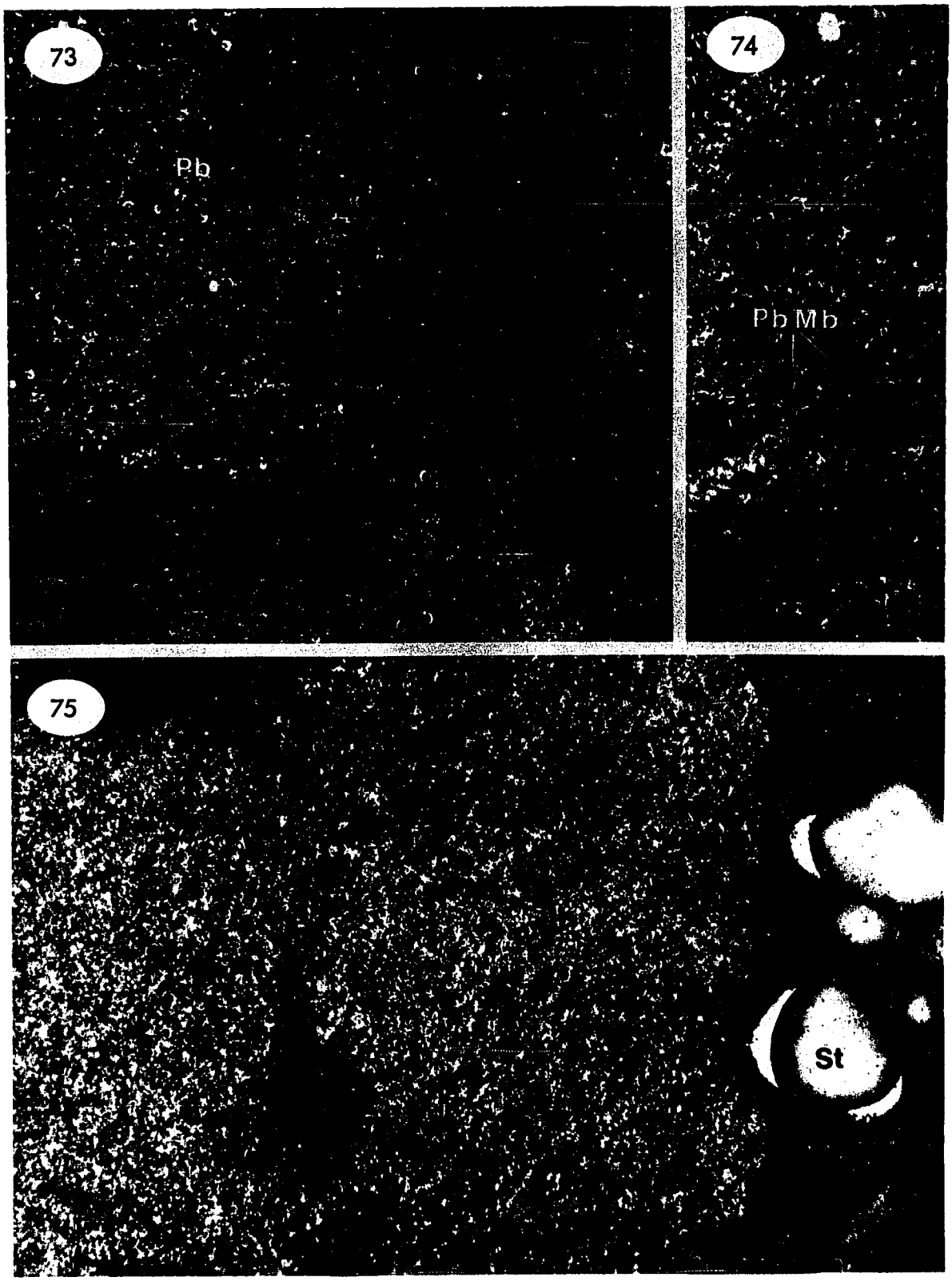

73
Pb
74
PbMb
75
St

Fig. 76. Percent composition of soybean cotyledons from 0 to 17 days after germination

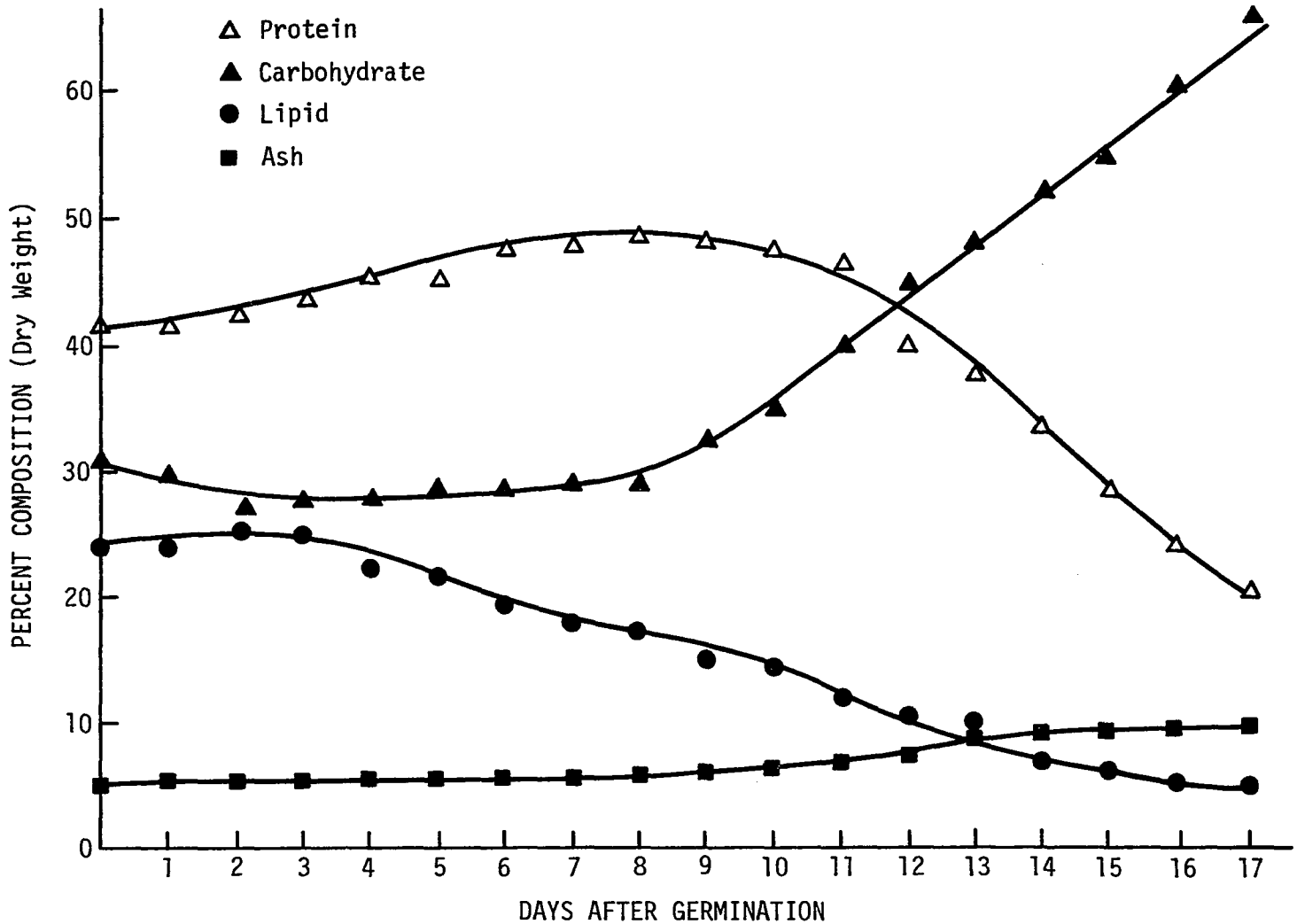
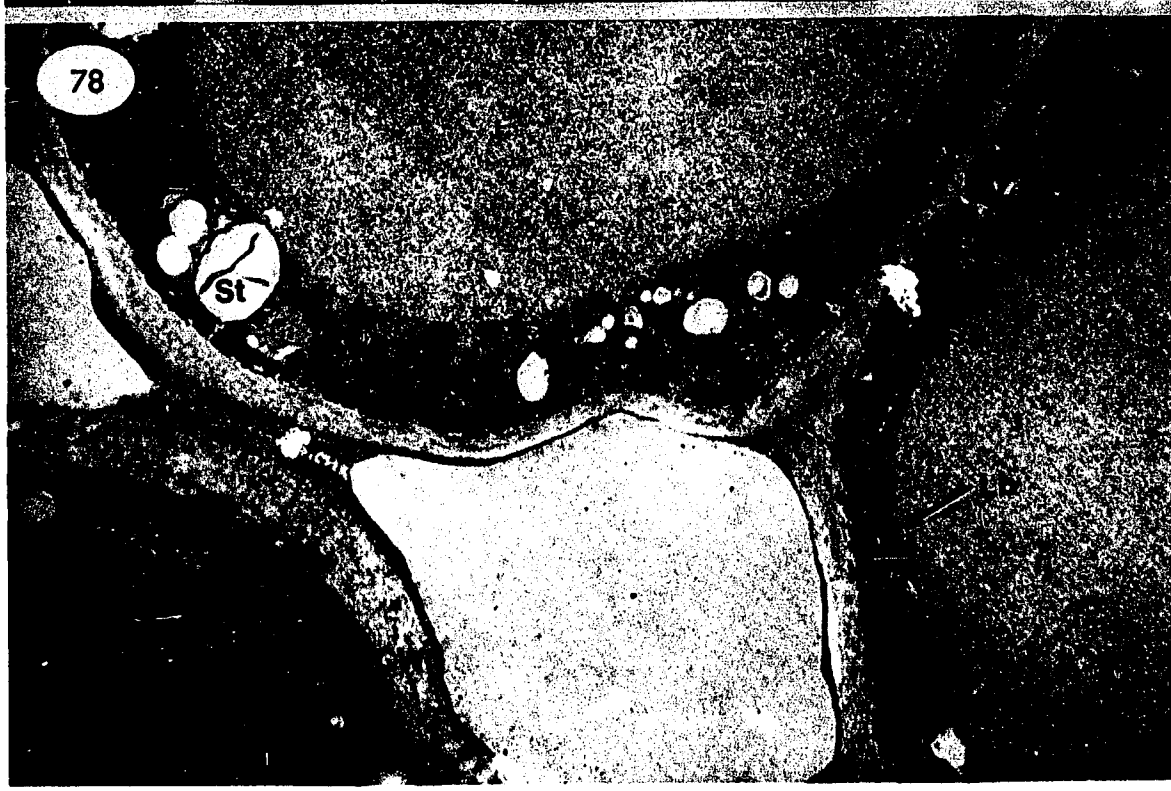

Protein
Carbohydrate
Lipid
Ash
PERCENT COMPOSITION (Dry Weight)
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
DAYS AFTER GERMINATION

Fig. 77. Plastic section of cotyledonary tissue from 4 day germinated soybean. Partial protein body fusion is seen (arrow). 1,275X

Fig. 78. TEM of cotyledonary tissue from 4 day germinated soybean. Center of cell appears as one large vacuole filled with an electron-permeable matrix. 4,589X


77


78
St

cells has modestly decreased and lipid bodies are distributed around the inside perimeter of the cells. Lipid analysis of the cotyledon tissue shows a decrease in total lipid (Fig. 76). Similarly, Kahn (1959) reported an accelerated loss of oil following a peak in lipase activity which was reached on the fifth day.

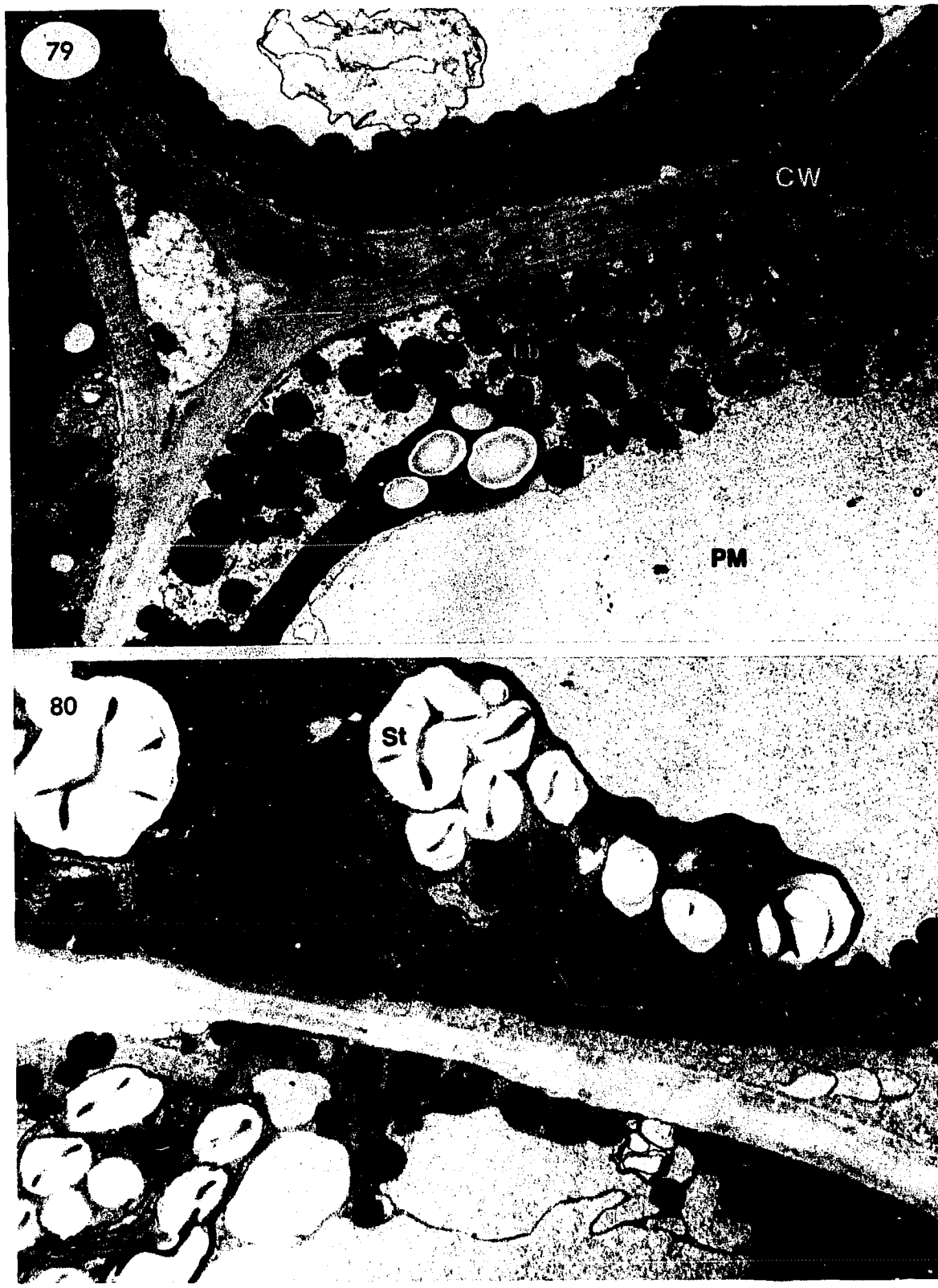
I attempted to localize lipase activity in some of the germinating soybean cotyledons and lipid body isolates using a localization technique described by Ory (1969). However, the experimental procedure failed to show positive results. Other concentrations of reaction mixture and combinations of incubation temperatures and times should be tried, before concluding that lipase activity is not associated with lipid bodies.

Protein masses in 6 day cotyledons are less electron-dense than in previous observations nevertheless, they still contained a granulated matrix easily recognized in electron micrographs (Figs. 79-80). In day 6 cotyledons, I noticed that lipid bodies continued to decrease with a concomitant proliferation of immature plastids and starch. Starch appears as clusters of spherical to oblong concentric granules and stains with both PAS and I_2KI. The occurrence and buildup of starch in the cotyledon cells must be due to the utilization of the food stores present there. Starch formation and accumulation predominantly occur in the cells shortly after germination and therefore, are most closely correlated with protein degradation. However, it is more probable that starch formation occurs from the breakdown of lipids, because lipid bodies show a slight decrease

Figs. 79-80. TEM of cotyledonary tissue from 6 day germinated soybeans

Fig. 79. Protein mass appears less electron dense 8,657X

Fig. 80. Numerous amyloplasts with associated starch granules 8,625X


79
CW
PM
80
St

number early in germination. Kornberg and Beevers (1957) and Beevers (1961) showed that fat is converted to carbohydrates by way of the glyoxalate cycle in castor beans while Oaks and Beevers (1964) found the same pathway operating in maize scutella. The indication is, with particular reference to oil seeds, that lipids are broken down into glycerol and fatty acids then converted to carbohydrates.

The most plausible explanation for the concomitant loss of lipid bodies and proteins as starch granules appear seems to be that the conversion of these reserve foods takes place at a rate greater than needed to satisfy the immediate needs of the developing seedling. Thus in order to maintain a satisfactory osmotic balance, the excess is converted to starch. Then this "secondary" reserve is used when other food reserves have been depleted.

Glyoxysomes have not previously been investigated in soybeans. Fig. 81 and 82 are electron micrographs of soybean cotyledon tissue following incubation with DAB (3,3'-diaminobenzidine), a reagent often used to localize catalase, an enzyme marker for glyoxysomes. The subcellular structures (indicated by the arrows in Figs. 81 and 82), appear to contain electron-dense deposits of DAB. They measure 0.4 μm to 0.9 μm in diameter, and are similar to those described in other tissues by numerous researchers (Breidenbach et al., 1968; Gruber et al., 1970; Mollenhauer and Totten, 1970; Trelease et al., 1971). Richardson (1974) found that glyoxysomes in various tissues have diameters ranging from 0.2 to

Figs. 81-82. TEM of cotyledonary tissue from 6 day germinated soybeans treated with DAB (3,3'diaminobenzidine) for localization of catalase in glyoxysomes

Fig. 81. Arrow indicates DAB localization 16,428X

Fig. 82. Arrows indicate DAB localization 28,755X


81


82

1.7 μm but are most commonly 0.3 to 0.4 μm. They also appear to be bound by a single-unit-membrane and contain a finely granular stroma which may have zones of different electron density. By changing the concentration of the reaction mixture and incubation times and temperatures used in this experiment, one might be able to increase the amount of DAB deposition in the glyoxysomes.

Eight days after germination, the protein content of the cotyledon cells begins to decrease (Fig. 76). Microscopically, the protein mass appears to be less electron-dense than previously observed (Figs. 83-85). Suryanarayana (1976), suggests that protein bodies in soybean cotyledons undergo progressive fragmentation into smaller particles and finally disappear from most cotyledon cells by 9 days after germination. However, observations made in this study show that protein bodies fuse together to form layer protein masses, and by 9 days after germination individual protein bodies are no longer seen. Lipid bodies in 8 day cotyledons are generally larger (0.8 μm to 1.3 μm) than normal, and sometimes appear to be more distorted and irregular in shape (Fig. 85). The structures indicated by the arrows in Fig. 85 appear to be glyoxysomes, based upon their size, shape and electron density. It is interesting to note that lipid bodies in close proximity to the glyoxysomes appear more irregular in shape and look as if they are undergoing degradation.

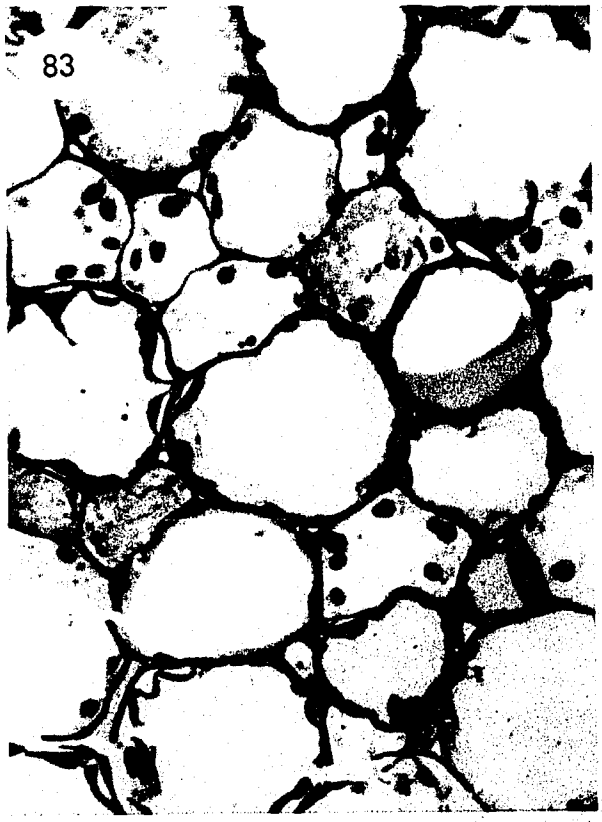
The lipid content has steadily decreased to about 18% by this time, while a marked increase in total carbohydrate is experienced in the soybean cotyledons.

