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Ultrastructural Localization of Solanidine in Potato Tubers

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ULTRASTRUCTURAL LOCALIZATION OF SOLANIDINE IN POTATO TUBERS

by

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Shau-Ron Han

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ABSTRACT

Ultrastructural Localization of Solanidine in Potato Tubers

by

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Department: Plant Science

Solanine, the green substance in potato, Solanum tuberosum L., tubers, is a toxic glycoalkaloid that is a potential human health hazard. To control the formation of this glycoalkaloid a greater understanding of its site of synthesis is needed. Labelling of solanidine, a direct precursor in the biosynthesis of solanine, with digitonin may indirectly locate the site of solanine synthesis in tubers. A study using ultrastructural cytochemical techniques was initiated to explore this possibility.

Sprouted tips and peridermal complex (periderm and cortex) tissue were fixed three different ways: (1) glutaraldehyde only, (2) osmium tetroxide and glutaraldehyde, and (3) glutaraldehyde-osmium tetroxide-digitonin mixture. The glutaraldehyde-osmium tetroxide-digitonin mixture provided the best fixation for this study and was used throughout.

An alkaloid extract of potato tissues incubated with digitonin resulted in a precipitate being formed. Application of the alkaloid extract to TLC

plates before and after incubating with digitonin indicated only solanidine was removed by the digitonin.

In the electron micrographs the solanidine-digitonin complex was recognized as darkly stained needles or spicules. These spicules were observed mostly in the vacuoles in the sprouted tips. However, a few spicules were also noted in the cytoplasm. Relatively few spicules were observed in the peridermal tissue.

(94 pages)

INTRODUCTION

In most supermarkets, potato tubers are displayed under artificial light to appeal to the customers. Such exposures cause greening, and an associated bitterness. The development of chlorophyll is responsible for greening, while the bitter taste is due to an excess of toxic glycoalkaloids, predominantly solanine (Hilton, 1951). Any mechanical injury during post harvest handling and marketing stimulates glycoalkaloids synthesis (Wu and Salunkhe, 1976). Sprouting of tubers is another problem confronted by the potato industry during their long-term storage. Initiation of sprouting leads to accumulation of glycoalkaloids in the eye region, and further growth develops the highest concentration of the glycoalkaloids in the sprouts (Wolf and Duggar, 1940). Formation of these components results in reduced quality of flavor, appearance, and nutritive value. It also makes them unfit for consumption even though they are still sold.

Ingestion of such potato tubers containing high amounts of solanine may cause severe illness, and occasionally death in humans and in livestock. Symptoms of poisoning generally include gastrointestinal and neurological disturbances. The instances of human and animal poisoning from the consumption of potato tubers are well documented (Hansen, 1925; Domon, 1928; Harris and Cookburn, 1918; Bömer and Mattis, 1923; Greibel, 1923; Willimott, 1933; Wilson, 1959; Zitnak, 1970. Renwick (1972) observed that the substances

present in potato tubers stressed by pathogens or other environmental agents can cause congenital malformations in newborn children.

The problem of potato greening and sprouting in thus both an economic problem and a potential health hazard. In recent years, this problem has become more serious because of the technological developments in handling, transporting and merchandising of potato tubers. Moreover, solanine is not destroyed by cooking, baking, or frying. Several physicochemical methods for the control of greening and solanine formation have been studied. The practical use of these methods has certain limitations because or marketing trends and primarily as potential health hazards. The only effective way to control this glycoalkaloid is to inhibit its synthesis in the tuber. A perusal of the literature indicates that the details of solanine synthesis are still being investigated. Attempts to localize the glycoalkaloids biosynthesis within plant cells are also underway (Toddick, 1977; Gizella, 1970). Williamson (1969), Napolitano and Scallen (1969), Gautheron and Chevallier (1971), Frühling, Penasse, and Claude (1969) and others have used the saponin digitonin to enhance cholesterol preservation in animal tissue. Since solanidine, a direct precursor in the biosynthesis of solanine, has a chemical structure similar to cholesterol there is the distinct possibility of using digitonin to mark the location of solanidine, and, thus, indirectly localize solanine. The work reported herein was undertaken to localize the free steroidal alkaloid, solanidine, by using ultrastructural cytochemical techniques.

REVIEW OF LITERATURE

The potato plant is a native of the highlands of South and Central America. A wild tuber-producing form, Solanum jamesii, is still found in the mountain of Arizona, Colorado, Texas, Utah, and Mexico (Hayward, 1938). The wild species resembles the cultivated Solanum tuberosum L., which is an annual herbaceous dicotyledon, a member of the Solanaceae. There are many poisonous plants in this family, such as, deadly nightshade, Solanum dueamara L., jimson weed, Datura stramonium L. (Jeppsen, 1974), and tomato, Lycopersicon esculentum Mill (Stecher, Windholz, and Leahy, 1968). The potato is also poisonous, especially in its above-ground parts. The edible tuber is much lower in concentrations of the poisonous glycoalkaloids than the foliage part of the potato plant. Abuses of light-exposing, wounding, brushing, dropping, bruising, or puncturing all induce the biosynthesis of these glycoalkaloids.

Anatomy of the Potato Tuber

The potato tuber is morphologically a shortened, thickened stem with scalelike leaves or leaf scars. Sections through the mature tuber show several zones. They are the periderm, cortex, vascular ring, and pith. The pith is the most prominent tissue of these three zones; with the vascular tissue being the least prominent (Artschwager, 1918).

Periderm

The outer skin consists of a layer of corky periderm, some 6-14 cells deep, which is a new tissue that replaces the epidermis of the tuber primordium. Periderm appears to retard loss of moisture and to resist attack by fungi. When a potato is cut or otherwise wounded, proliferation of cells at the surface forms "wound periderm" which is said to be even more effective than normal periderm in performing these functions (Schwimmer and Burr, 1967).