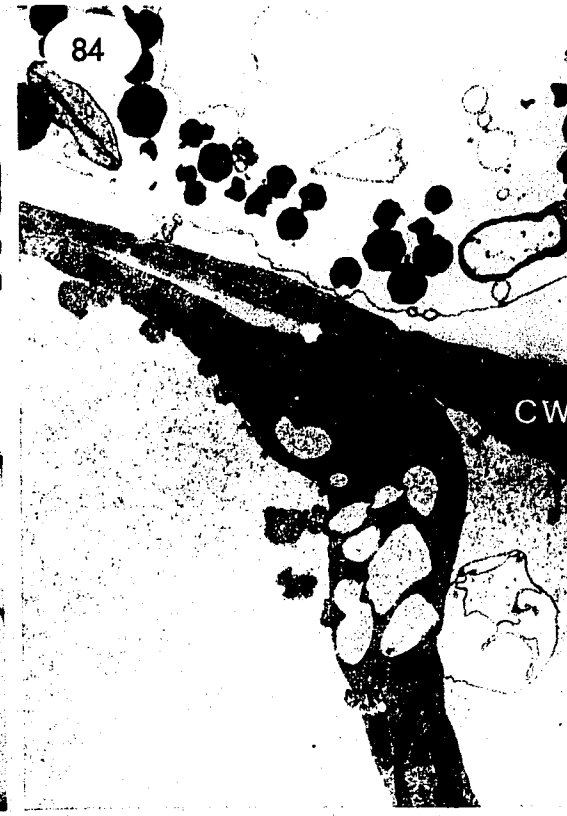
Fig. 83. Plastic section of cotyledonary tissue from 8 day germinated soybean stained with toludine blue O 1,275X

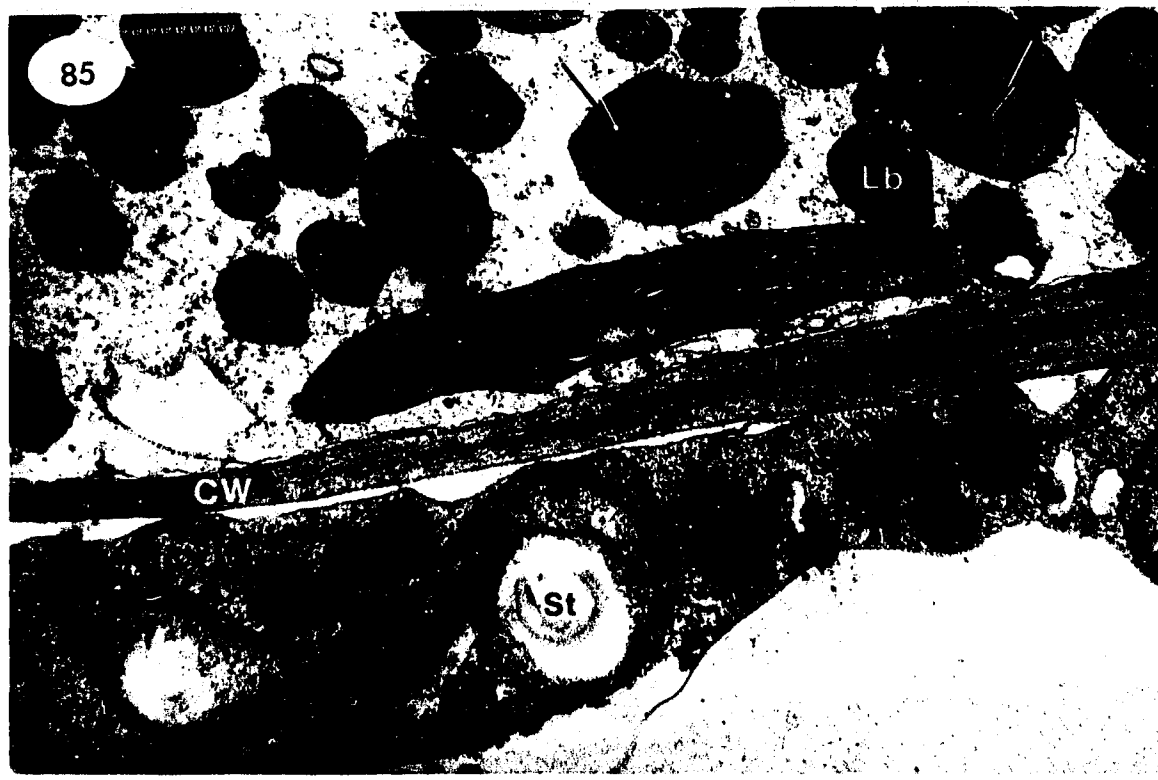
Fig. 84-85. TEM of cotyledonary tissue from 8 day germinated soybeans

Fig. 84. 5,865X

Fig. 85. Arrows indicate possible glyoxysomes 19,090X


83


84
CW


85
Lb
CW
St

This increase is most likely due to the concomitant decrease in both protein and lipid reserves. Also the amount of ash shows a slight increase in the cotyledons.

Figs. 86 and 87 are electron micrographs of 10 day cotyledon cells. By this time, the granular appearance of the protein mass has almost completely disappeared and appears as an electron-translucent vacuole. A chloroplast with its characteristic grana is shown in Fig. 87 along with numerous lipid bodies. The lipid bodies measure 0.8 to 1.2 μm in diameter, and their delimiting single-line-membranes are easily seen (indicated by arrow in Fig. 87).

Most of the recognizable cellular components of cotyledons, except for cell walls, are absent by day 12 (Figs. 88 and 89). However, chemical analysis of the tissue reveals that the cotyledonary cells still contain 38% protein and 10% lipid. In 14 day cotyledons some lipid bodies are seen in the highly vacuolated tissue (Fig. 90) and by day 16 to day 17 (Figs. 91-93), only irregular-shaped cell walls remain visible in the tissue. Holman (1948) found that the oil content of soybean cotyledons rapidly decreased during germination and after 15 days was only about 2 percent. In this study, I found that 17 day cotyledons contained 5% oil and 19% protein.

Figs. 94 and 95 are of 2 day germinated soybean cotyledon tissue treated with a lead containing reagent used to localize the enzyme acid phosphatase. This experiment was performed to answer the question of which subcellular constituent contained the enzyme. In a number of reports, lipid bodies or

Fig. 86-87. TEM of cotyledonary tissue from 10 day germinated soybeans

Fig. 86. 6,630X

Fig. 87. Arrow indicates lipid body membrane 27,780X

Fig. 88. Plastic section of cotyledonary tissue from 12 day germinated soybean 1,275X

Fig. 89. TEM of cotyledonary tissue from 12 day germinated soybean 5,175X

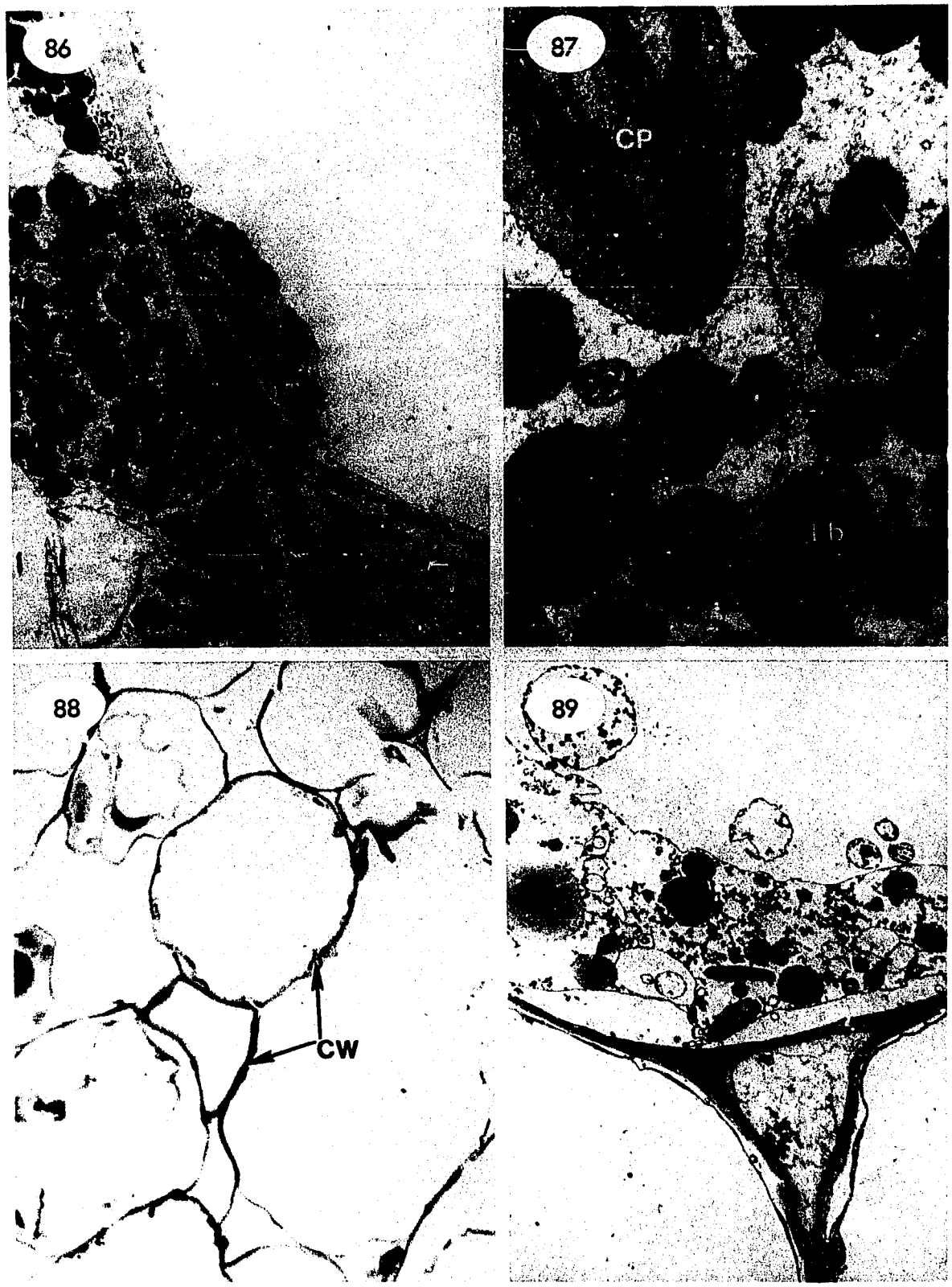

86
87
CP
88
CW
89

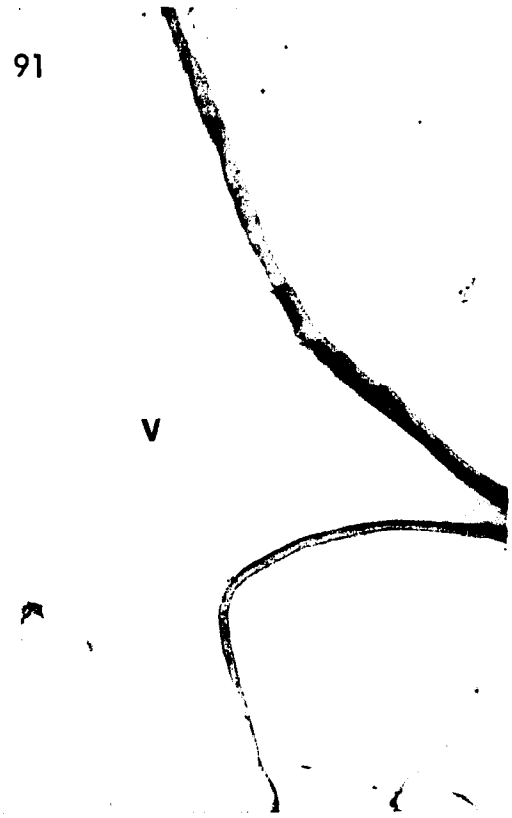
Fig. 90. TEM of cotyledonary tissue from 14 day germinated soybean 3,968X

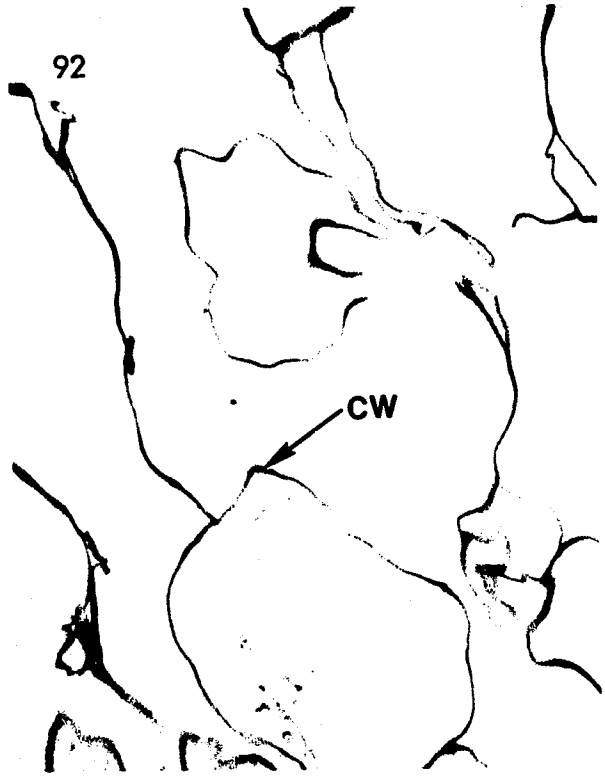
Fig. 91. TEM of cotyledonary tissue from 16 day germinated soybean showing highly vacuolated tissue 5,520X

Fig. 92. Plastic section of cotyledonary tissue from 17 day germinated soybean stained with toludine blue O 1,275X

Fig. 93. TEM of cotyledonary tissue from 17 day germinated soybean 3,968X


90
C


91
V


92
CW

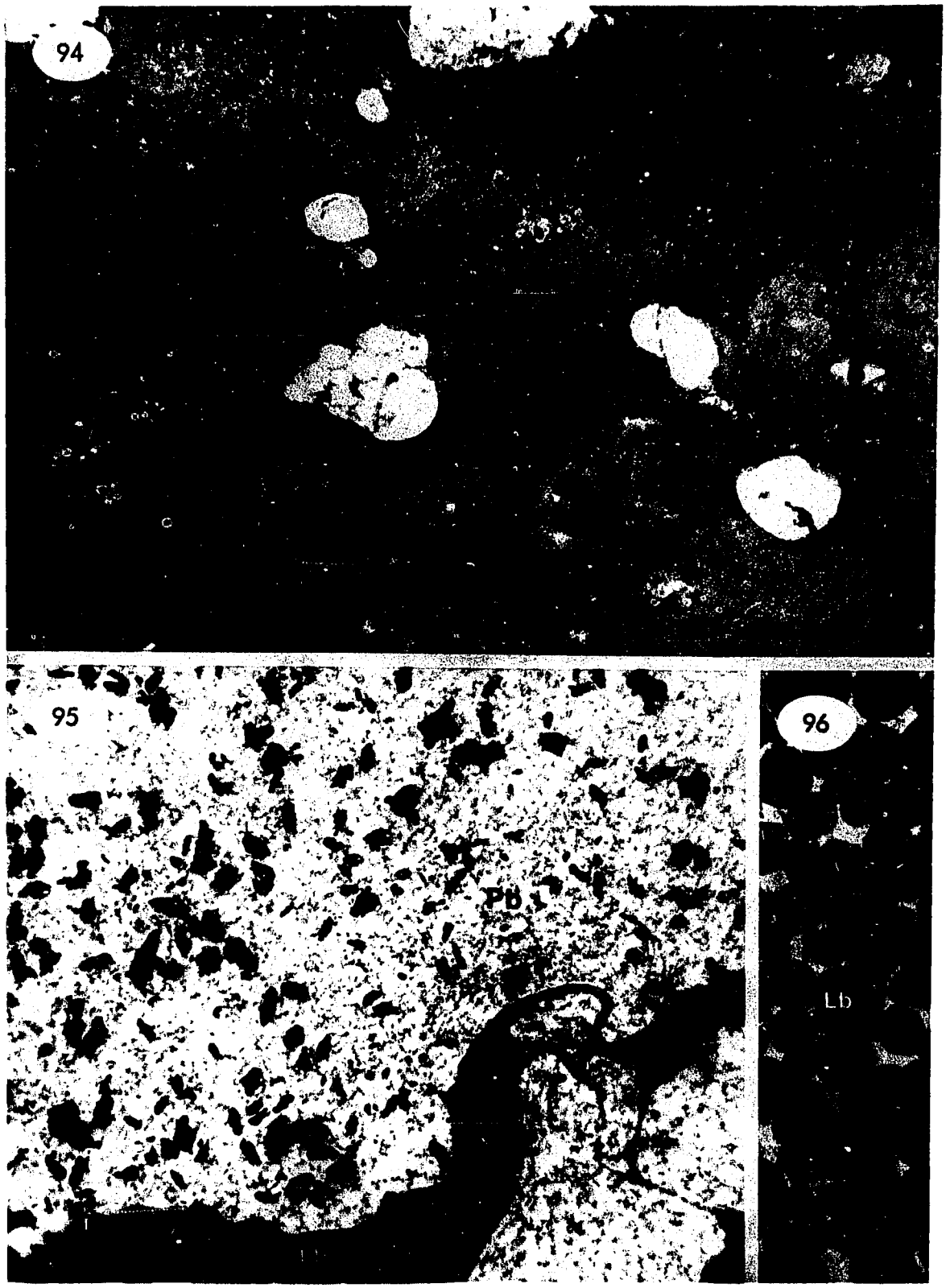

93

Figs. 94-95. TEM of cotyledonary tissue from 2 day germinated soybeans showing electron-dense deposits of lead soaps at the site of acid phosphatase activity

Fig. 94. Lead deposits in protein bodies but not in lipid bodies 4,589X

Fig. 95. Lead deposits in protein bodies at site of acid phosphatase activity 14,660X

Fig. 96. TEM of lipid body isolate prepared by the procedure of Yatsu and Jacks (1972) and treated with the lead reagent for localization of acid phosphatase activity. No deposits are observed 15,630X


94
95
PB
96
Lb

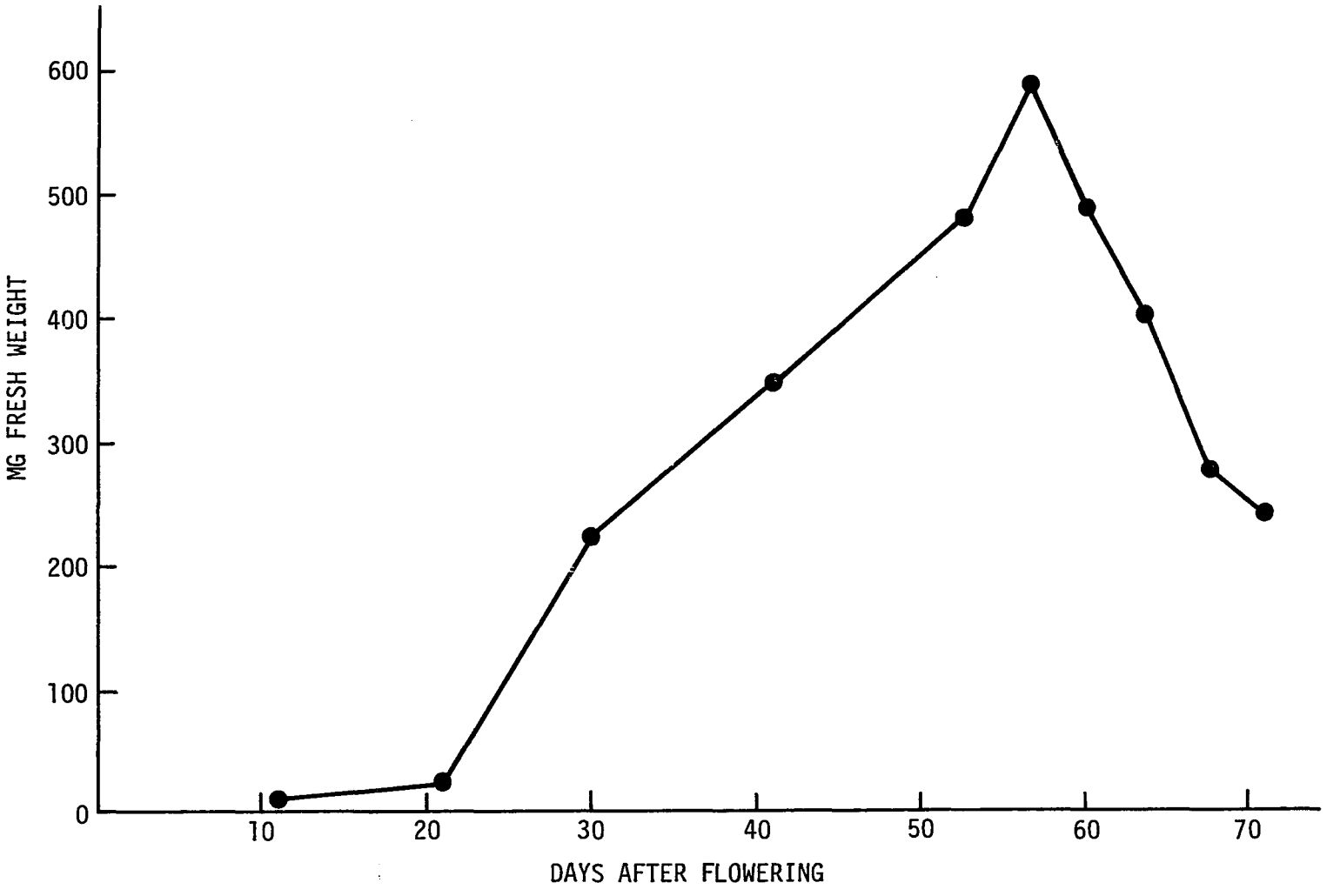
spherosomes were reported to contain various enzymes, especially acid phosphatase (Gomori, 1952; Matile et al., 1965; Sorokin, 1967). However, following incubation, electron-dense lead soaps were found concentrated in protein bodies and not in the surrounding lipid bodies. Furthermore, when lipid body isolates from germinated cotyledons were treated for acid phosphatase localization, no apparent deposition of lead soaps were observed. Protein bodies in other tissues have been reported to contain acid phosphatase (Poux, 1963; Yatsu and Jacks, 1968). My results confirm that protein bodies rather than lipid bodies contain acid phosphatase.