Cortex

Underlying the periderm is the cortex, a narrow layer of parenchyma tissue. The parenchyma cell in potato tubers shows an average diameter of 160-240 micrometers (Reeve, 1971). The mature cells of the cortex, like those of the pith, are polyhedral, with a median diameter of about 180 micrometers. The periclinal walls are larger, because of the great peripheral growth of the organ. Compared to the cells of the pith, however, the cortex shows a greater degree of specialization. Some of the cells, especially in the region of the eyes, have greatly thickened walls, forming typical stone cells or sclereids. Not all varieties develop these stone cells. The peripheral cortical cells either abut abruptly on the periderm or form a transition zone three to eight cells wide. The cells in this transition region are smaller than the typical cortical cells, more elongated, and nearly rectangular. They are almost devoid of starch, but contain proteins, especially in the form of

cubical crystals, and, in the case of colored varieties also the pigment (Artschwager, 1924). The cortex and the pith differ mainly in the relative amount of cellular density, the cortex being more dense on account of the smaller size of its cells and larger amount of cell content other than starch (Artschwager, 1918).

Vascular storage parenchyma cells

A central pith with its lateral branches, and the vascular ring are surrounded on either side by thick storage parenchyma. Vascular storage parenchyma, high in starch content, lies within the shell of the cortex (Schwimmer and Burr, 1967).

Vascular ring

Xylem and phloem are found in minute strands or bundles, most of which form a narrow, discontinuous ring ("Vascular ring") somewhat within the boundary between the cortex and the vascular area (Schwimmer and Burr, 1967).

Pith

Forming a small central core but radiating narrow branches to each of the eyes, is the pith, sometimes called the "water core."

It consists primarily of large cells containing less starch than cells in the vascular area and the innermost part of the cortex (Schwimmer and Burr, 1967).

Eye

The potato eye is a rudimentary scale leaf or leaf scar with its subtended axil, which contains a suppressed lateral bud and undeveloped internodes. As on the stem, these are arranged in a right-handed or left-handed spiral around the tuber, according to the variety and the individual. Thirteen eyes to five turns of the helix (5/13 phyllotaxy). Each eye contains at least three buds arranged in the form of an obtuse triangle and protected by more or less conspicuous scales. Often, however, a larger number of buds are present which then form a secondary spiral to the opposite direction, that is, to the left if the main spiral of the eyes is dextrose, or conversely if the latter is sinistrorse.

The eyes of the tuber vary greatly in size and form. In a given tuber the lowest basal eye is small and inconspicuous; the eyes in immediate succession are larger and may protrude. After the first or second turn of the spiral the eyes become uniform and characteristic of the variety. The apical eye cluster is commonly not in a direct line with the main axis, but excentric; the eyes in it are small and contain fewer buds than those of the body of the tuber. There is, furthermore, a variation in the depth of these eyes. Some are very protuberant, but most are quite shallow.

As the tuber enlarges, new buds are constantly differentiated from the growing apex, while the older ones gradually develop to maturity. The apical region, well protected by overlapping scales, is composed of a mass of cells rich in protoplasm. The walls are thin and of cellulose except for

the outer wall of the epidermis, which is covered by a thin cuticle where the bud merges into the tuber. Differentiation into the characteristic tissue region takes place and the vascular tissue of the bud and bud scales joins the vascular ring of the tuber, which in the region of the eyes closely approaches the periphery. Like the vascular ring, the pith retains its connection with the lateral members of the tuber in that branches of the pit continue directly into the eyes. Since all the eyes thus maintain a connection with the central pith, sections through any part of the tuber show a system of such connecting strands of pith radiating from the center toward the periphery (Artschwager, 1924).

Sprouting of Potato Tubers

Freshly harvested potato tubers usually undergo a dormant period for several months during which time there is little or no sprout growth regardless of environmental conditions. Following the rest period sprout growth occurs at temperatures of 4.4°C or above (Jadhav, 1973). When a normal tuber sprouts, it is the eye at the bud end, corresponding to the growing tip of a stem, which develops first. Sprouts develop at few, if any of the other eyes under normal circumstances. If a tuber has been cut into pieces, as when potatoes are planted, sprouts will appear at one or more eyes on each piece (Schwimmer and Burr, 1967).

Ultrastructural Studies on Potatoes

Tsutsumi, Olivas, and Alvarez-Fuertes (1972) studied the ultrastructure of the phloem of leaves in healthy potato plants. This was carried out as a preliminary step for further identification of pathogenic agents in diseased plants. Their attention was focused on the disease called "purple top" (Olivas, Tsutsumi, and Alvarez-Fuertes, 1973).

Another ultrastructural study of the interaction between Phytophthora infestans and leaves of a susceptible (Majestic) and a resistant (Orion) potato variety was conducted by Shimony and Friend (1975). Shortly after penetration, an infection vesicle was formed in both varieties but the subsequent stages of infection differed. In Majestic, hyphae spread both intra- and inter-cellularly and even 48 hours after inoculation there were still juvenile-type hyphae inside mesophyll cells which appeared undamaged. In Orion, the juvenile parts of hyphae were not seen in or near the cells later than 7-12 hours after inoculation. Orion cells died at an early stage, but death of the fungus only occurred more than 12 hours after inoculation.

Rodionov, Kozubov, Tikhova, and Sulimova (1975) concluded that decrease in the concentration of monogalactosyl diglyceride, digalactosyl diglyceride in a mixture with sulfolipid, phosphatidyl glycerin, schlorphylls a and b, and carotinoids in potato leaves, began after 18 hours of shading. It was accompanied by thinning of the lamellar membranes, decrease in the number of granal and stromal lamellae, and formation of the plastoglobules

and electron-impermeable inclusions characteristic of chromoplasts. The concentrations of phosphatidyl ethanolamine, phosphatidyl inositol, diphosphatidyl glycerin, phosphatidyl serine, and phosphatidic acid did not change significantly in the course of 121 hours shading. After which a thickening of mitochondrial cristae packing was observed. The content of phosphatidyl choline decreased by approximately one-third. Verleur, van der Velde and Sminia (1970) reported that an increase of the mitochondrial population was observed both in wounded and diseased potato tuber tissue. Heterogeneity of the mitochondrial population was demonstrated by differences in sedimentation velocities and in biochemical properties after differential centrifugation. Electron micrographs showed particles with different complexity in structure. The distribution of protein, cytochrome oxidase activity and succinate dehydrogenase activity coincided with the number of particles observed in the fractions. Another electron microscopic study has shown that the spread of Erwinia carotovora var. atroseptica through potato tuber tissue occurred primarily via the intercellular spaces of the storage parenchyma. Restricted infection of the xylem and phloem elements also occurred, leading to localized pectolysis and melanin formation limited by the suberised vascular parenchyma. The symptoms were a maceration of the cell wall, disorganization of the cytoplasm, and a rupturing of various cell membranes which were associated with the enlargement of the microbodies (Fox, Manners, and Myers, 1972).

Barckhausen and Rosenstock (1973b) reported that in nucleoli of aging tuber disks of potato a rapid increase of the pars granulosa began shortly after slicing. At the same time, vacuoles of different sizes could be observed. In some nucleoli, however, only one central vacuole was formed, which increased continuously. Maximal size of this vacuole was reached 48 hours after wounding. At this time the remaining material of the nucleolus was arranged in a small ring around this structure. The size of these giant vacuoles, which contained RNA, was reduced in the subsequent period of woundhealing. Barckhausen and Rosenstock (1973a) also reported that within large invaginations of the nucleus as well as in protoplasmic strands the formation of smooth, tubular endoplasmic reticulum would be increased within a short time after wounding. This effect was correlated in time with the suberinization of the cells immediately bordering the wounded area.

Potato tuber buds were examined during dormancy and the initial stages of sprouting (Marinos, 1967). Under certain physiological conditions, such as dormancy, plastids in meristems are potentially multifunctional organelles, capable of performing diversified biochemical tasks resulting in the accumulation and storage of a variety of materials that can be utilized upon commencement of sprouting.

Glycoalkaloids in Potato Tubers

A mixture of the poisonous glycoalkaloids was first discovered by Desfosses (1820) in the juice of expressed berries of the black nightshade (Solanum nigrum L.). Shortly thereafter, Baup (1826) isolated them from potato plants (Solanum tuberosum L.). These include solanines, chaconines, solacauline, leptinines, and leptines (Zitnak, 1964). Until 1954, it had been considered that the cultivated form of potato contained only one alkaloid, solanine, discovered nearly 150 years ago. Kuhn and Löw (1954) reported the discovery of another glycoalkaloid, α-chaconine, in the leaves of the wild potato (Solanum chacoense), from which it was named. The occurrence of leptinines and leptines (hydroxy- and acetoxy derivative of solanine and chaconines) have been reported only in the wild potato, Solanum chacoense (Schreiber, (1968).

Zitnak (1968) found several unknown alkaloids that could be obtained from the flower of potato plants by different extraction procedures. It is interesting to note that kennebec potato tubers, when sliced and aged at room temperature for 48 hours, synthesized two new alkaloids, identified as α and β solamarine, not previously present (Shih, 1972). However, the several alkaloids existing in potatoes tubers may have arisen through hybridization of such species with the cultivated potato plant (Jadhav, 1973).

Distribution of Glycoalkaloids

In the potato plant, most of the tissues, including leaves, shoots, stems, blossoms, tubers, tuber-eyes, peels and sprouts contain the major glycoalkaloids. The alkaloid concentration is high in the shoot tip, and the flowers are particularly rich in solanine (Lampitt, Bushill, Rooke, and Jackson, 1943). Usually the glycoalkaloids are disposed in regions of high metabolic activity; i.e., meristematic regions and areas of recent chlorophyll formation (Jeppsen, 1974). Wolf and Duggar (1940) noticed high glycoalkaloid contents in leaf buds and young leaves down to about the eighth node, and beyond which there was a marked decrease. Glycoalkaloid levels in the leaves and stems of the potato plant fluctuated throughout the growing season, but decreased sharply at maturity (Wolf and Duggar, 1946). The tubers were the only organs to show a steady accumulation of total glycoalkaloids over the entire period.

Solanine, as the total alkaloid fraction, is present in the normal whole tuber to the extent of 0.01 to 0.10 percent of the dry weight (Schwimmer and Burr, 1967). The alkaloid is formed in the parenchyma cells of the periderm and cortex of the tubers and in areas of high metabolic activity such as the eye regions (Wolf and Duggar, 1940; Hilton, 1951; Reeve, Hautala, and Weaver, 1969). The concentration is arranged in a descending gradient from the outside inward. Little or none is found in the pith while only small amounts are present in the intermediate region (Lampitt et al., 1943).

Schwimmer and Burr (1967) reported that there is at least twice as much of solanine alkaloid in the peel as in the flesh. Potato periderms contained solanidine in amounts equal to those in the peeled tuber, although the periderm represented only one-seventh of the whole tuber weight (Zitnak, 1961). It is known that the maximum amount of alkaloid is found in sprouts (Wolf and Duggar, 1940). Values as high as 4.3 percent solanine (dry weight basis) have been reported for sprouts (Schwimmer and Burr, 1967). According to Guseva, Borikhina, and Paseshnichenko (1960), α-solanine and α-chaconine represent about 40 percent and 60 percent of the total glycoalkaloids of sprouts respectively.