Maturation

Fig. 97 shows the fresh weights of developing soybean cotyledons from 10 days after flowering (DAF) to maturity (about 70 DAF). The cotyledons reached a maximum fresh weight of 590 mg about two weeks before maturity, then lost a considerable amount of water, so that they had fresh weights of 200 to 210 mg at maturity. The seeds were considered to be mature when the pods had become ripe, as evidenced by their dry appearance (Weiss et al., 1950). Developing soybean cotyledons were microscopically and chemically examined from 10 to 70 DAF.

A section of a 10 DAF (about 10 mg) soybean cotyledon is shown in Fig. 98. All the cells of the cotyledon are irregular in shape and contain a variety of densely-stained cellular inclusions (Fig. 99). However, the prominent structures

Fig. 97. Fresh weights of soybean cotyledons from 10 to 70 days after plant flowering


MG FRESH WEIGHT
600
500
400
300
200
100
0
10
20
30
40
50
60
70
DAYS AFTER FLOWERING

Figs. 98-99. Cotyledonary tissue from soybeans 10 DAF

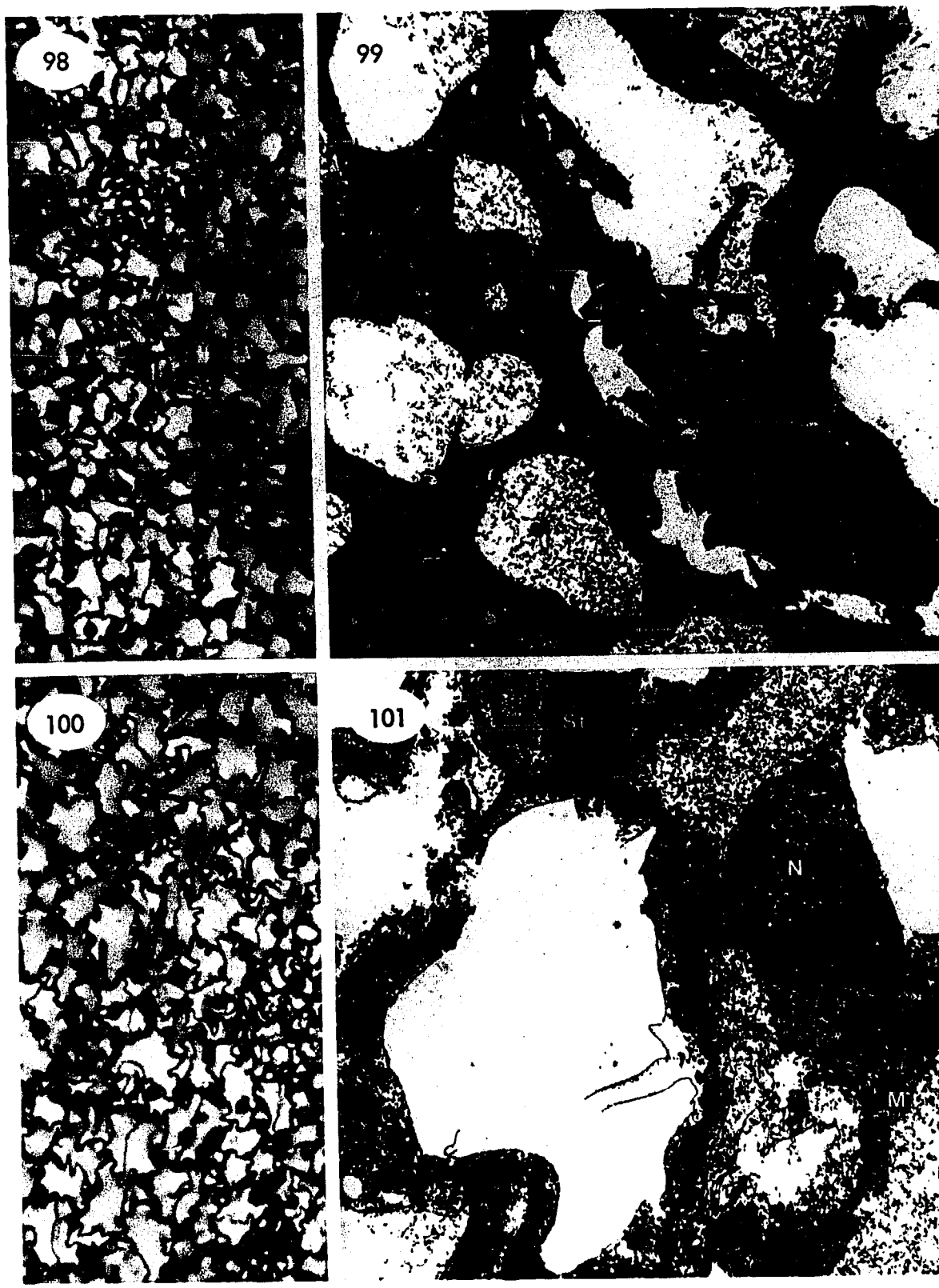
Fig. 98. Plastic section stained with toludine blue O 1,275X

Fig. 99. TEM micrograph 4,216

Figs. 100-101. Cotyledonary tissue from soybeans 20 DAF

Fig. 100. Plastic section stained with toludine blue O 1,275X

Fig. 101. TEM micrograph 6,690X


98
99
100
101
N

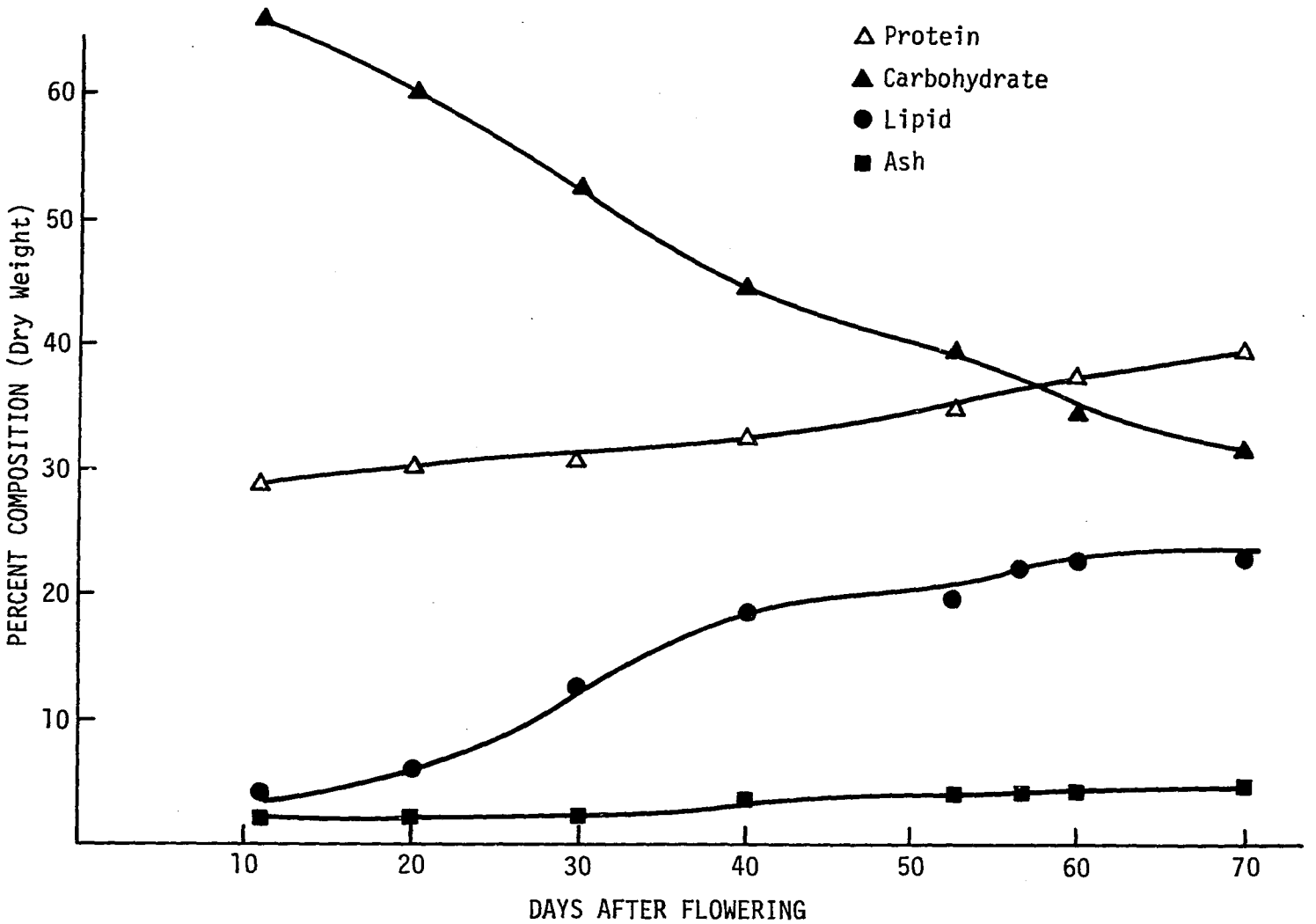
of the mature cotyledon, protein bodies and lipid bodies are absent. Bils and Howell (1963) reported similar observations for maturing soybean cotyledons.

By 20 DAF the cytoplasm is composed mainly of scattered masses of ribonucleoprotein particles (Figs. 100 and 101), which are similar to those described by Bils and Howell (1963). Endoplasmic reticulum appears to be associated with many of the ribonucleoprotein particles. Some nuclei and starch granules are also present at this developmental stage, but mitochondria are the most prevalent subcellular organelles. The protein content of 20-day cotyledons is 29%, while lipid and ash account for 7% and 3% of the cotyledonary tissue respectively (Fig. 102).

A large increase in cell size occurs from 20 to 30 DAF. The general appearance of the cotyledon cells does not change appreciably from 30 DAF to maturity; however, prominent changes do occur in cellular components. Figs. 103-108 show the developmental changes in cotyledon cells 30 DAF. In Fig. 104 the palisade cells show the first sign of lipid bodies. Lipid bodies are predominantly distributed around the inside perimeter of the cell wall, but some are often seen scattered throughout the cell cytoplasm.

An attempt was made to trace the origin and development of lipid bodies in the cotyledon cells. I found that lipid bodies appeared to start as minute vesicles originating from the ends of endoplasmic reticulum (Figs. 105-108). Numerous other researchers (Frey-Wyssling et al., 1963; Schwarzenbach, 1971;

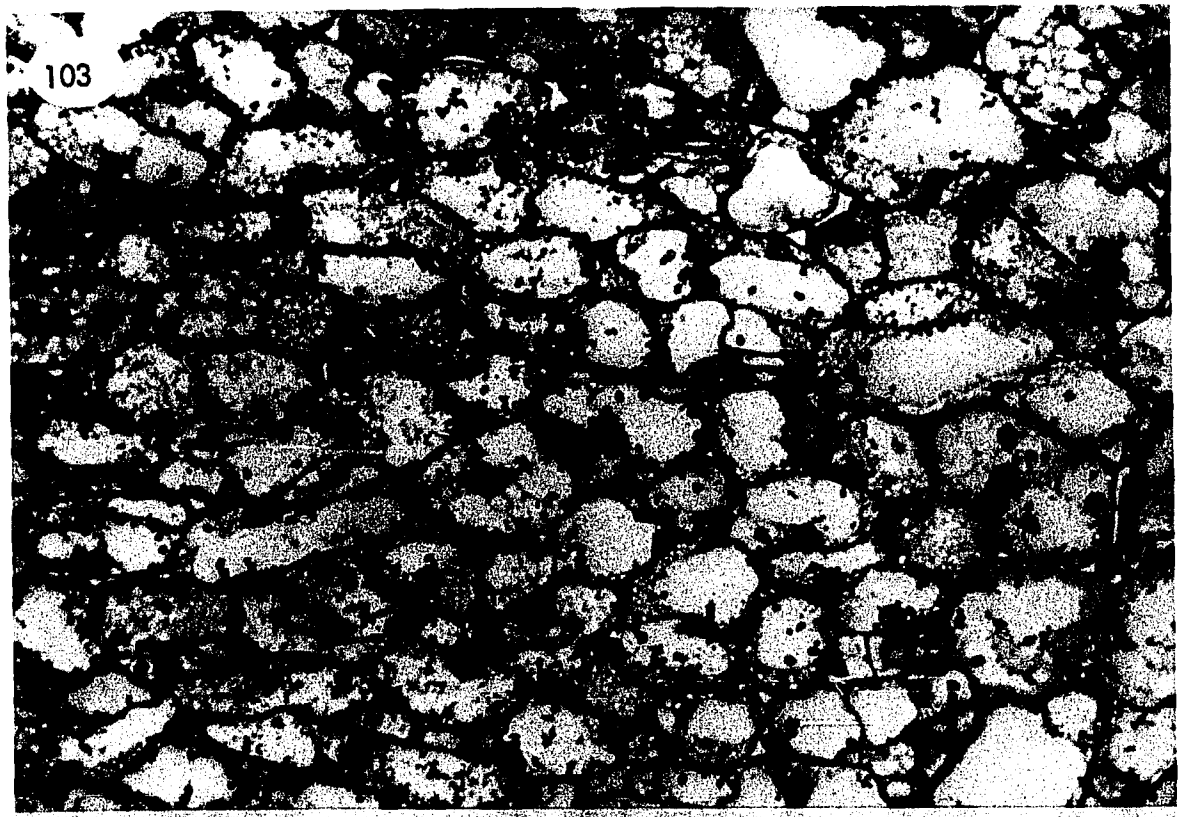
Fig. 102. Percent composition of soybean cotyledons from 10 to 70 DAF

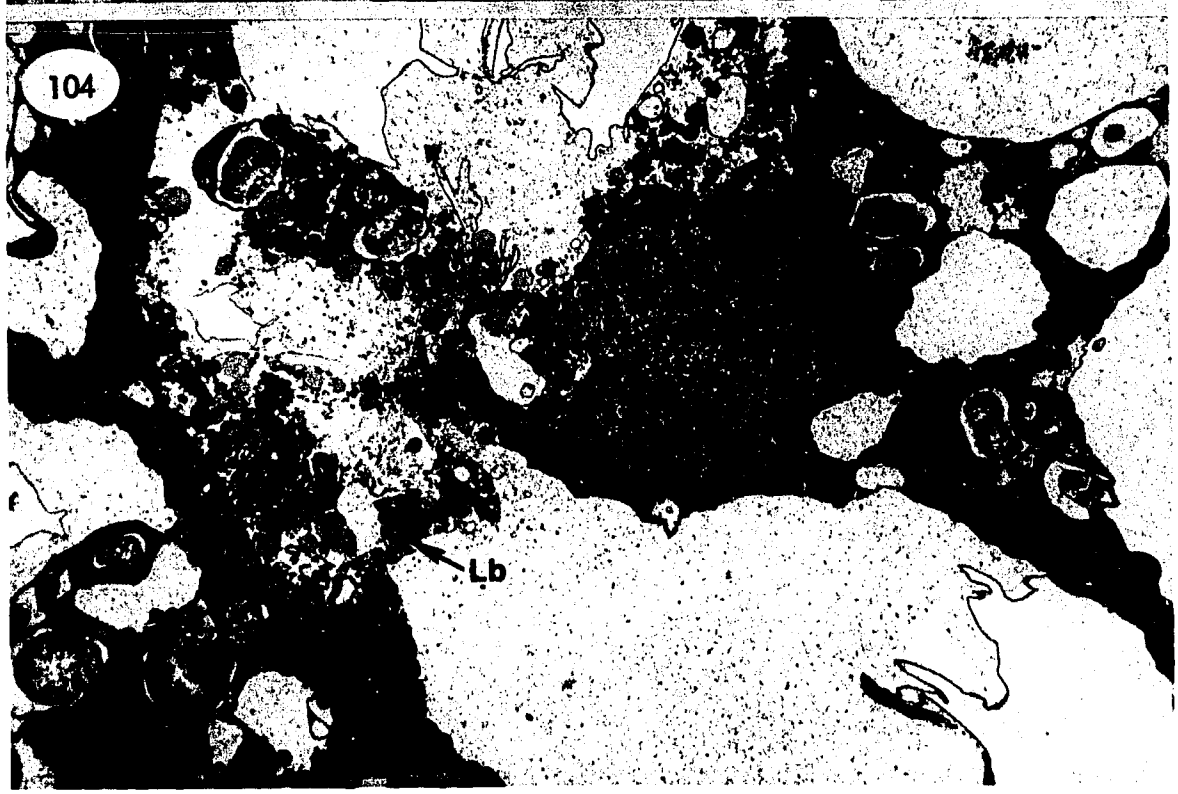

Protein
Carbohydrate
Lipid
Ash
PERCENT COMPOSITION (Dry Weight)
60
50
40
30
20
10
10
20
30
40
50
60
70
DAYS AFTER FLOWERING

Figs. 103-104. Cotyledonary tissue from soybeans 30 DAF

Fig. 103. Plastic section stained with toludine blue O 1,275X

Fig. 104. TEM micrograph 4,589X


103

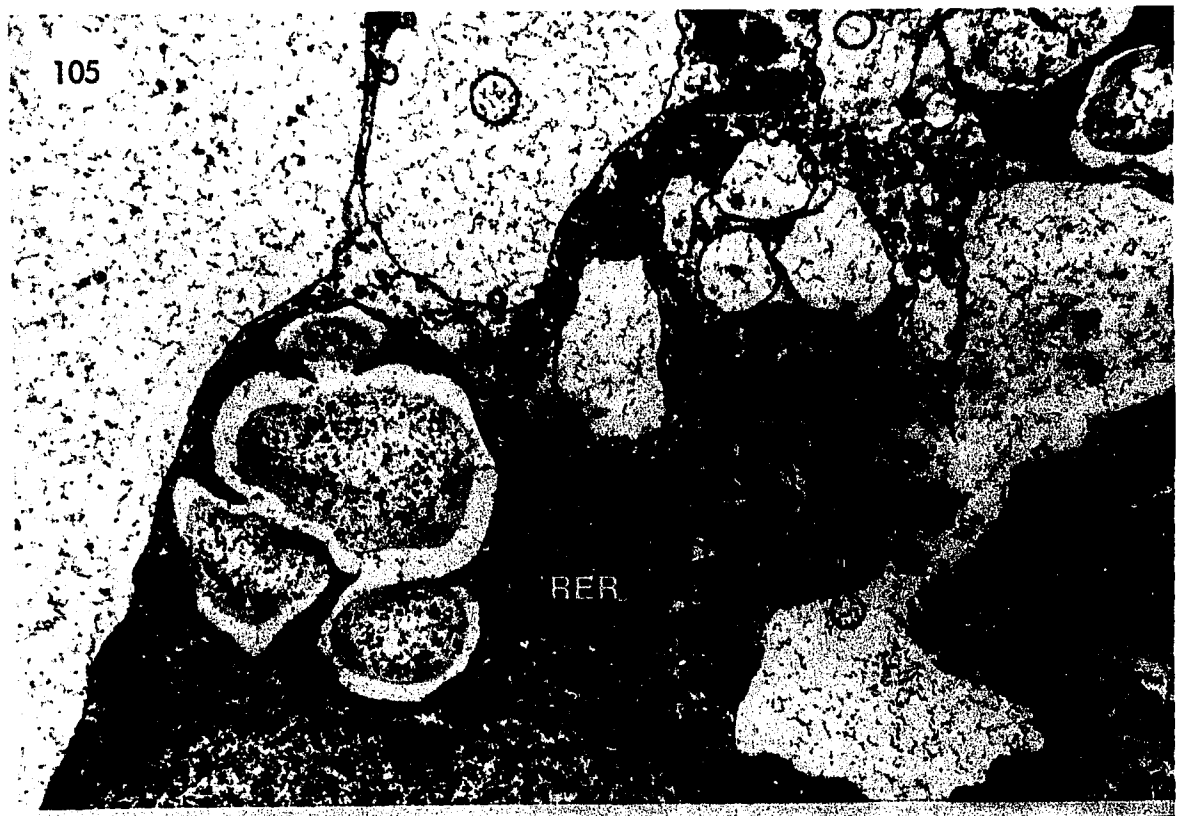

104
Lb

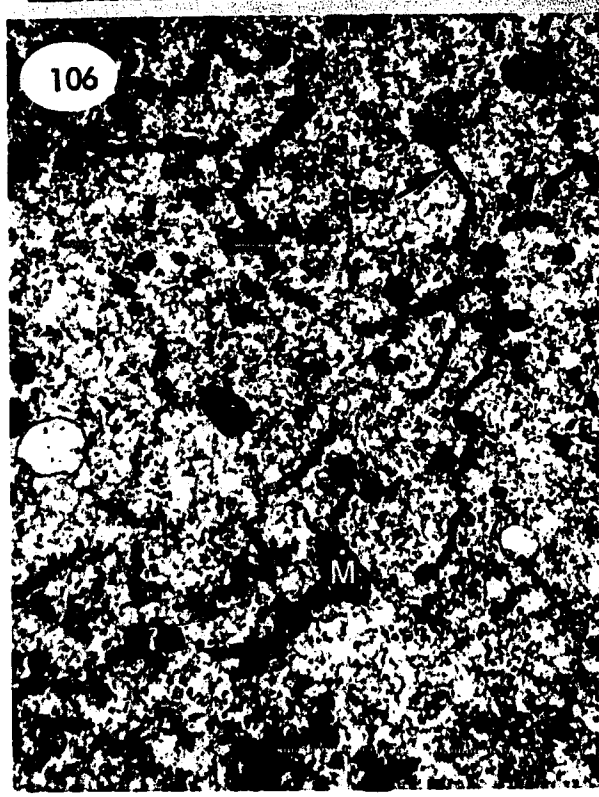
Figs. 105-107. TEM of cotyledonary tissue from soybeans 30 DAF

Fig. 105. 16,126X

Fig. 106. Close association or budding of lipid from the ends of endoplasmic reticulum 15,062X

Fig. 107. Lipid budding from endoplasmic reticulum 15,062X


105
RER


106
M

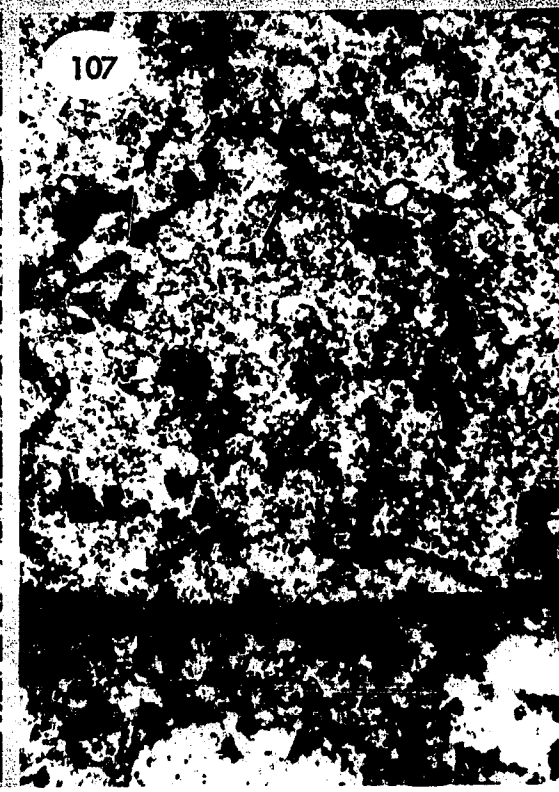
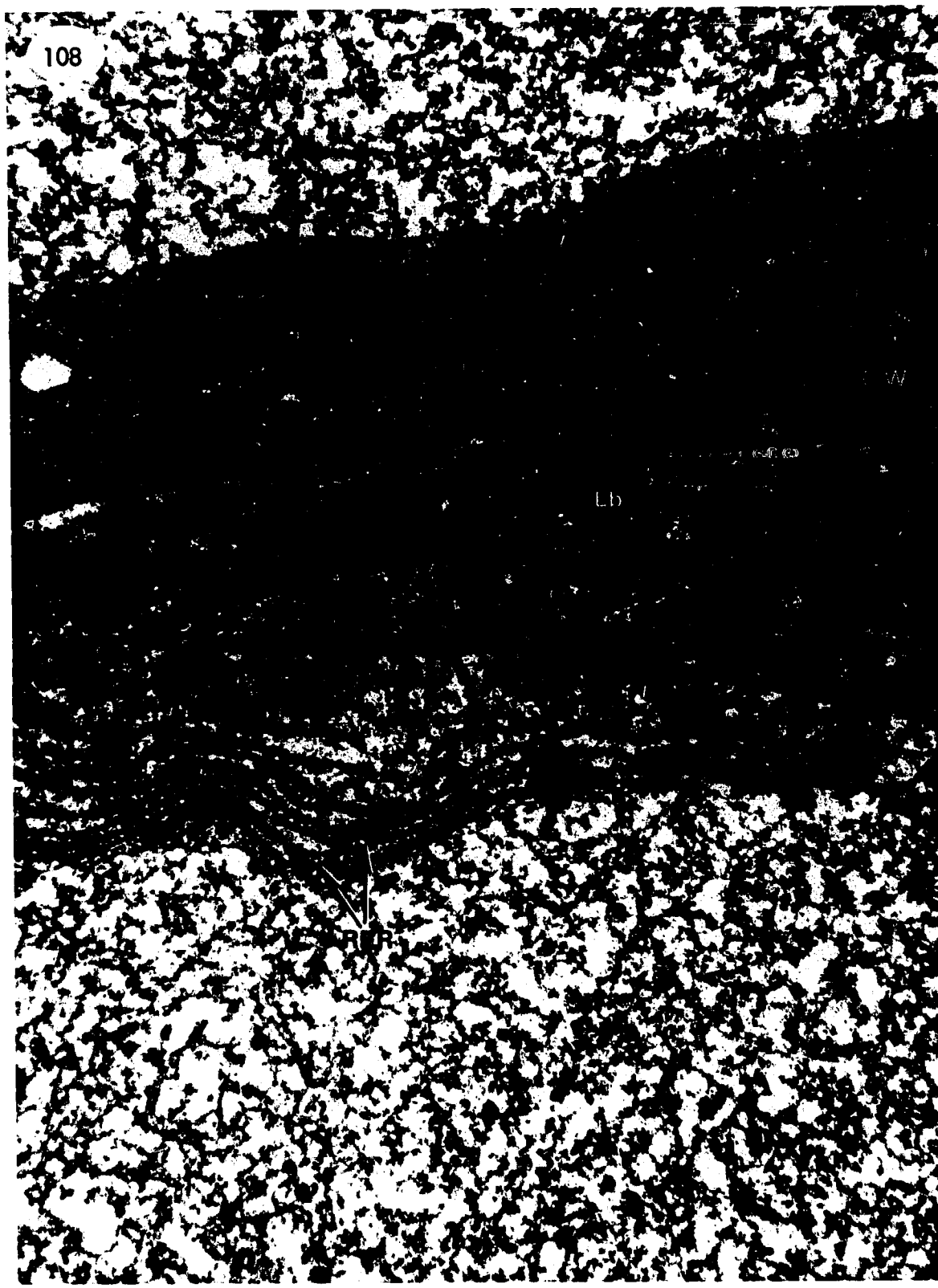

107

Fig. 108. TEM of cotyledonary tissue from a soybean 30 DAF, showing the close association of lipid bodies and rough endoplasmic reticulum 19,080X


108
Lb

Simola, 1969), working with various tissues have reported that spherosomes originate from enlarged fragments of the endoplasmic reticulum. Smith (1974), working with Crambe abyssinica, also observed that spherosomes developed as small terminal vesicles formed on the endoplasmic reticulum which subsequently increased in size and became filled with osmiophilic material. Eventually the spherosomes abscised from the endoplasmic reticulum and developed into oil bodies. In my soybean preparation, however, it was difficult to distinguish whether the lipid bodies were continuous with or appeared to be budding from the endoplasmic reticulum. From some observations, it may also be reasonable to assume that lipid synthesis occurs external to, or on the surface of the endoplasmic reticulum. Before establishing a definite connection between the endoplasmic reticulum and lipid bodies, further verification is needed. Nevertheless, a close association of the two was noticed in developing soybean cotyledons (Figs. 106-108) as well as in sections of mature cotyledons (Figs. 44 and 45). The proliferation of lipid bodies observed in the 30 DAF cotyledons, parallels the increased lipid content of the tissue (Fig. 102).

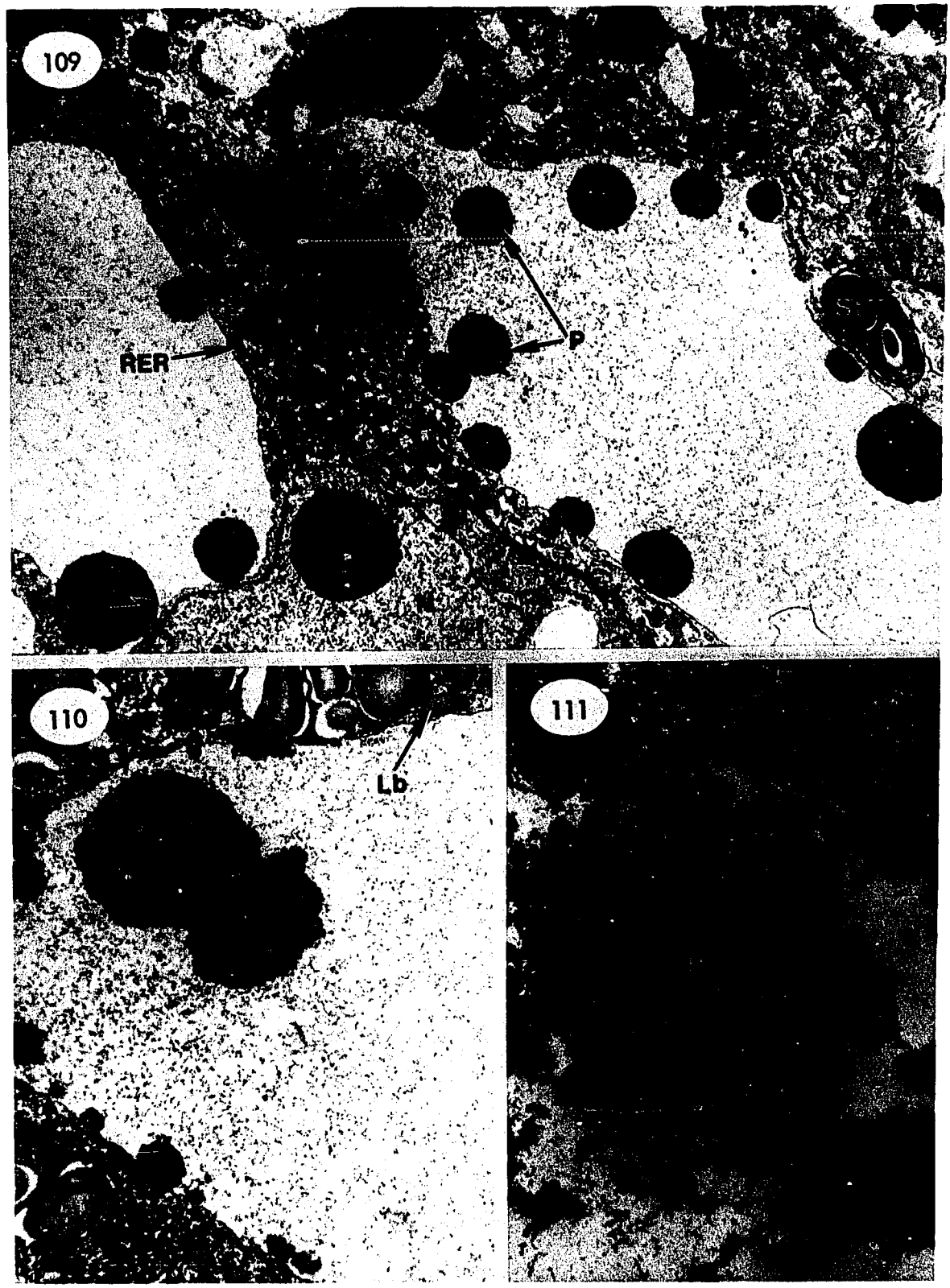
Figs. 109-111 show cotyledonary tissue at 40 DAF. In some of the vacuolated areas, small electron-dense spheroids closely align the vacuole wall. The dark spheres are presumed to be synthesized protein. Although most of the protein appears in single spheres, the protein in Fig. 110 appears to be dividing or budding into two separate entities. In some cells protein exhibits a more dispersed

Figs. 109-111. TEM of cotyledonary tissue from soybeans 40 DAF

Fig. 109. Cotyledon cells showing formation of protein 7,590X

Fig. 110. Protein material appears to be separating 12,321X

Fig. 111. Protein material appears to be diffusing 28,584X


109
RER
P
110
Lb
111

or less-ordered appearance. The nature of these spheres and their metabolic significance is presently unclear. Even though there is a noticeable appearance of ·' is material in the cotyledon cells, there is no apparent marked increase in the protein content of the tissue (Fig. 102).

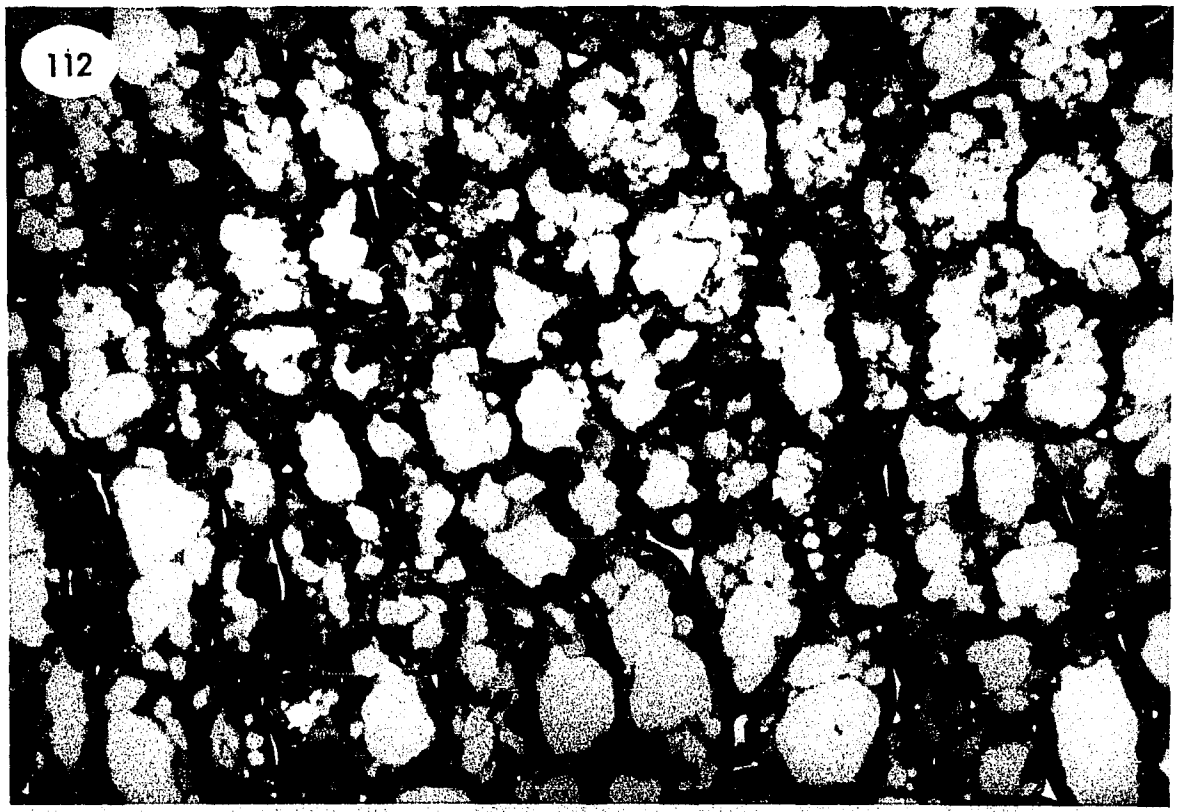
By 50 DAF, rudimentary outlines of protein bodies are observed within the cells (Figs. 112 and 113). At this developmental stage, the dark, electron-dense spheres are no longer seen. Possibly they became completely dispersed and represent the material contained in the vacuolated spaces of the cells as shown in Fig. 113. Numerous starch granules and lipid bodies are found throughout the immature cotyledons. Bils and Howell (1963) and Tada and Kawamura reported similar observations in developing soybean cotyledons.

Individual protein bodies are readily distinguished in the palisade cells of 60 DAF cotyledons (Fig. 114). Protein bodies appear circular to irregular in shape and consist of a matrix of finely granulated electron-dense material. By this developmental stage, the lipid, protein, carbohydrate and ash content of the cotyledon has reached a level comparable to that of the mature cotyledon. The cotyledon undergoes further dehydration and develops into a mature cotyledon as shown in Fig. 115.

Figs. 112-113. Cotyledonary tissue from soybeans 50 DAF

Fig. 112. Plastic section stained with toludine blue O 1,275X

Fig. 113. TEM micrograph showing granulated protein and other cellular components 4,589X


112

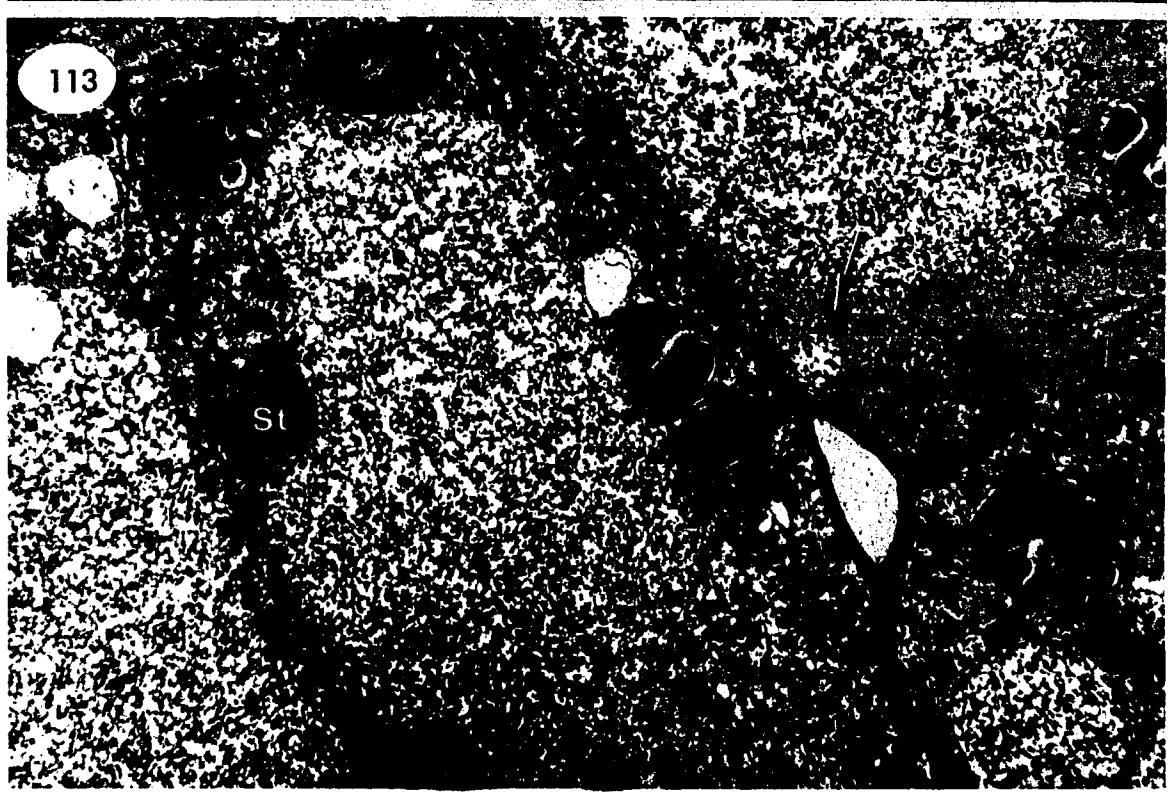
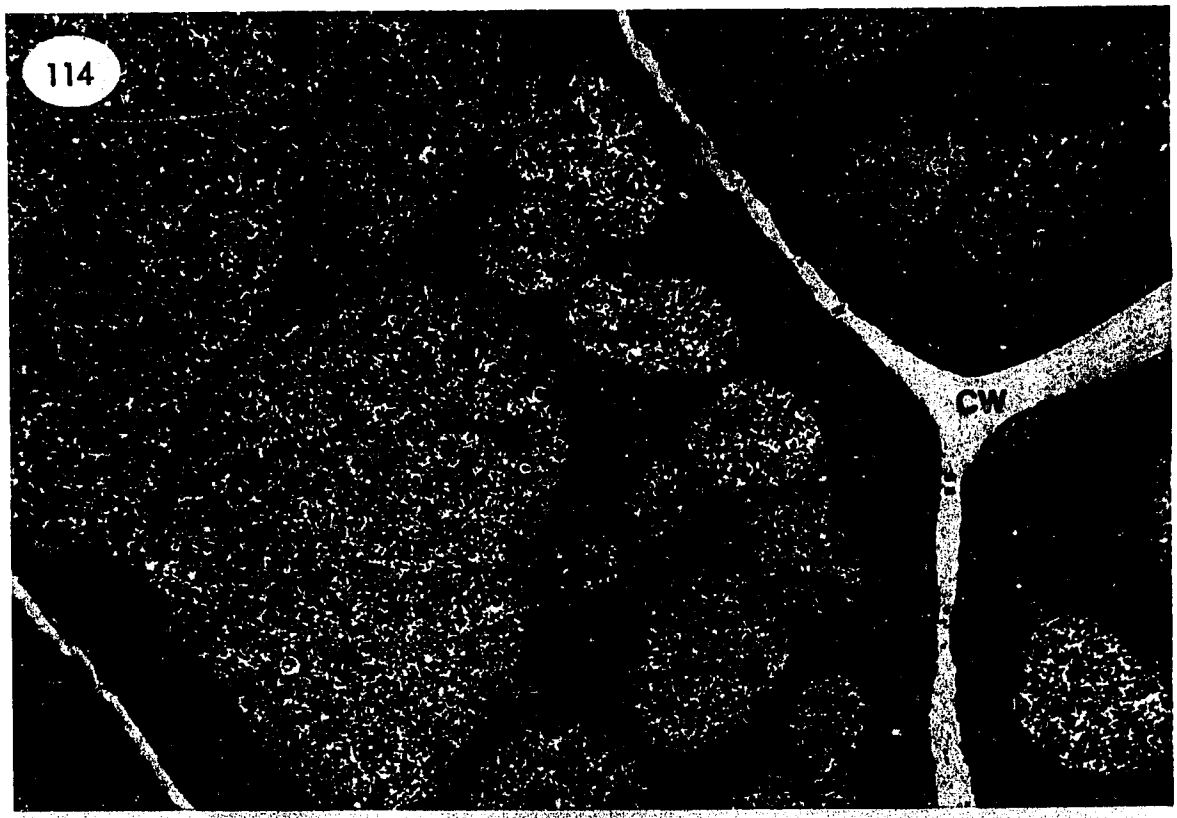
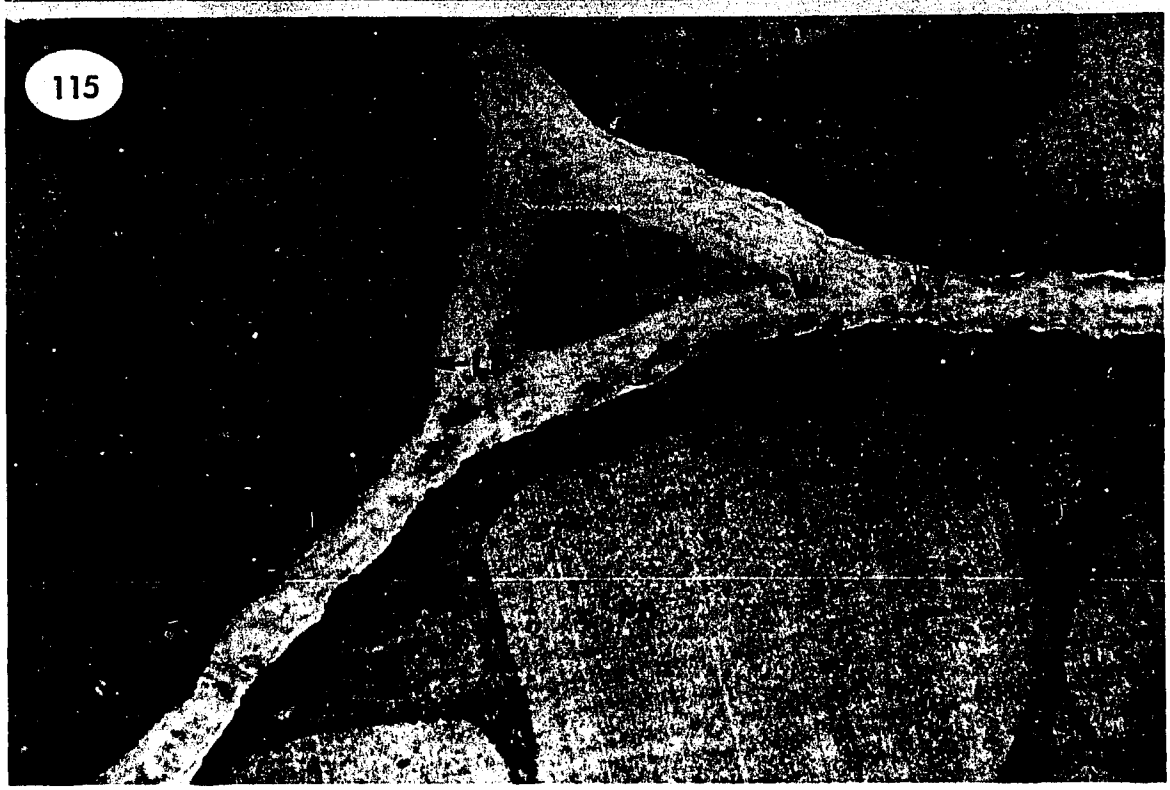