Chemistry of Glycoalkaloids

α-Solanine is the major glycoalkaloid in potato tubers. Currently, solanine is known as a mixture of α-solanine and α-chaconine, which represent up to 95 percent of the total alkaloids. β and γ forms of both solanine and chaconine possessed a shortened chain (Kuhn and Löw, 1955a and 1955b) and were found in leaves of Solanum tuberosum and Solanum chacoense. Solanidine was mainly found in Solanaceae (Wojciechowska and Pasciak, 1973) and also isolated from Cestrum purpureum (Karawya, 1972).

α-Solanine ($C_{45}H_{73}NO_{15}$) and α-chaconine ($C_{45}H_{73}NO_{14}$) both have the same aglycone, solanidine, but different carbohydrate residues. The sugar portion of α-solanine is condensed by galactose, glucose, and rhamnose while glucose-rhamnose are present in α-chaconine (Figure 3). The sugar

sequences of solanidine glycosides also present in potato tubers are as follows: β-solanine, galactose-glucose; γ-solanine, galactose; β-chaconine, glucose-rhamnose; γ-chaconine, and glucose (Schwimmer and Burr, 1967). Solanine is soluble in acidified alcohol and insoluble in slightly alkaline aqueous solution.

Solanidine ($C_{27}H_{43}NO$), an aglycone of α-solanine or α-chaconine, is derived from cholesterol (Tschesche and Julpke, 1967). This compound is a direct precursor of the solanine alkaloid. Its structural formula is shown in Figure 2.

Biosynthesis of Glycoalkaloids

Biosynthetically, all steroidal compounds such as sterols, certain sapogenins, terpenes, hormones, and alkaloids are interrelated and pathways leading to a synthesis of a structurally similar compound could be postulated on the basis of known ones. Thus, the regular pathway starting from acetate via mevalonate, isopentyl pyrophosphate, farnesyl pyrophosphate, squalene, and cholesterol is applicable to steroidal alkaloids. Reviews of biochemical and possible biogenetic relationships of steroidal alkaloids of the Solanum group have been conducted by several authors (Heftmann and Mosettig, 1960; Heftman, 1963; Clayton, 1955; Willuhn, 1965; Schreiber, 1966; 1968).

Through several precursor studies utilizing radioactive acetate in potato sprouts, Guseva and Paseshuichenko (1958), Guseva, Paseshuichenko, and Borikhina (1961, 1963), determined that the glycoalkaloids isolated from

sprouts grown under conditions of normal illumination had the labeled carbon chiefly in the aglycone, and from sprouts grown in the dark it was in the sugar portion of the glycoalkaloids. Moreover, they observed that the aglycone could be synthesized from units of mevalonic acid which passed through a squalene stage before fruition in its steroidal form. Also mevalonic acid was more effectively utilized for the biosynthesis of glycoalkaloids of potato seedlings than was acetate. Guseva, Borikhina, and Paseshnichenko (1960) found that the specific activity of α-chaconine was higher than that of α-solanine. The activity of both the alkaloids rose progressively to a maximum at the end of 2 days after incorporation, and then fell steeply. They also found that the specific activity of solanidine prepared by hydrolysis of α-chaconine considerably exceeded that of solanidine from α-solanine. Thus, they concluded that the rate of formation and replacement of chaconine was greater than that of solanine.

Mevalonic acid is formed by condensation of three molecules of acetyl-Co A (Jadhav, 1973). The key intermediate in this process is β-hydrozy-β-methylglutaryl-Co A (HMG-Co A). The sequence of reaction begins with the phosphorylation of mevalonate by ATP and forms 3-isopentenyl pyrophosphate (Jadhav, 1973). Isopentenyl pyrophosphate is the original "isoprene" C-5 unit, from which polyisoprene compounds are formed (Guseva and Paseshuichenko, 1962).

Squalene consists of six isoprene units. The monoterpene derivative, geranyl pyrophosphate, then reacts with another molecule of isopentenyl

pyrophosphate to yield the sesquiterpene derivative, farnesyl pyrophosphate which then forms squalene. Squalene then undergoes cyclization to lanosterol and is converted into cholesterol (Jadhav, 1973). Ripperger, Moritz, and Schreiber (1971) also reported that after application of labelled lanosterol, the radioactivity was detected in the Solanum alkaloid, solanidine. Cholesterol has been shown to be metabolized to solanidine when applied to leaf surfaces of potato plants (Tschesche and Hulpke, 1967).

Cholesterol hydroxylated at C-22 prior to hydroxylation at C-16 and verazine was produced from either dormantinone or dormantinol. The resulting verazine would be converted to solanidine after hydroxylation at C-16 (Kaneko, 1975). Moreover, Canonica, Ronchetti, Russo, and Sportoletti (1977) reported that the 16β-hydrogen atom of cholesterol was lost during the biosynthesis of solanidine in Solanum tuberosum. However, according to the hypothesis of Heftman (1967), cholesterol may be undergoing cyclization in the side chain subsequent to the formation of 27-hydroxycholesterol followed by a direct replacement of the hydroxyl group by an amine function.

Kaneko, Tanaka, and Mitsuhashi (1976) conducted feeding experiments with budding Veratrum and suggested that L-arginine is the most likely nitrogen source for solanidine biosynthesis, as L-arginine-(^{15}N) was incorporated into solanidine 20 times more efficiently than $^{15}NH_4Cl$.