113
St

Fig. 114. TEM of cotyledonary tissue from soybean 60 DAF. Protein bodies start appearing as separate entities 6,900X

Fig. 115. TEM of cotyledonary tissue from soybean 70 DAF. The cotyledon is mature 7,935X


114
CW


115

Relationship of Processing and Soybean Ultrastructure

Ultrastructural changes in soybeans during processing to yield desolventized-toasted meal

Table 2 shows the proximate compositions of samples taken from the seven different stages of soybean processing. Field beans, dried beans and cracked and conditioned beans, showed no apparent changes in their composition or microscopic appearance. The surface of a cracked and conditioned bean as viewed by SEM is shown in Fig. 116 and a LM section of the same tissue appears in Fig. 120. The cotyledon cells appear to be intact with little overall cellular disruption. The appearance and distribution of subcellular components (protein bodies and lipid bodies) remain unchanged by the cracking process and are indicative of intact and normal cells. Examinations of other sections and surfaces of field beans, dried beans and cracked and conditioned beans showed no differences from Figs. 116 and 120.

Flaking the cracked pieces of soybean did not alter the proximate composition, but did have a dramatic destructive effect on the cellular structure. Fig. 117 shows the freeze-fractured surface of a flake in which the regular cellular arrangement, indicative of unprocessed tissue has disappeared. Fig. 124 is a higher magnification of Fig. 117 and shows the lack of intact cells. A photomicrograph of a cross section of a soybean flake is shown in Fig. 121. The extensive cellular disruption is evident and leads to the conclusion that flaking

Table 2. Proximate composition and PDI values of soybean samples taken at seven different stages of processing.

Sample	Moisture (%)	Protein[a] (%)	Lipid[a] (%)	Carbohydrate[a] (%)	Ash[a] (%)	PDI
Field bean	10.4	38.8	21.8	34.5	4.9	85
Dried bean	9.5	39.0	22.1	34.1	4.8	81
Cracked and conditioned bean	9.5	41.8	22.6	30.7	4.8	81
Full-fat flake	9.1	40.9	22.3	32.2	4.7	80
Defatted flake	11.0	52.9	0.9	40.0	6.2	80
Desolventized toasted flake	15.8	52.8	0.9	40.1	6.1	52
Dried meal	11.0	53.1	0.8	39.9	6.2	52

[a]Dry weight basis.

of soybeans does disrupt most of the cells. A protein body, visible in Fig. 125, is partially covered with a cytoplasmic network that contains lipid bodies. Where protein bodies are not covered by the cytoplasmic network a very smooth surface is evident (Fig. 126). In freeze-fractured surfaces studied by Wolf and Baker (1975), protein bodies were wholly covered by a sponge-like cytoplasmic

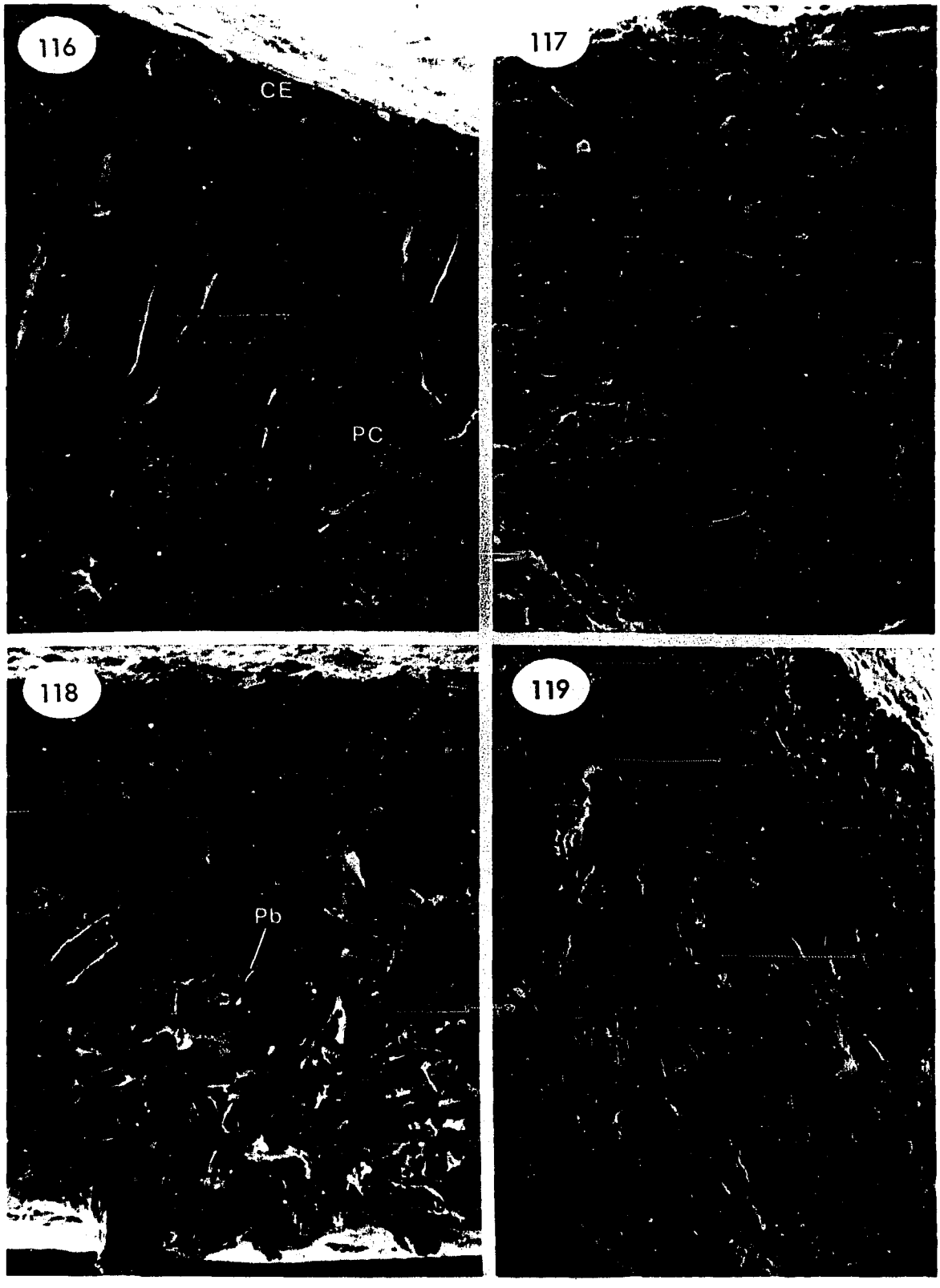
Figs. 116-119. SEM of soybean samples at various stages of processing

Fig. 116. Surface of a cracked and conditioned bean 660X

Fig. 117. Freeze fractured full-fat flake 400X

Fig. 118. Freeze fractured defatted flake 400X

Fig. 119. Freeze fractured desolventized-toasted flake 400X


116
CE
PC
117
118
Pb
119

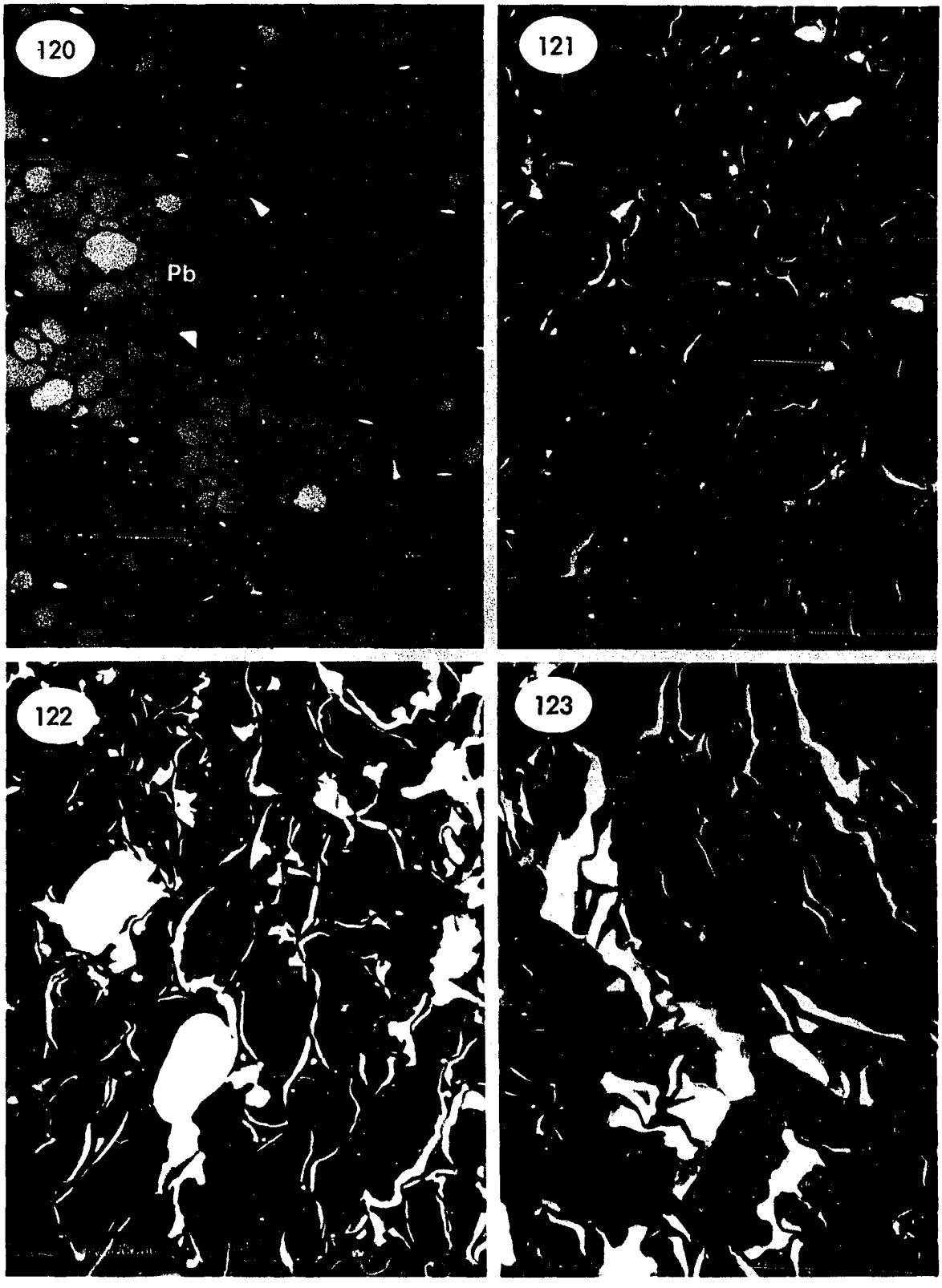
Figs. 120–123. Plastic sections of soybean samples taken from various stages of processing

Fig. 120. Cracked and conditioned bean 1,275X

Fig. 121. Full-fat flake 1,275X

Fig. 122. Defatted flake 1,275X

Fig. 123. Desolventized-toasted flake 1,275X

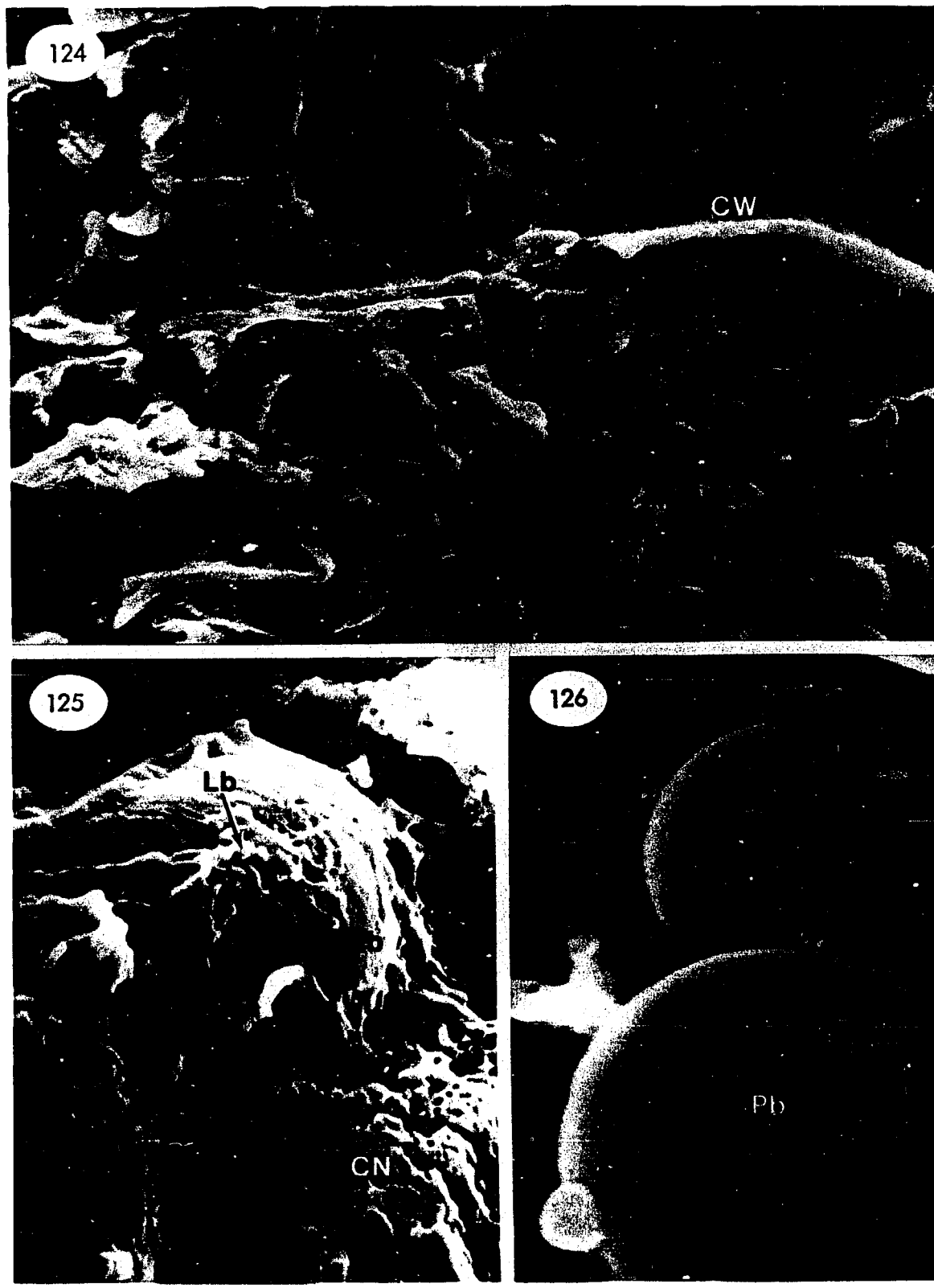

120
Pb
121
122
123

Figs. 124-126. SEM of freeze fractured full-fat soybean flakes

Fig. 124. Cellular disruption caused by flaking 1,320X

Fig. 125. Protein body covered by cytoplasmic network 11,220X

Fig. 126. Protein bodies showing their smooth surface 11,220X


124
CW
125
Lb
CN
126
Pb

network and the smooth surface of the protein body was not evident. The general appearances of protein and lipid bodies in full-fat flakes are unaltered during flaking; however, cell walls are extensively fractured (Figs. 127 and 128, arrow indicates a fractured cell wall).

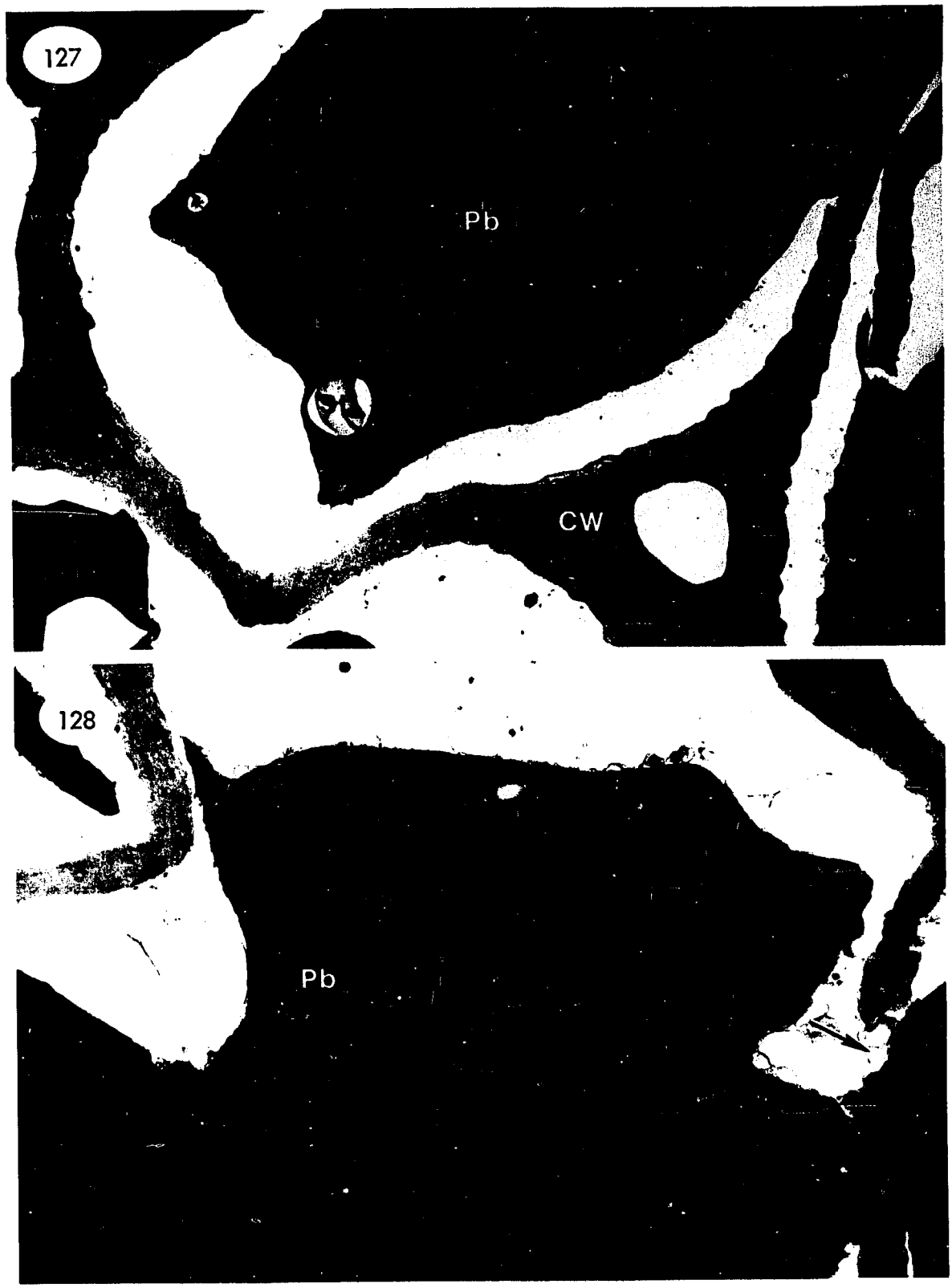
After hexane extraction, the defatted flakes have less than 1% lipid and the percentages of protein, carbohydrate and ash increase correspondingly (Table 2). The gross structure of the defatted flake is shown in Fig. 118. The protein bodies in the defatted flake are more prominent than those in full-fat flakes (compare Figs. 118 and 117), Wolf and Baker (1975) made a similar observation. A photomicrograph of a cross section of a defatted flake (Fig. 122) shows an increase in intra- and inter-cellular spaces, most likely caused by the extraction of oil. Hexane extraction appears to be an effective and efficient process for the removal of the oil from lipid bodies. Figs. 129 and 130 show electron-permeable spaces where oil was extracted from the tissue. There appears to be some coalescence of lipid before or during extraction.