Schreiber (1968) suggested a hypothetical pathway of solanidine biosynthesis in budding Veratrum grandiflorum, which most actively synthesized solanidine. Cholesterol is converted to dormantinol ((25^S)-cholest-5-en-3β,

22α, 26-triol), dormantinone ((25S)-3β, 26-dihydroxycholest-5-en-22-one), verazine, etioline ((25S)-22,26-epiminocholesta-5,22(N)-diene 3β, 16α-diol), then teinemine ((25S)-22,26-epiminocholest-5-en-3β,16α-diol) to yield solanidine.

A sterically unhindered 3β-hydroxyl group of steroids could be glucosylated if the steroids belong to the 5α-H or Δ5 series (Procházka, 1971). Enzyme preparations obtained from the juice of potato sprouts caused hydrolysis of α-solanine by stepwise splitting of the sugars. The same enzyme preparation produced hydrolysis of α-chaconine, but in a different way (Guseva and Paseshnichenko, 1957). Kuhn and Low (1955a) isolated two glycoalkaloids from the leaves of Solanum avivulare, solasonine and solamargine. The same sugars were found in the sugar portion of these glycoalkaloids as in the glycoalkaloids of the cultivated potato. α-Solasonine contains glucose, galactose, and rhamnose, and α-solamargine contains galactose and two molecules of rhamnose. The hypothesis is that the distribution of labeled carbon atoms in all the steroidal rings of solasodine, synthesized from radioactive acetate or mevalonate by Solanum avivulare, agrees with that expected on the basis of the known biosynthetic and cyclization scheme of squalene (Guseva and Paseshnichenko, 1962) and may be applied to solanidine because of their structural similarity with solasodine. Great similarity was observed between the biosynthesis of polyisoprene compounds in the animal organism and the biosynthesis of solasodine characteristic for plants of the genus Solanum. Experiments by Liljegren

(1971) also agreed and sugar substitution was the last step in the synthesis of solasonine and solamargine. Jadhav, Salunkhe, Wyse, and Dalsi (1973) further indicated that the end products of photosynthesis were not direct precursors of the steroidal moiety, but did participate as substituted sugars in the glycoalkaloids by studying with D-glucose-U-C^{14}. Potato tubers are capable of glucosylating the 3β-hydroxyl group of solanidine when UDP-glucose-U-^{14}C serves as a donor. The distribution of label from D-glucose-U-^{14}C administered to potato sprouts was localized more in the glycoside moiety than that in the aglycone portion of the glycoalkaloids. Since α, β and γ forms of solanine and chaconine occur in potato tubers and sprouts, stepwise synthesis of α-solanine and α-chaconine from solanidine seems possible (Jadhav and Salunkhe, 1973a).

Ramaswamy, Behere, and Nair (1976) reported that chlorophyll synthesis and photosynthetic activity of the chloroplast were essential prerequisites for solanine synthesis in the green peels of potato. The potato chloroplast photosynthetic system differed in many respects from the conventional photosynthetic systems isolated from green plants. Since the potato tubers are rich in starch, the need for the synthesis of starch or other carbohydrates by photosynthesis is limited. The amount of CO_2 fixed in potato tubers was comparatively much less than that fixed in normal plants. Normal Calvin cycle intermediates are not involved in the production of mevalonic acid. In the potato system, CO_2 is directly reduced to formate and channelled to the synthesis of acetate (Ramaswamy, Behere, and Nair, 1976).

Formate, glycine, serine, and pyruvate have been identified as intermediates. Ramaswamy, Behere, and Nair (1976) also suggested that a CO_2 oxidoreductase enzyme might participate in the reduction of CO_2 in potato chloroplasts. The chloroplast excretes 3-C photosynthetic products which are converted to pyruvate in the cytoplasm and pyruvate is converted to 2-C units in the mitochondria, which are transferred back to chloroplast to form solanidine.

Factors Affecting Glycoalkaloid Formation

Solanine develops in potato tubers in variable amounts depending upon the variety, stage of development and environmental conditions (Larsen, 1949; Gull and Isenberg, 1958, 1960; Liljemark and Widoff, 1960; Yamaguchi, Hughes, and Howard, 1960a; 1960b; Patil, Salunkhe, and Singh, 1971). Light, however, exerts the most critical influence. Such factors as light intensity and quality, and duration of exposure stimulate the development of this compound (Hardenburg, 1964).

Though the synthesis of both chlorophyll and solanine in potato tubers is activated by light, the formation of each is an independent process (Conner, 1937). This finding was supported by Gull and Isenberg (1960) and Patil, Sharma, Salunkhe, and Salunkhe (1972). They further noted the parallel increase between solanine end of the spectrum was most efficient for chlorophyll but did not increase solanine (Conner, 1937). Thus, pink, blue and daylight fluorescent lights may be used to reduce greening Isenberg and Gull, 1959; Yamaguchi, Hughes, and Howard, 1960b; Liljemark and Widoff, 1960).

The exposure time is an important factor in greening because of its cumulative effect. The length of exposure required to cause greening and an increase in solanine formation varies in the literature reports (Larsen, 1949; Gull and Isenberg, 1960; Patil, Singh, and Salunkhe, 1971; Yamaguchi, Hughes, and Howard, 1960b; Howard, Yamaguchi, and Timm, 1957).

Potato cultivars differ markedly in solanine content on exposure to light (Wintgen, 1906; Morgenstern, 1907; Bömer and Mattis, 1923; Wolf and Duggar, 1946; Patchett, Cunningham, and Lill, 1977). Investigations conducted by Bömer and Mattis (1924), Lepper (1949), Zitnak (1970), Sandford and Sinden (1972), and Sinden and Webb (1972) revealed that cultivars with high glycoalkaloid contents are more likely to produce excessive glycoalkaloid than the cultivars with low glycoalkaloid contents when subjected to less than ideal environmental conditions or to improper handling.

Immature potato tubers were more susceptible to greening (Yamaguchi, Hughes, and Howard, 1960a and 1960b) and had a higher initial glycoalkaloid content than mature tubers (Patchett, Cunningham, and Lill, 1977). Moreover, immature tubers accumulated more glycoalkaloids over the period of exposure to light.

Low-temperature storage maintained or caused more bitterness of the tubers than did storage temperatures above $10^{o}C$ (Wolf and Duggar, 1946; Hilton, 1951; Zitnak, 1953).

The production of solanine alkaloid has been reported in wounded potato tubers by McKee (1955). Blighten potato tubers contain higher

concentrations of glycoalkaloids (Poswillo, Sopher, and Mitchell, 1972). Injury of tubers caused by either bruising or mechanical grading after harvesting induced alkaloid synthesis (Sinden and Webb, 1972). Furthermore, Wu and Salunkhe (1976) reported that mechanical injuries of potato tubers such as brushing, cutting, dropping, puncturing and hammering greatly stimulated glycoalkaloid synthesis in both periderm and flesh of tubers.

Toxicology of Solanine Glycoalkaloids

Since solanine is a poisonous alkaloid, its presence in increased amounts in the potato tuber has, in the past, been considered to be a health hazard. Potato tubers containing more than 0.1 percent solanine are considered to be unfit for human consumption. However, most of the authenticated reports of solanine poisoning are in connection with the use of sprouts as edible shoots (Schwimmer and Burr, 1967) and the consumption of blighted potato tubers (Poswillo, Sopher, and Mitchell, 1972; Kirk and Mittwoch (1975) reported that low concentrations of α-solanine stimulated the growth of cultured human fibroblasts, while higher concentrations ($>3\mu g ml^{-1}$) had a markedly inhibitory effect. Autoradiographic studies indicated that the stimulation of cell growth was due to a shortening of the G_1 phase. Feulgen microdensity of cells treated with high doses of α-solanine revealed an abnormal accumulation of cells in G_2. By virtue of either its stimulatory or its inhibitory effect on cell growth, α-solanine could act as a human teratogen. Further, Bell, Gibson, McCarroll, and McClean (1976) stated that solanine

was embryotoxic and its toxicity was potentiated by aspirin. The toxic effect is apparently cumulative and depends on a critical drug level in an animal.

According to U.S. standards for potato tubers issued on July 15, 1958, greening is defined as "damage" if more than 5 percent of the total weight of the potato must be removed to eliminate the greened tissue, and as "serious damage" if the loss is over 10 percent. Several animals have been tested for differences in sensitivity to a total potato alkaloid as well as to α-solanine.

Information on the toxicology of α-chaconine is meager. Nishie, Norred, and Swain (1975) reported that the pharmacological responses produced by α-chaconine showed no essential differences from those produced by α-solanine. Another study with labelled chaconine had been conducted by Norred, Nishie, and Osman (1976). They concluded that ^{3}H-α-chaconine was poorly absorbed from the gastrointestinal tract and rapidly excreted in feces when administered orally to male rats. High, toxic, i.p. doses (15-25 mg/kg) depressed fecal and urinary elimination, and resulted in accumulation of ^{3}H in various tissues. The major metabolite appeared to be the aglycone, solanidine. α-Chaconine is very similar to α-solanine in its elimination by and distribution in tissues of rats.

Toxicological properties of trace glycoalkaloids, leptines and leptinines, of potato are almost unknown.

Digitonin

Digitonin, $C_{56}H_{92}O_{29}$ (Figure 1), is a glycoside belonging to the saponin group and occurring in leaves and seeds of Digitalis purpurea L., Scrophulariaceae. The commercial product contains 70-80 percent digitonin, 10-20 percent gitonin and tigonin, and 5-15 percent minor saponins (Stecher, Windholz, and Leahy, 1968).

Windaus (1910) was the first to describe the digitonin reaction for the estimation of free cholesterol. Essentially, the method is based on the formation of a 1:1 additional compound by digitonin and free 3β-hydroxysterol, which is immediately condensed in the common medium in the form of crystalline needles (Ökrös, 1968). It has since been found that this insoluble complex formation is nearly restricted to unesterified 3β-hydroxysteroids in either the 5α-or the 5β-series, for reasons that are not clear. The 3β-hydroxysteroids are also formed with other sapogenins, e.g., tigonin, the 2,15-bisdesoxy derivative of digitonin. The introduction of an acyloxy group at C-4 or C-7 prevents complex formation, as does inversion at C-10 (Nes and McKean, 1977).

The technique was first employed in light microscopy by Brunswick (1922) on frozen sections. Leulier and Revol (1930) successfully utilized the digitonin reaction not only in frozen sections but also in unfrozen tissues. As the digitonin-cholesterol crystals show double refraction they can be studied by polarization microscopy, allowing the reaction to be used in

histochemical studies. The technique has been modified by Grundland, Bulliard, and Maillet (1949) and Feigin (1956).

The digitonin-cholesterol crystalline needles formed during the reaction process possess strong osmiophilous properties, permitting the localization and structure of the crystals to be studied in ultrathin section (Ökrös, 1968). Furthermore, this cholesterol complex is relatively insoluble both in aqueous solutions and in the solvents required for dehydration for electron microscopy. Precipitation by digitonin has been widely used as a means of separating free cholesterol from esterified cholesterol and other lipid mixtures (Williamson, 1969).

The digitonin-cholesterol crystalline needles under the electron microscope gave a picture similar to that revealed by the light microscope: cylindrical structures with slightly tapering ends, sectioned in different levels. The crystals are made up of electron-dense and electron-transparent coaxial lamellae (Ökrös, 1968). Albert and Rucker (1975) also demonstrated the localization of free cholesterol in atheromatous aorta of New Zealand rabbits with the electron microscope by the use of digitonin.