I found a distinct difference in protein bodies of defatted flakes. The protein bodies in Figs. 131-133 show a particulate surface. The small particles measure 0.1 to 0.5 μm in diameter and appear to extend into the protein body (Figs. 132 and 133). Tombs (1967) found that when defatted soybean meal was suspended in water, the protein bodies swelled, often doubling in diameter. Eventually they ruptured, releasing numerous small granules of less than 0.5 μm

Figs. 127-128. TEM of full-fat soybean flakes

Fig. 127. 4,389X

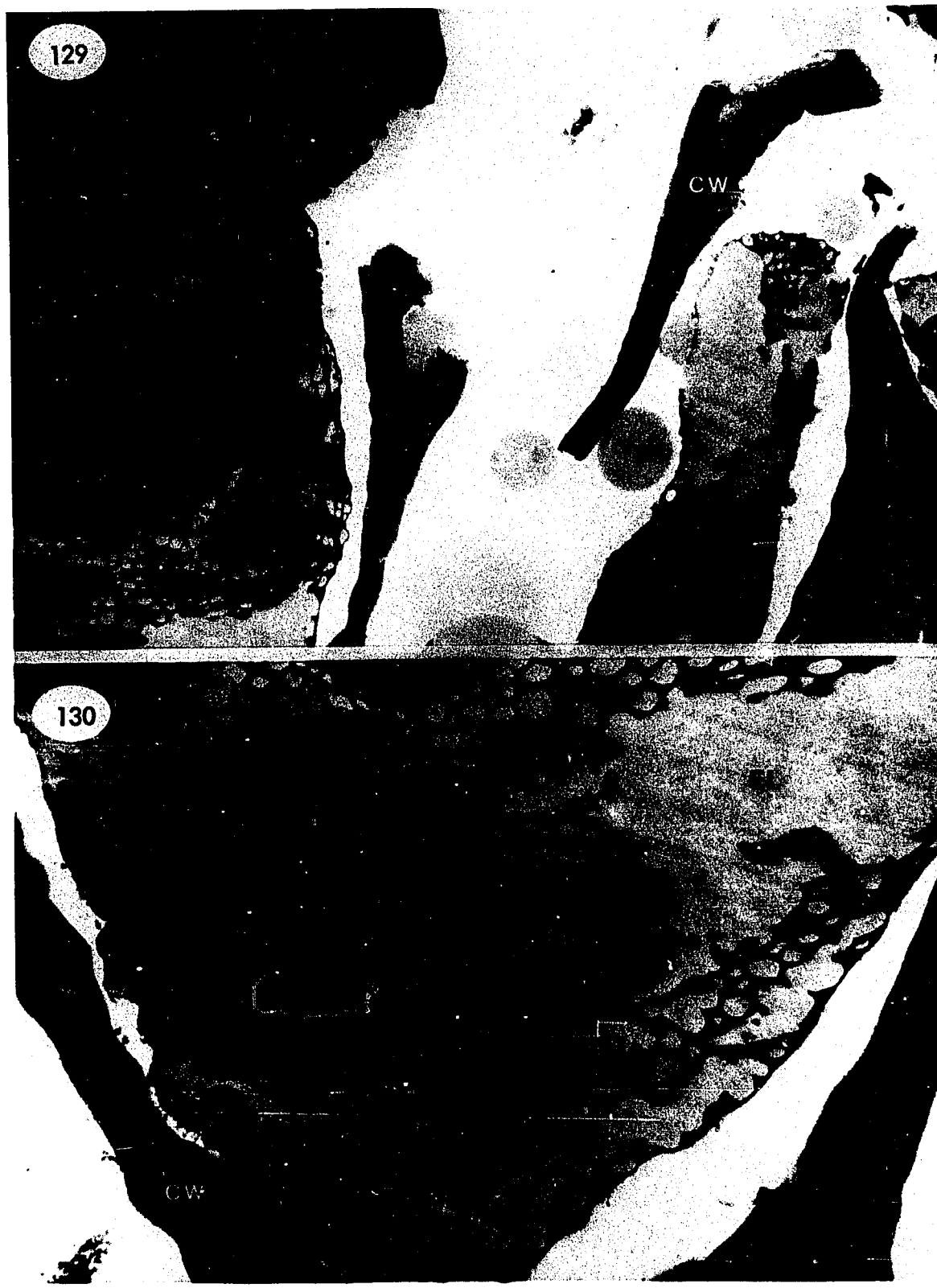
Fig. 128. Arrow indicates fractured cell wall 7,590X


127
Pb
CW
128
Pb

Figs. 129-130. TEM of defatted soybean flakes

Fig. 129. Shows fragmented cell walls and extracted lipid bodies 5,456X

Fig. 130. Lipid is effectively extracted from lipid bodies. Some lipid is coalesced during flaking or extraction 12,925X

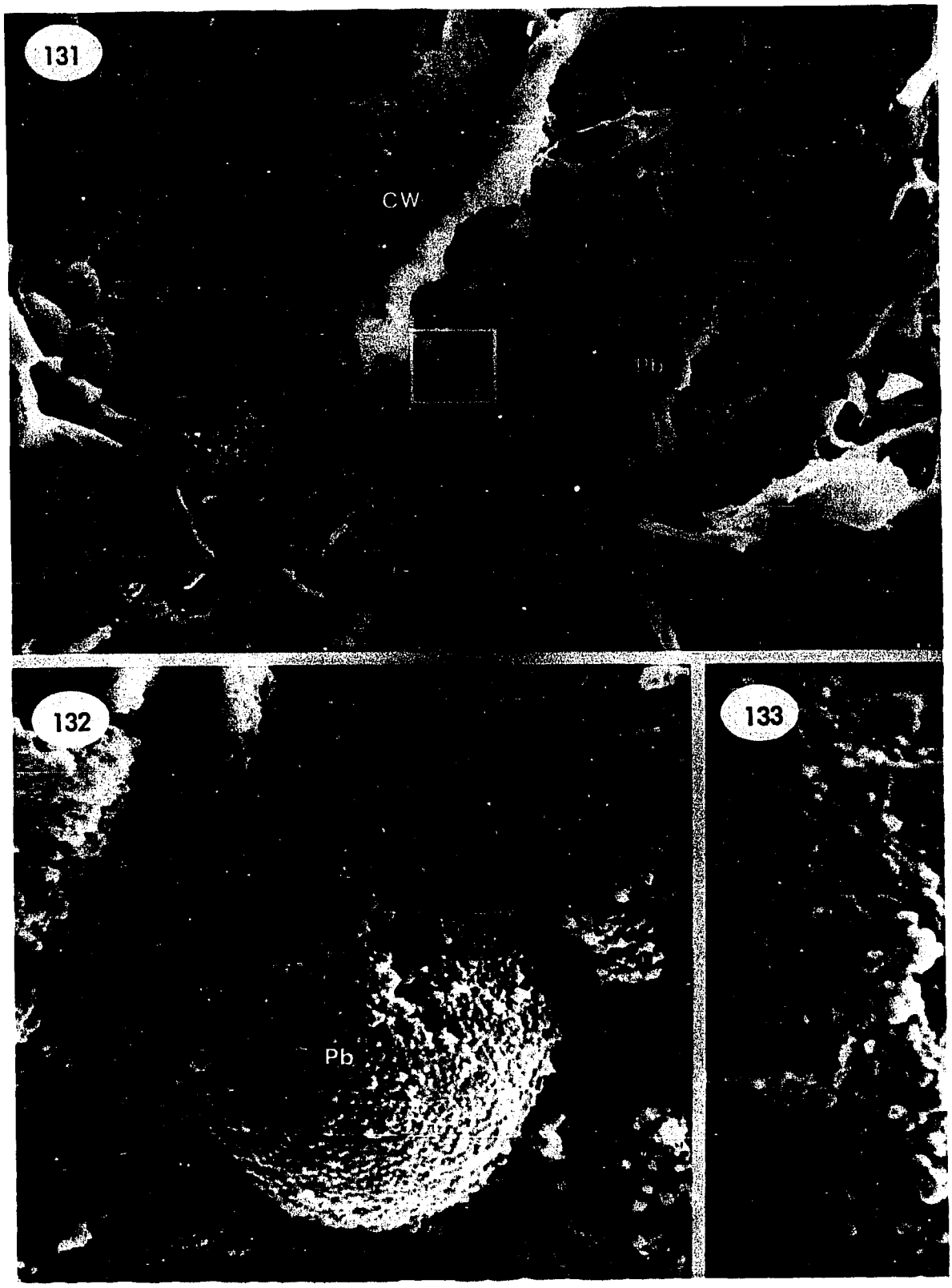

129
CW
130
CW

Figs. 131-133. SEM of freeze fractured defatted soybean flake

Fig. 131. Protein bodies appear more exposed in defatted flakes 1,320X

Fig. 132. Higher magnification of protein body appearing in enclosed box in Fig. 131. Protein body is made up of particulate material 11,700X

Fig. 133. Higher magnification of protein body showing particulate structure 20,475X


131
CW
Pb
132
Pb
133

in diameter. However, buffering the medium to pH 5, the pH of minimum solubility of glycinin prevented rupture of protein bodies. Wolf and Baker (1975), reported that protein bodies from intact defatted cells showed a sponge-like texture with many depressions where lipid bodies had been. The TEM views (Figs. 129 and 130) of protein bodies after lipid extraction, however, do not show any particles of the size (0.1 to 0.5 μm) seen in Figs. 132 and 133.

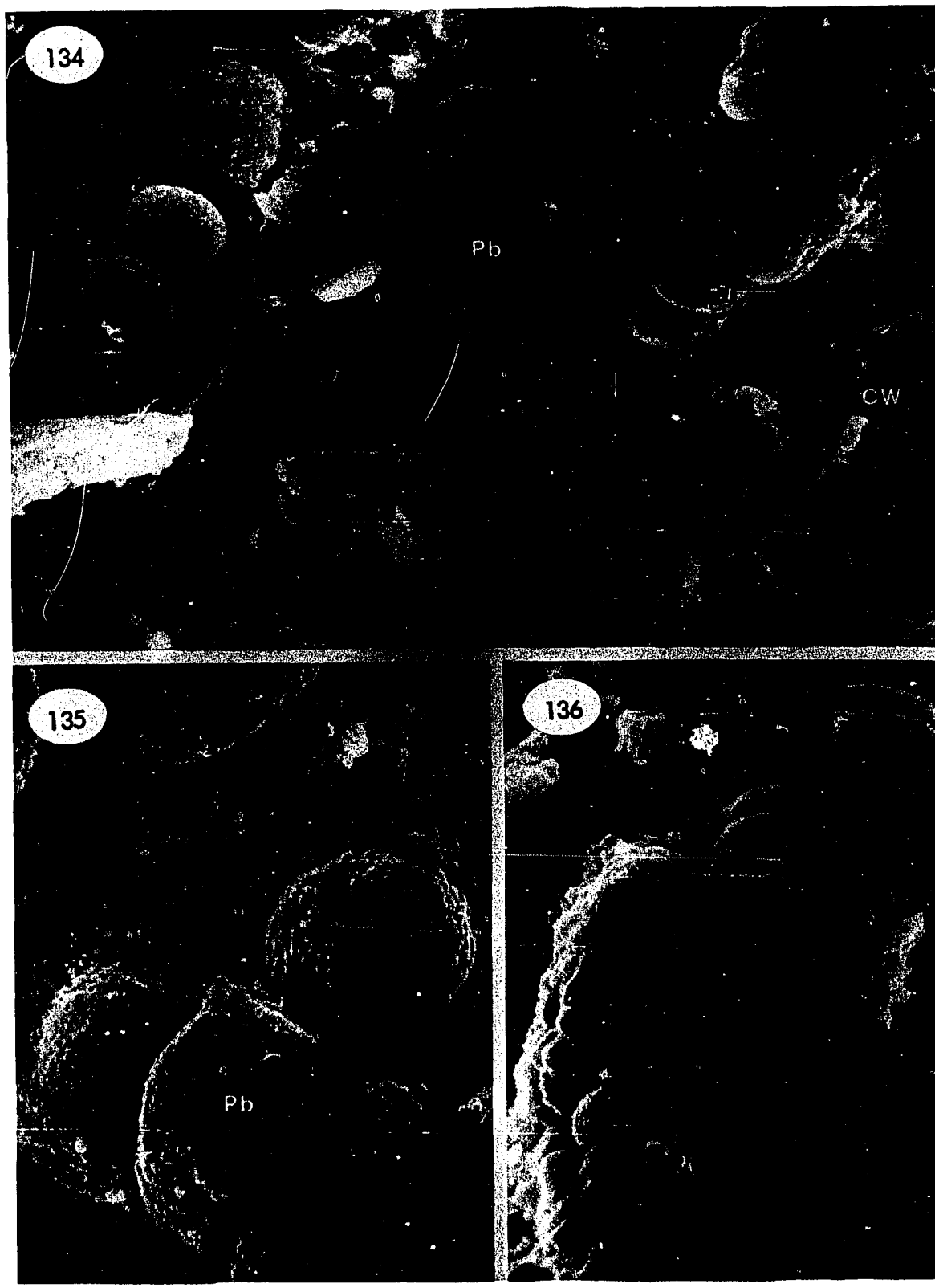
Desolventizing-toasting is another step in soybean processing that has noticeable effects on the cellular and subcellular structure. Fig. 119 shows the gross flake structure with a flowing or melted appearance due probably to the heating and rapid escape of hexane during the process. Sipos and Witte (1961) reported that rapid heating of solvent laden flakes was responsible for most cellular disruption. However, my study indicates that flaking plays the major role in cellular disruption. The photomicrograph in Fig. 123 shows the flowing appearance of a desolventized-toasted flake. Protein bodies are still evident in desolventized-toasted flakes, but are aggregated or coalesced into masses (Figs. 134-136). They are somewhat distorted or elongated in shape, while the surface of the protein bodies still show granularity, but more prominently in some than others. Also evident in Figs. 134-136 are numerous thin fibers that appear to originate from cell walls. Figs. 137 and 138 are TEM views of desolventized-toasted flakes and show the distorted and elongated appearance of

Figs. 134-136. SEM of freeze fractured desolventized-toasted soybean flakes

Fig. 134. Protein bodies are distorted, elongated and massed together 3,800X

Fig. 135. Coalesced protein bodies, still showing particulate surface 9,400X

Fig. 136. Protein body massed together 5,100X


134
Pb
CW
135
Pb
136

Figs. 137-138. TEM of desolventized-toasted soybean flakes

Fig. 137. Protein bodies have a flowing appearance 4,389X

Fig. 138. Protein bodies massed or coalesced 6,523X


137
CW
138
PD
CW

protein bodies. Fig. 138 shows considerable shrinkage of the cytoplasm away from the cell wall. Little difference in cellular and subcellular structure was observed upon drying the desolventized-toasted flakes.

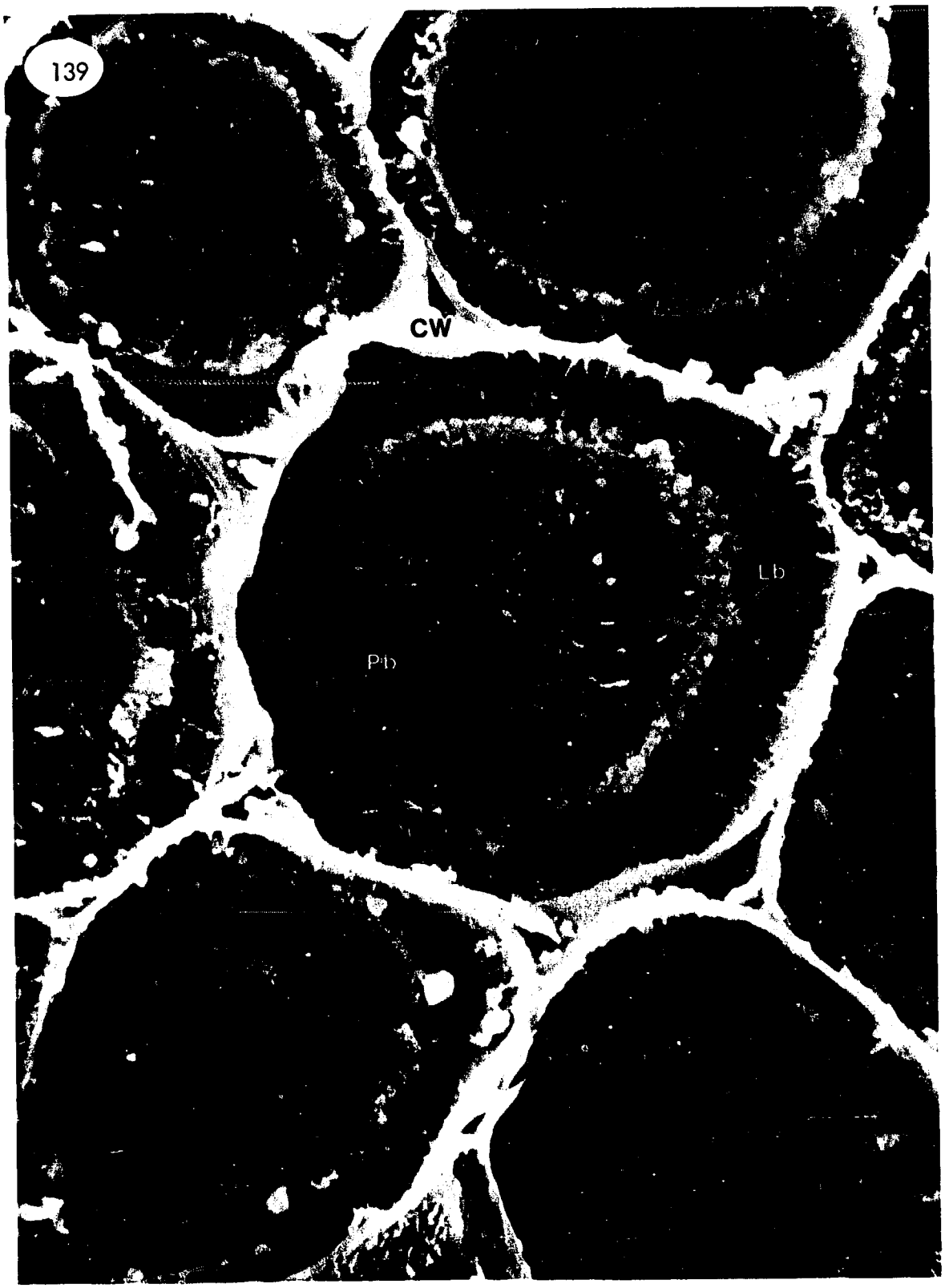
PDI values for the desolventized-toasted flakes were 52 compared to 80-85 for unheated flakes (Table 2). This indicates a mild desolventizing-toasting since usual PDI values are about 20 (Becker, 1971).

Effect of boiling

Fig. 139 is a cross section of a freeze-fractured soybean cotyledon which was boiled 15 min in water. The most apparent difference in cellular structure, as compared with unheated tissue, is the shrunken cytoplasm. The distance of cytoplasm shrinkage from cell wall various from cell-to-cell, but an average of 5.0 μm is found. Also numerous fibers are seen connecting cell wall and cytoplasm. The dark spherical bodies located within the cytoplasm are protein bodies and range in size from 5.0 to 10.0 μm in diameter. Some lipid bodies are seen towards the perimeter of the cytoplasm, however few are distinguished on the fractured surface. Cell walls appear to be intact in the boiled tissue.

Longitudinal freeze fractures of boiled cotyledons (Figs. 140 and 141), show coagulated masses of protein bodies, fused together in a lipid-cytoplasm matrix. However, the outlines of some individual protein bodies are still recognized throughout the tissue (Fig. 141). In some boiled soybean cotyledons, however, protein bodies and lipid bodies are extensively intermeshed and few

Fig. 139. SEM of soybean cotyledon boiled 15 min in water. Cell cytoplasm is shrunken away from cell wall 2,750X


139
CW
Lb
Pb

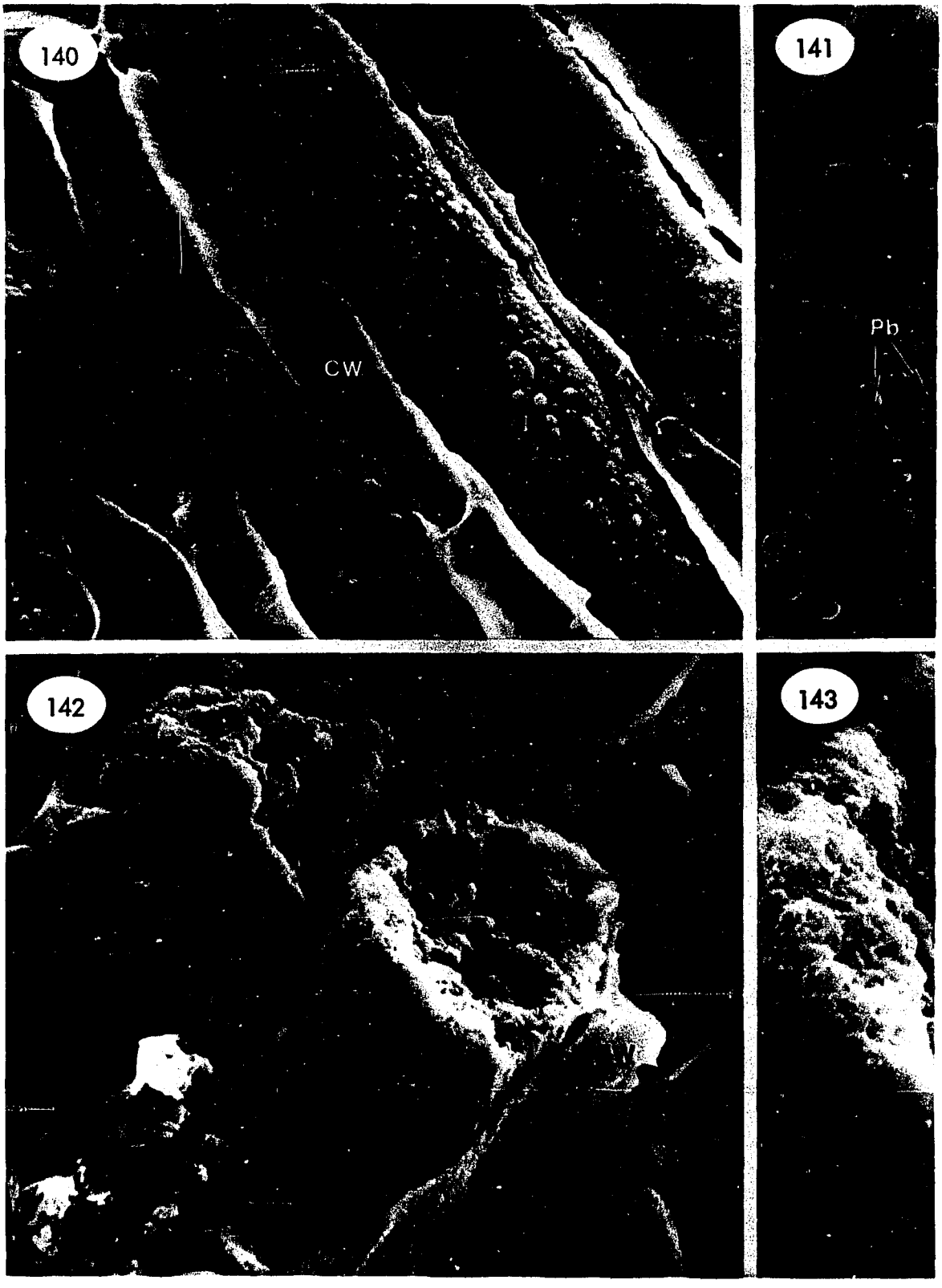
Figs. 140-143. SEM of soybean cotyledons boiled 15 min in water

Fig. 140. Longitudinal freeze fracture 1,224X

Fig. 141. Higher magnification of protein bodies 2,550X

Fig. 142. Fractured cotyledon cells 2,210X

Fig. 143. Higher magnification of Fig. 142 9,000X


140
CW
141
Pb
142
143

appear as separate distinct bodies (Figs. 142 and 143).

TEM reveals lipid bodies which are irregularly-shaped and distorted, unlike those observed in unheated preparations (Fig. 144). They range in size from 0.2 to 3.0 μm and sometimes appear as coalesced aggregates of two or more lipid bodies. Saio and Watanabe (1968), also using TEM, noted that when soybeans were steamed at 115° C for 30 min the lipid bodies broke-up and the released oil appeared as droplets 1.0 to 3.0 μm in diameter. Hexane extraction of boiled cotyledonary tissue (Figs. 145 and 146) reveals electron-permeable spaces indicative of extracted lipid bodies.

Effect of trypsin treatment

Trypsin treatment of unheated soybean cotyledons caused changes in protein bodies and lipid bodies. The typical granular and electron-dense matrix of protein bodies was no longer observed, instead they appeared lighter in electron-density with only a small amount of scattered dark material (Figs. 147 and 148). Lipid bodies were slightly distorted and instances of coalescence were noted.

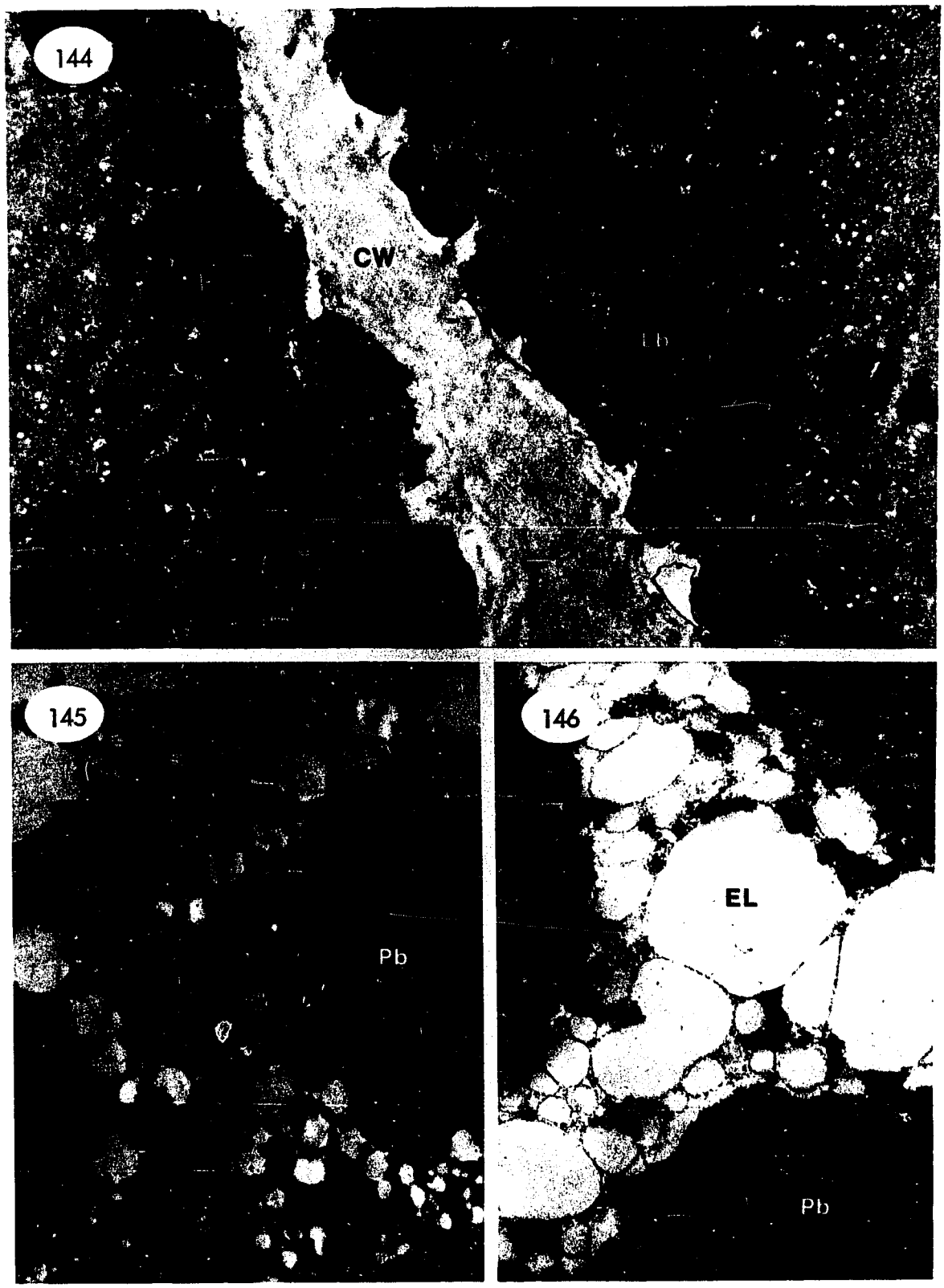
Cotyledons boiled before treatment with trypsin retained the characteristic electron-dense appearance of protein bodies (Figs. 149 and 150). Johnson and Snyder (1978) found that when soybeans were heated prior to cellular disruption, intact protein bodies (2.0 to 10.0 μm in diameter) could be observed. However, intact protein bodies were not observed in slurries of unheated soybeans.

Figs. 144-146. TEM of soybean cotyledons boiled 15 min in water

Fig. 144. Disruption of lipid bodies 12,936X

Fig. 145. Hexane extracted tissue 8,625X

Fig. 146. Hexane extracted tissue 22,100X


144
CW
Lb
145
Pb
146
EL
Pb

Figs. 147-148. TEM of trypsin treated unheated soybean cotyledons

Fig. 147. Protein bodies appear degraded 7,956X

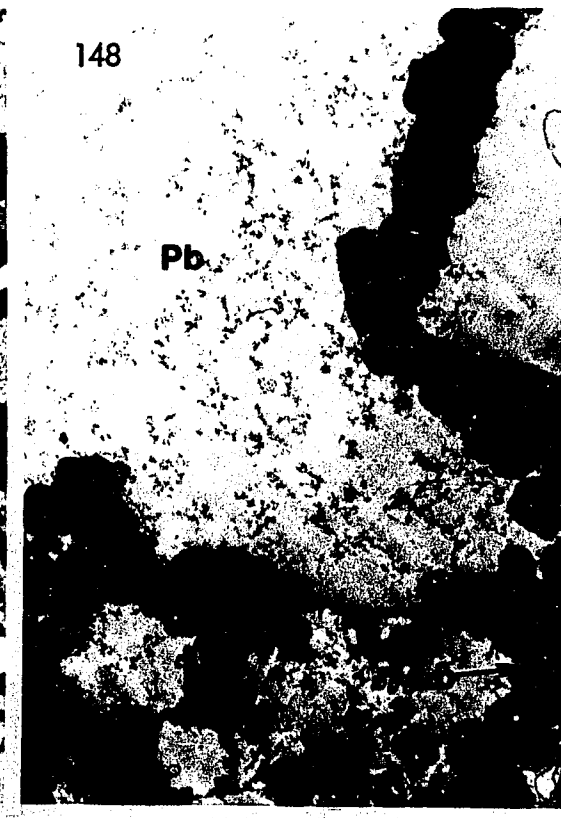
Fig. 148. Higher magnification of protein bodies 14,940X

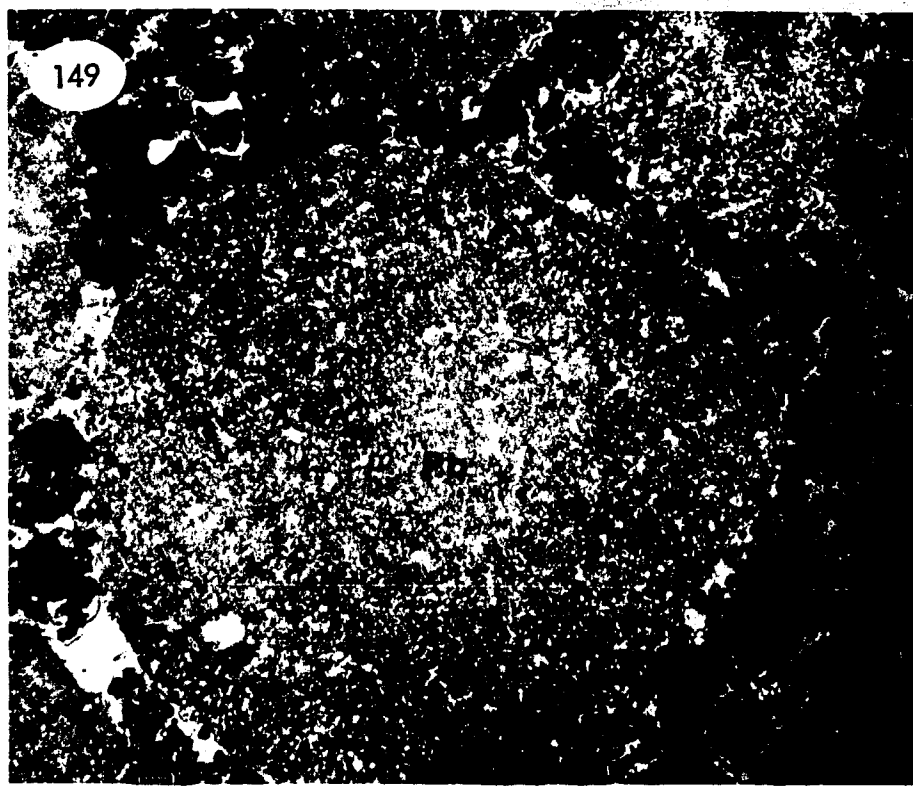
Figs. 149-150. TEM of soybean cotyledons boiled 15 min in water before trypsin treatment

Fig. 149. Protein bodies appear intact 9,988X

Fig. 150. Higher magnification 26,818X


147
CW


148
Pb


149


150

The ineffective action of trypsin on the heated protein bodies is contrary to that expected. Normally, soybeans are heated during processing to inactivate trypsin inhibitors which are inherent to the tissue. Without these inhibitors, the enzyme trypsin would be able to attack the protein uninhibited. Furthermore, heating the tissue should denature the protein, rendering it more susceptible to enzyme attack.

Effect of freezing-thawing

The process of freezing and thawing is disruptive to intact soybean cotyledons (Figs. 151-154). Lipid bodies are often seen coalesced into larger masses as shown in Figs. 151 and 154. The lipid appears less electron-dense than that normally observed in the tissue, and often looks partially extracted. The freezing-thawing process may modify the lipid bodies by disrupting their limiting membranes causing the lipid to be subsequently removed during dehydration of the tissue for TEM viewing. Little difference in protein body structure is observed. However, the cytoplasmic network containing protein bodies and lipid bodies appears shrunken from the cell walls (Figs. 151-154). Fig. 152 shows a fractured cell wall, a common observation made in tissue treated in this manner. Preliminary studies have shown that a larger fat pad and a higher percentage of lipid is obtained during lipid body isolation of frozen and thawed soybeans.

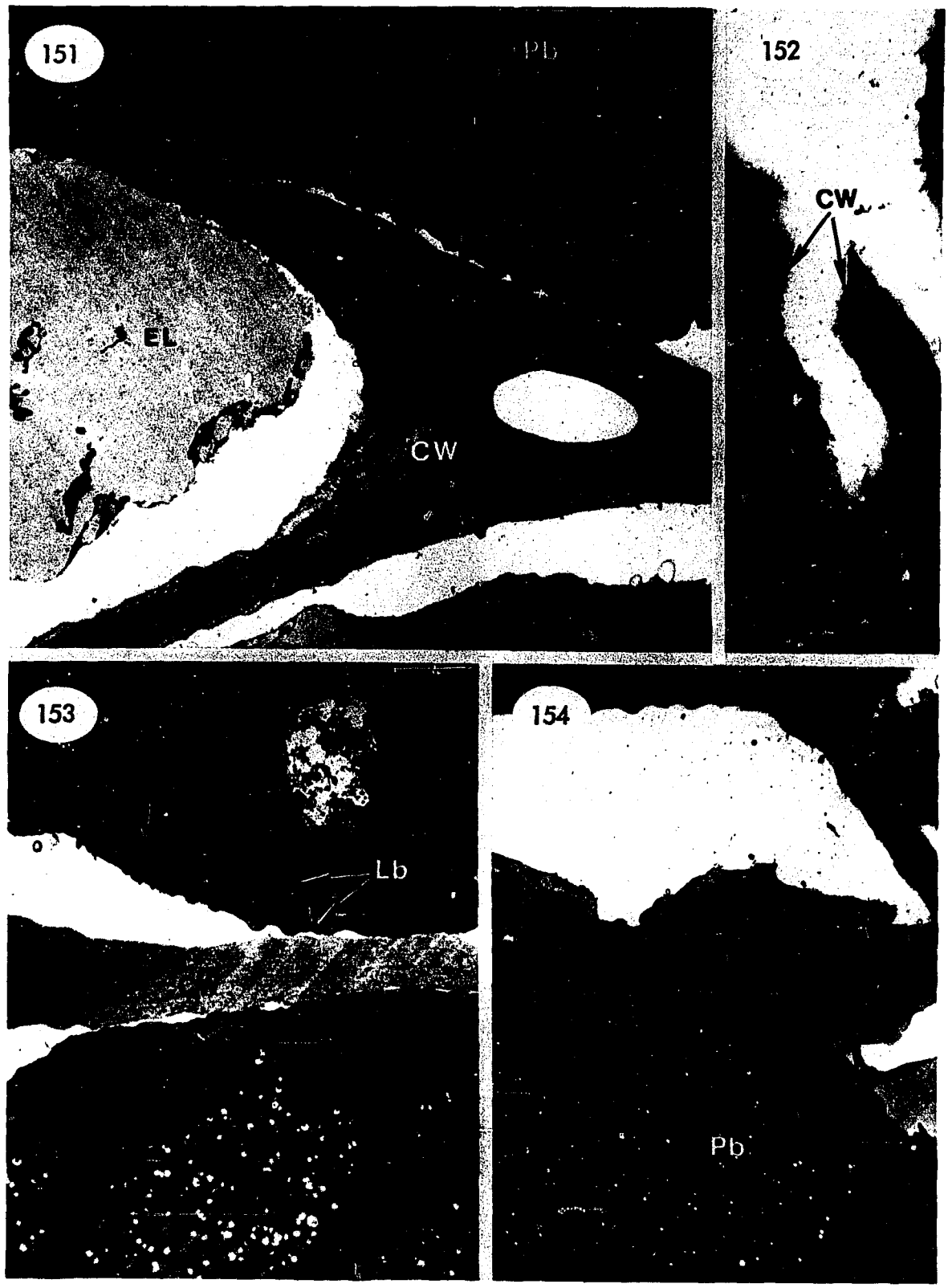
Figs. 151-154. TEM of soybean cotyledons subjected to freezing-thawing treatment

Fig. 151. Cell disruption showing coalesced lipid bodies 7,935X

Fig. 152. Fractured cell wall 17,975X

Fig. 153. Cellular disruption 6,210X

Fig. 154. Cellular disruption showing coalesced lipid bodies 7,956X


151
Pb
EL
CW
152
CW
153
Lb
154
Pb

Effect of solvent extraction

Solvent extraction is the most common form of removal of oil from soybeans. In the United States, the petroleum hydrocarbon mixture called hexane (bp. 66 to 69° C) is widely used. Cotyledonary tissue extracted with hexane is shown in Figs. 155 and 156. Small (0.1 to 0.5 μm) electron-permeable spaces observed throughout the tissue are lipid bodies which have been extracted of their oil. All other cellular components appear to be unaltered by hexane extraction. Proximate compositions of soybean cotyledons extracted with various solvents are shown in Fig. 157. When acetone was used as the extracting solvent, no difference in proximate composition or microscopical appearance was noticed.

Fig. 158 is a TEM micrograph of a soybean cotyledon following extraction with chloroform. Lipid bodies are effectively extracted; however, some lipid bodies appear distorted (Fig. 158, arrows), atypical of nonextracted tissue. Proximate composition of the extracted tissue was similar to those already reported.