MATERIALS AND METHODS

Storage Environment

Russet Burbank, Solanum tuberosum L., potato tubers were sampled and stored in a dark room at 16°C and 60 percent relative humidity for 10 days to serve as control.

Potato tubers were exposed to light in order to induce the formation of glycoalkaloids. Fluorescent lights (Sylvania F40 CW (Wool White)) were employed as the illumination source. Light intensities were measured in foot-candles with a Weston illumination meter, Model 603, No. 610. Tubers were exposed to 200 ft-c (2152 lux)[1] of light at 16°C and 60 percent relative humidity for 10 days.

Potato tubers were placed in darkness at 16°C and 60 percent relative humidity to develop sprouts void of chlorophyll.

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[1] Definitions of photometric units:

Foot-candle (ft-c) = the illumination on a surface 1 ft^2 in area on which there is a uniformly distributed flux of 1 lumen. (1 ft-c = 10.76 lux)

Lux = The International System (SI) unit of illumination defining the illumination on a surface 1 m^2 in area on which there is a uniformly distributed flux of 1 lumen.

Lumen = the luminous flux on a unit surface all points of which are at unit distance from a uniform source of one candela.

Candela = The SI unit of luminous intensity comprising 1/60 cm^2 of the projected area of a black body radiator operating at the temperature of freezing platimum (2042K).

Source: Weast, 1973. Handbook of Chemistry and Physics, 53rd Edition. p. E-191.

Plant Samples

In the initial experiments, sprouted tips, unsprouted tips, peridermal tissue and cortex were used. The sprouted tip was cut at 2 mm from the tip of the sprout. Unsprouted tips were obtained from the eye region of the potato tuber. Tissue (1 mm thick) was removed for use as the peridermal and cortex tissue. From these pilot studies, it was determined that sprouted tips and the peridermal complex were the most easily handled and contained relatively amounts of glycoalkaloids.

Chemicals

Chemicals used in this study were obtained as follows: α-Solanine from K and K Laboratories, Incorporated, Plainview, New York, and Sigma Chemical Company, St. Louis, Missouri; Solanidine from Schwarz/Mann, Division of Becton, Dickinson and Company (B. D.), Orangeburg, New York; Silica gel G absorbant from Warner-Chilcott Laboratories Instrument Division, Richmond, California; Digitonin from Fisher Scientific Company, Fair Lawn, New Jersey; Sodium cacodylate from Sigma Chemical Company, St. Louis, Missouri; Glutaraldehyde (25 percent EM grade) from Taab Laboratories, Reading, England; Osmium tetroxide from Englehard Co., Newark, New Jersey; Propylene oxide from Eastman Organic Chemicals, Rochester, New York; ERL-4206 (Vinylcyclohexene dioxide), D.E.R.-736 (Diglycidyl ether of polypropylene glycol), NSA (Nonenyl succinic anhydride), and DMAE

(Dimethylaminoethanol) from Electron Microscopy Sciences, Fort Washington, Pennsylvania; Lead nitrate from Mallinckrodt Chemical Works, St. Louis, Missouri; and Sodium citrate from J. T. Baker Chemical Company, Phillipsburg, New Jersey, respectively.

Extraction of Glycoalkaloids

Extraction of the Solanum alkaloids were made according to the methods of Gull and Isenberg (1960).

The potato sprouts (20 g) were cut into small pieces, macerated with 120 ml of 95 percent ethanol in a Waring blendor at high speed for 4 minutes. The extract and grinding were transferred to a beaker along with two washes of the blendor cup with 95 percent ethanol (15 ml each). Three ml glacial acetic acid were added to halt bacterial action. The beaker was covered with parafilm and placed in a refrigerator overnight.

The acidified alcoholic extract was filtered into a Soxhlet flask and the beaker was rinsed twice with 15 ml of 95 percent ethanol. Extra ethanol was added to bring the total volume up to 150 ml (filling half of the round-bottomed Soxhlet flask). The filter paper containing the grindings was folded and stapled and inserted into the collection tower to act as the Soxhlet thimble. The reflux apparatus was assembled, heat was applied, and the Soxhlet extraction was conducted for 24 hours (minimum of 16 hours required). Following extraction, the grindings were discarded, while the hot extract was poured into an evaporating dish. After two rinses of alcohol from the

round-bottomed flask, the alcohol extract was evaporated to dyrness. The dry residue was washed with 15 ml of 5 percent sulfuric acid (H_2SO_4). The residue layers were scraped off the sides of the porcelain dish with a glass rod. The suspension was poured through a fluted filter paper into a 50 ml glass centrifuge tube. The dish was again rinsed with 10 ml of 5 percent H_2SO_4 and the rinse was also filtered into the centrifuge tube. After placing the tube into an ice-water bath, the acidic solution was neutralized with the addition of concentrated ammonium hydroxide (NH_4OH). Then excess concentrated NH_4OH was added to raise the pH above 9.5 and precipate the glycoalkaloids. The total requirement of NH_4OH approximated 10 ml. Following the pH adjustment, the solution was heated at 80°C in a water bath for 1 hour in order to flocculate the glycoalkaloids; the tube with the glycoalkaloid solution was then covered with parafilm and kept overnight at 4°C.

The sample was removed from the refrigerator and centrifuged at high speed for 20 minutes. Following this sedimentation, the supernatant was poured off, and the precipitate was washed with 25 ml of 1 percent NH^4OH. After another 20 minutes of centrifugation, the supernatant was again discarded. The alkaloid fraction was acidified, reprecipitated, and washed with 1 percent NH_4OH as described previously, followed by ether.