When tissues were treated with methanol, cellular structure appeared disrupted and distorted (Fig. 159). Lipid bodies were affected the most and were more electron-dense than in tissues extracted with the other solvents. Lipid analysis (Fig. 157) revealed that 48% lipid was extracted from the tissue which was obviously greater than that normally found in the cotyledons. Therefore, other components, probably sugars were extracted along with the lipid, which caused the lipid content to appear high. Proximate composition of the tissue (Fig. 157)

Figs. 155–156. TEM of soybean cotyledons treated with hexane

Fig. 155. Tissue showing extracted lipid bodies 6,200X

Fig. 156. Tissue showing extracted lipid bodies 22,150X

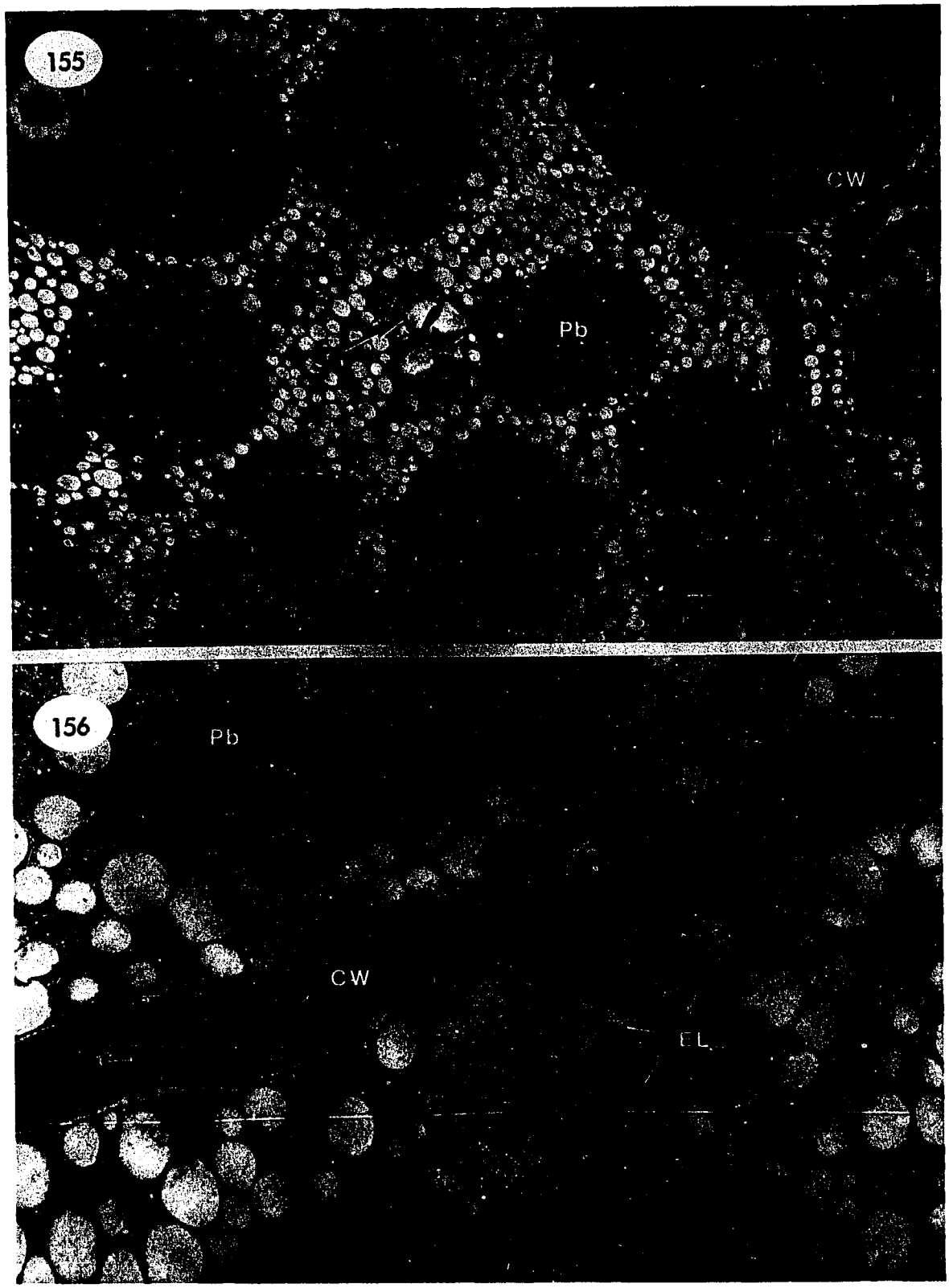
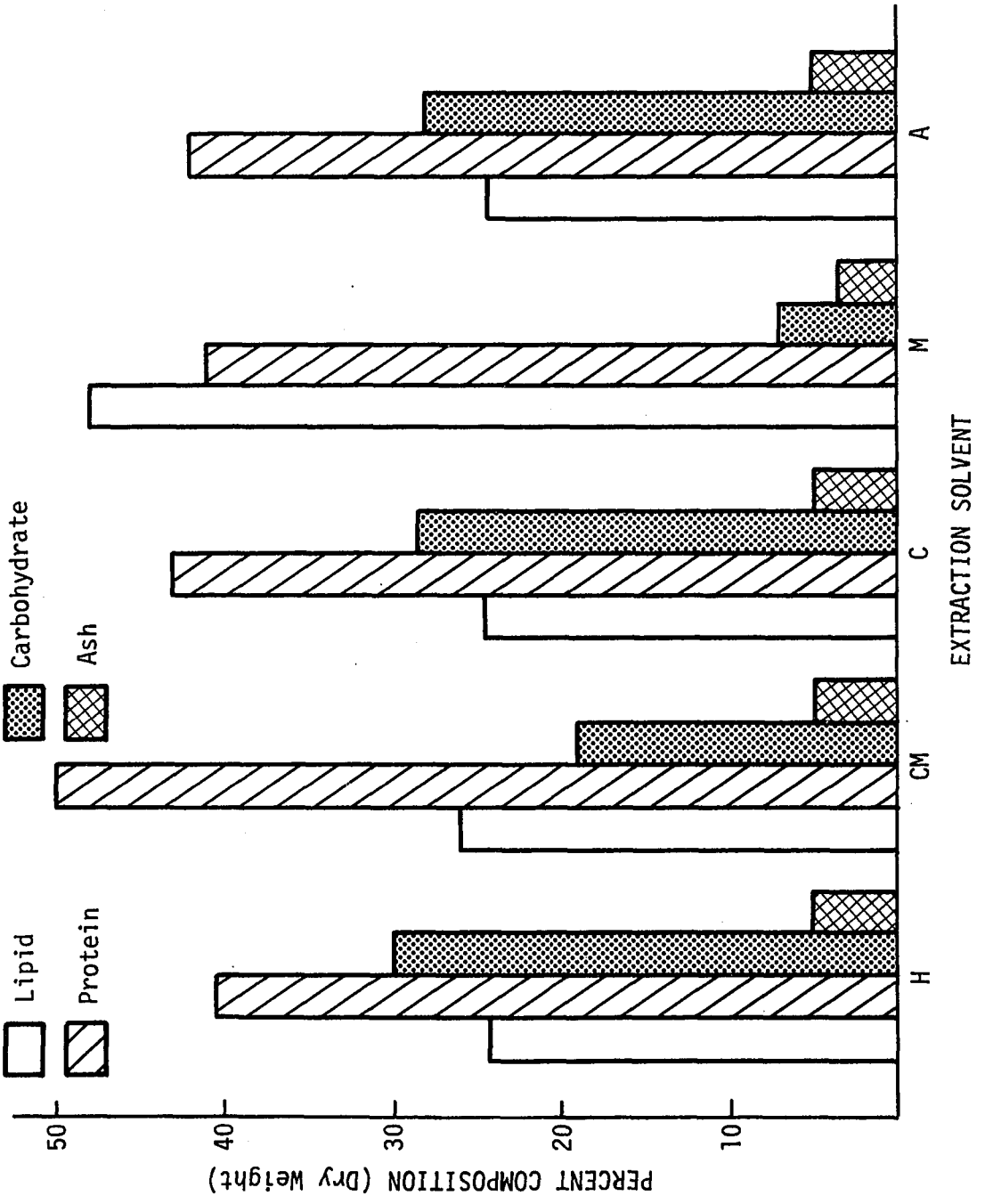

155
CW
Pb
156
Pb
CW
EL

Fig. 157. Percent composition of soybean cotyledons following extraction with various solvents. Protein, ash and carbohydrate were analyzed on extracted cotyledons. (H) hexane, (CM) chloroform; methanol 2:1 (v/v), (C) chloroform, (M) methanol and (A) acetone


Lipid
Protein
Carbohydrate
Ash
PERCENT COMPOSITION (Dry Weight)
50
40
30
20
10
H
CM
C
M
A
EXTRACTION SOLVENT

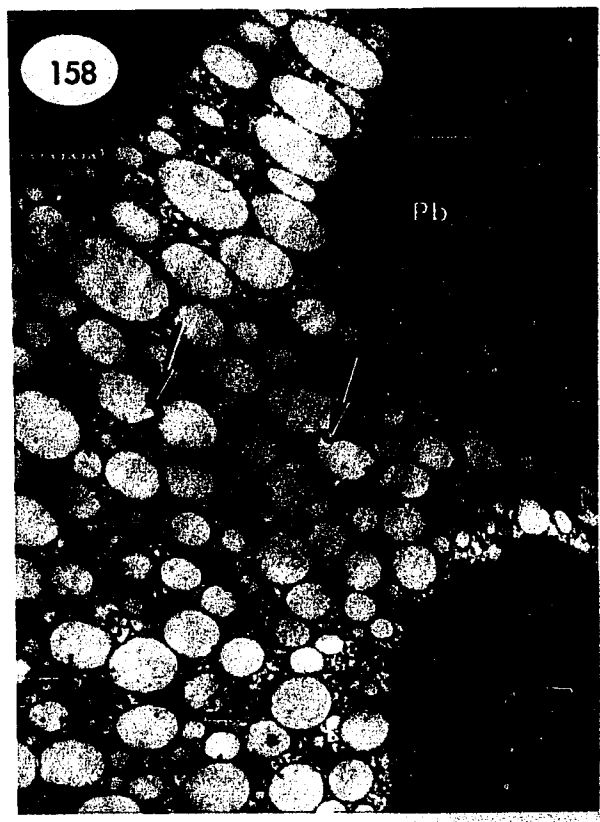
Figs. 158-161. TEM of soybean cotyledons treated with various solvents

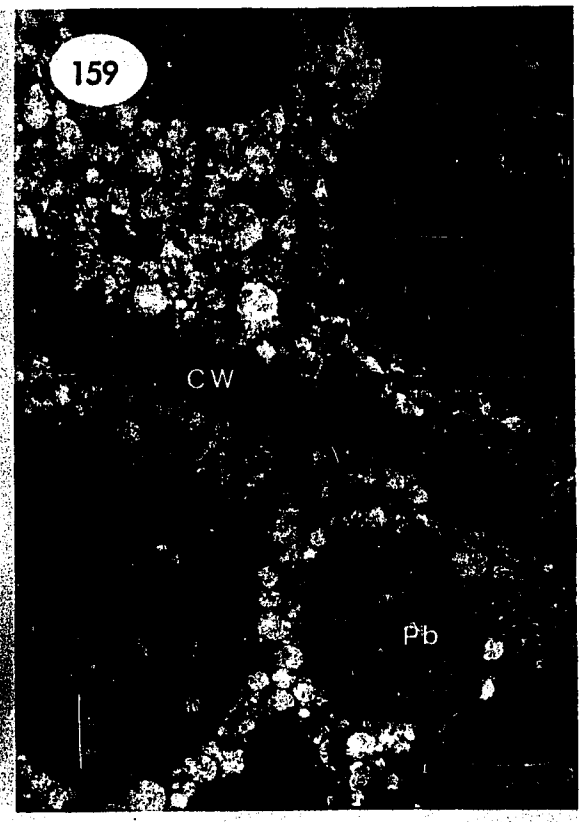
Fig. 158. Chloroform treated tissue, showing disrupted lipid bodies (arrows) 16,956X

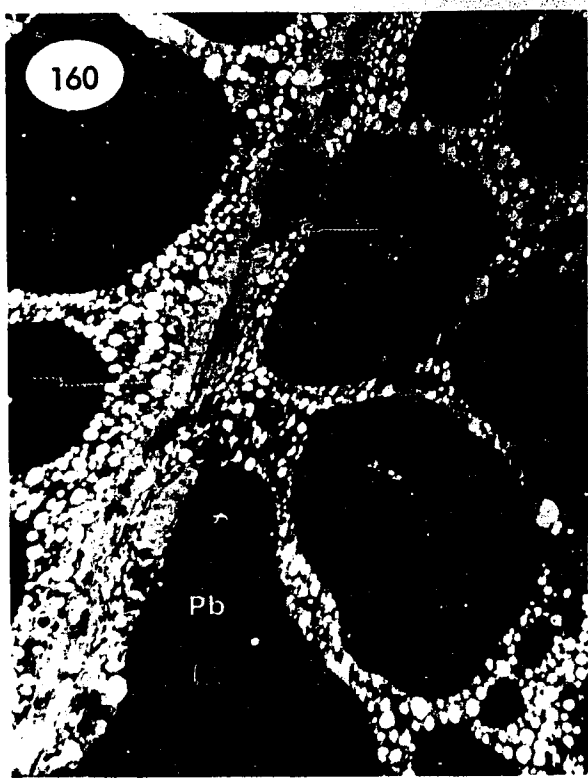
Fig. 159. Methanol treated tissue 10,575X

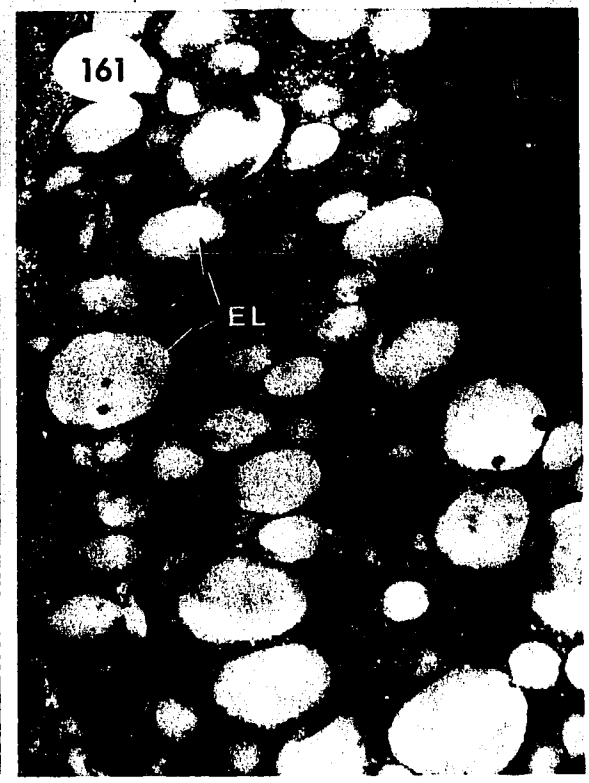
Fig. 160. Chloroform:methanol 2:1 (v/v) treated tissue 3,591X

Fig. 161. Higher magnification of chloroform:methanol 2:1 (v/v) treated tissue 29,772X


158
Pb


159
CW
Pb


160
Pb


161
EL

shows that protein and ash content are similar to those analyzed in the other tissues; however, the amount of carbohydrate has been considerably reduced from 30% found in hexane extracted tissue to 17% in the methanol treated cotyledons. When soybean cotyledons were treated with chloroform-methanol 2:1 (v/v) (Figs. 160 and 161), some cellular disruption of lipid bodies was noticed; however, it was not as extensive as with methanol alone. Proximate analysis of tissues extracted with chloroform-methanol showed a surprisingly large amount of protein, 10% higher than normally found, and carbohydrates were decreased to 18%.

Effect of sonication

Sonication of soybeans had no effect on the structural appearance of the cotyledonary tissue.

FIGURE ABBREVIATIONS

AP	amyloplast	Lb M	lipid body mass
C	crystal	M	mitochondrion
Cot E	cotyledon epidermis	Mb	membrane
Ch	chloroplast	N	nucleus
CL	coalesced lipid	Nu	nucleolus
CN	cytoplasmic network	P	protein
CW	cell wall	Pb	protein body
D	dictyosome	Pb D	protein body depression
EL	extracted lipid	PC	palisade cell
GC	globoid crystal	PM	protein mass
Hp	hypocotyl	RER	rough endoplasmic reticulum
Lb	lipid body	SG	soft globoid
		St	starch

Fig. 162. Explanation of figure abbreviations

SUMMARY

TEM reveals that some soybean protein bodies contain globoid inclusions. These globoids vary in their appearance and electron-density. Electron-dense globoid crystals were observed in some protein bodies and upon X-ray microprobe (EDX) analysis, high levels of P were detected. Similar globoid crystals in other tissues have been determined to be phytin. Other globoids in soybean protein bodies appear electron-translucent (soft globoids), electron-translucent with an inner fringe and some which are similar in appearance and electron-density to surrounding lipid bodies.

Contrary to most reports, starch granules were found in the mature soybean cotyledon. They were spherical to elongate in shape, ranged in size from 0.5 to 3.0 μm in length and displayed centric polarization crosses when viewed with polarized optics. Starch granules were absent at the periphery of the cotyledons and appeared concentrated towards the cotyledon mid-line.

Crystals of calcium oxalate were photographed for the first time in soybeans. They ranged in size from 8.0 to 10.0 μm in length and 2.0 to 3.0 μm in width and appeared as highly polarizing particles displaying a variety of shapes when viewed under polarized optics. Calcium oxalate crystals were not observed in hypocotyl tissue but were distributed throughout the cotyledons. The amount of oxalate and its nutritional significance in soybean cotyledons remains unknown. Lipid bodies appear as small (0.05 μm to 1.0 μm in diameter) subcellular inclusions

that are predominantly spherical and extremely electron-dense in TEM preparations. Most appear to be delimited by single-line (half-unit) membranes measuring 2.5 nm to 3.5 nm in thickness, although some with thicker boundaries (9.0 nm to 10.0 nm) have been observed. Soybean lipid bodies closely align or associate with single-membrane bound structures (protein bodies, cell wall, unidentified structures) but never with double-membrane bound components (mitochondrion, amyloplasts and nuclei).

Isolated lipid bodies from mature soybean cotyledons appear to be relatively sturdy and easily survive homogenization and centrifugation. Isolation procedure, isolation medium and the initial bean:isolation medium ratio affected the proximate composition of the floating fat pad layer; however, centrifugation temperature was not critical for their isolation. Preparations containing 80% lipid and 17% protein were obtained using 0.5 M saline as the isolation medium. Higher percentages of lipid were attained when soybeans were heated prior to lipid body isolation. Soybean lipid bodies have different densities, as evidenced by their appearance in continuous and discontinuous sucrose density gradients. They must be a mixture rather than pure lipid. Trypsin treatment causes isolated lipid bodies to coalesce and appear as irregularly-shaped masses with scattered electron-dense perimeters. When isolated lipid bodies are extracted with hexane, only electron-translucent "ghosts" and their delimiting electron-dense membranes remain.

The germinating soybean cotyledon undergoes a substantial increase in fresh

weight and reaches a maximum of 750 mg about a week after germination. By 2 days after germination, the protein bodies are the first of the reserve food substances to exhibit change. They become granular in appearance and more irregular in shape and begin to coalesce to form larger protein masses. Protein bodies apparently lose their limiting membranes upon coalescence. A proliferation of starch granules is also apparent in 2 day cotyledons.

Cotyledon cells of 4 day germinated soybeans have large vacuolated regions occupying their central portions, filled with material of uniformly low electron-density, presumably protein. The number of lipid bodies in the cells has modestly decreased, and lipid bodies are distributed around the inside perimeter of the cells. A decrease in lipid composition from 24% to 21% was found in the tissue. Lipase activity failed to show positive localization in intact tissue or in isolated lipid bodies.

In 6 day cotyledons, protein masses and lipid bodies continued to decrease with a concomitant proliferation of immature plastids and starch. Probably the conversion of lipid and protein takes place at a rate greater than needed to satisfy the immediate needs of the developing seedling. Thus to maintain a satisfactory osmotic balance, the excess is converted to starch.

Glyoxysomes were identified in germinating soybean cotyledons as small (0.4 μm to 0.9 μm in diameter), spherical, electron-dense inclusions. Their role in the degradation of lipid bodies in soybean cotyledons remains unsolved.

By 8 days after germination the lipid content decreased to about 18% and total carbohydrate markedly increased. This increase is due to the concomitant decrease in both protein and lipid. Localization of acid phosphatase in 8 day cotyledons was found in protein bodies and not in surrounding lipid bodies.

Most of the recognizable cellular components of cotyledons, except for cell walls, are absent by day 12. However, chemical analysis of the tissue gives 38% protein and 10% lipid.

Fresh weights of developing soybean cotyledons from 10 days after flowering (DAF) to maturity (70 DAF) reached a maximum of 590 mg two weeks before maturity. Then they lost a considerable amount of water, so that they had fresh weights of 200 to 210 mg at maturity. Soybean cotyledons 10 DAF contained irregularly-shaped cells and a variety of densely-stained cellular inclusions. However, the prominent structures of the mature cotyledon (protein bodies and lipid bodies) are absent.

A large increase in cell size occurs from 20 to 30 DAF. The palisade cells of the cotyledon show the first sign of lipid bodies. Lipid bodies appear to start as minute vesicles originating from the ends of endoplasmic reticulum; however, it may also be reasonable to assume that lipid synthesis occurs external to, or on the surface of the endoplasmic reticulum.

Cotyledonary tissue at 40 DAF showed electron-dense spheroids closely aligning the inside wall of vacuolated regions in the cell. The dark spheres are

presumed to be synthesized protein. By 50 DAF, rudimentary outlines of protein bodies are observed within the cells. Numerous starch granules and lipid bodies are found throughout the immature cotyledons.

Individual protein bodies are readily distinguished in the palisade cells of 60 DAF cotyledons. By this developmental stage, the lipid, protein, carbohydrate and ash content of the cotyledon have reached a level comparable to that of the mature cotyledon.

Ultrastructural changes in soybeans during processing to yield desolventized-toasted meal were examined. Flaking the cracked pieces of soybean did not alter the proximate composition but did disrupt most of the cells. Protein bodies, which were not covered by the cytoplasmic network, had smooth surfaces. The general appearance of protein and lipid bodies in ful-fat flakes are unaltered during flaking.

Defatted flakes have less than 1% lipid, and microscopically show more exposed protein bodies. The protein bodies in defatted flakes showed a particulate surface with small particles measuring 0.1 to 0.5 μm in diameter extending into the protein body.

Desolventized-toasted flakes have a flowing or melted appearance probably due to the heating and rapid escape of hexane during the process. Protein bodies are still evident in desolventized-toasted flakes, but are aggregated or coalesced into masses. Also evident are numerous thin fibers that appear to originate from cell walls.

Boiling soybean cotyledons causes the cytoplasm to shrink away from the cell wall and causes aggregation of protein bodies. In TEM preparations lipid bodies are irregularly-shaped and distorted, unlike those observed in unheated preparations.

Trypsin treatment of unheated soybean cotyledons causes the typical granular and electron-dense matrix of protein bodies to disappear and causes lipid bodies to be slightly distorted with some coalescence. However, cotyledons boiled before treatment with trypsin retained the characteristic electron-dense appearance of protein bodies.

The process of freezing and thawing caused lipid bodies to coalesce and to appear less electron-dense than those normally observed.

Cotyledonary tissue extracted with hexane leaves numerous electron-permeable spaces which once contained the oil. All other cellular components appear to be unaltered by hexane. Chloroform also extracts the oil from the lipid bodies; however, some of them appear distorted and atypical. When tissues are treated with methanol, cellular structure appears disrupted and distorted. Lipid analysis revealed that 48% lipid was extracted, which was greater than that normally found in the cotyledons. Protein and ash content were normal but carbohydrate was only 17%.

When soybean cotyledons were treated with chloroform-methanol 2:1 (v/v), some cellular disruption of lipid bodies was noticed, but was not as extensive as with methanol alone. Acetone extraction showed no differences in cellular structure from that observed in the hexane extracted soybean cotyledons.

Sonication of soybeans had no effect on the structural appearance of the cotyledonary tissue.

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