Thin-layer Chromatographic Analysis I

Rectangular glass plates (5 cm x 20 cm) were coated with a 250 mμ layer of silica gel G absorbant. In preparation for spotting, the TLC plates

were marked with an etching on the side 2.5 cm from the bottom, and again 15 cm up from the first etching. A line was scored through the silica gel at the second mark extending the width of the plate demarcating the endpoint for solvent advancement up the plate.

Just prior to spotting and placing in solvent chambers, the plates were activated at 120°C in dry heat for 1 hour. After a short cooling period the plates were spotted with four spots per plate. The left hand spot contained commercially prepared solanine, chaconine, and solanidine, while the right hand spot contained an unknown glycoalkaloid mixture extracted from potato sprouts. The TLC plates were placed at a vertical slant in a glass chamber that had been saturated overnight with a solvent comprised of methanol:1-butanol:acetic acid:water in a ratio of 60:15:15:10, respectively. About 2 hours were required for solvent ascension to the 15 cm demarcation line. After removal from the chambers, the TLC plates were dried under a hood. Spot separation on plates were developed in about 4 hours in a glass chamber containing iodine fumes. The centers of the separated spots were determined to the nearest mm and R_f values were calculated.

Qualitative Precipitation Test

Extracted glycoalkaloid mixture was dissolved in 1 percent acetic acid to make a 1 percent solution in a centrifuge tube. Saturated solution of digitonin in 0.1 M cacodylate buffer was added to this solution in a 1 to 1 ratio. The tube was covered with parafilm and left overnight at room

temperature. The next day the tube was removed and centrifuged at 1600 rpm for 10 minutes with an ICE table top centrifuge.

Thin-layer Chromatographic Analysis II

The same TLC analytical procedure described previously was followed except the unknown had been changed. The supernatant obtained from qualitative precipitation test was used as an unknown glycoalkaloid mixture.

Determination in Fixative Combination

Three different kinds of electron microscopic fixative combinations were tried:

(I) Samples were fixed in 3 percent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hours at room temperature. This was followed by two rinses (20 minutes each) in cacodylate buffer and then by immersion in saturated solution of digitonin in the same buffer for 4 hours, and postfixed with 1 percent osmium tetroxide (also in cacodylate buffer) for 1 hour (Williamson, 1969).

(II) Samples were fixed with these three fixatives at room temperature but in different order. Samples were immersed in saturated digitonin for 4 hours, and fixed in 3 percent glutaraldehyde for 2 hours, followed by postfixation in 1 percent

osium tetroxide. All these fixatives were made in the same buffer, o.1 M cacodylate buffer pH 7.4.

(III) Samples were fixed in a mixture of 2 percent glutaraldehyde, 1 percent osium tetroxide and 0.2 percent digitonin (also in 0.1 M cacodylate buffer pH 7.4) for 30 minutes at room temperature (modification of Napolitano and Scallen, 1969).

Fixation

Samples were cut into small pieces (1 mm^3). With the aid of a wooden toothpick, they were transferred to fresh fixative in a labelled vial. All samples were fixed three different ways:

(1) Tissues were fixed in 2.5 percent glutaraldehyde in 0.1 M phosphate buffer for 2 hours at room temperature.

(2) Tissues were incubated in 2.5 percent osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature, and washed with two rinses of cacodylate buffer (20 minutes each), then back to (1).

(3) Tissues were fixed by glutaraldehyde-osmium tetroxide-digitonin mixture for 30 minutes at room temperature. This fixative composed of glutaraldehyde (2 percent), osmium tetroxide (1 percent), and digitonin (0.2 percent) adjusted to pH 7.4 with cacodylate buffer (modification of Napolitano and Scallen, 1969).

All samples were then thoroughly washed with 0.1 M cacodylate buffer by three rinses for 20 minutes each, transferred into fresh cacodylate buffer and left overnight. All fixatives and buffers were at pH 7.4. After washing in buffer, tissues were dehydrated once in 30 percent ethanol for 7 minutes and in 50 percent, 70 percent, 85 percent, 95 percent ethanol for 30 minutes each, followed by three changes of dry absolute alcohol and three changes of 100 percent propylene oxide for 10 minutes each, embedded after two changes of 50 and 75 percent Spurr's Epon (Spurr, 1969) in propylene oxide for 1 and 18 hours. Polymerization was carried out by placing the specimens in Beem capsules (Beem Company). Blocks were cured at 40°C for 24 hours and then at 60°C for 48 hours.

Sectioning, Staining, and Examining

Thick sections for light microscopy

The blocks were properly trimmed down to the specimen and with a block face of 1 mm^2. One μm sections were cut with a glass knife (LKB Instruments, Inc.) on a Sorvall porter-Blum MT-2 ultramicrotome. Sections were examined under the light microscope. This insured that the tissue had been exposed, and allowed for preliminary orientation of the portion to be thin-sectioned.

Slides previously cleaned and stored in 70 percent ethanol with a few drops of 0.1 N HCl were air-dried. Sections were floated on a drop of distilled water. A camel's hairbrush was wetted and used to transfer the

sections from the water trough of the knife to the slide. The slide was placed on a hot plate (40°C). After drying, Richardson's stain (Richardson, Jarett, and Finke, 1960) was applied on the sections and the slide was covered with a petri dish to minimize dust contamination. Excess stain was rinsed from the slide with a stream of deionized water after 1 or 2 minutes. The slide was again dried on the hot plate and then examined with a light microscope.

Thin sections for electron microscopy

Blocks were trimmed to approximately 0.5 mm on a side. Sections were cut with a diamond knife on a Sorvall porter-Blum MT-2 ultramicrotome. Silver or gray sections were picked up by touching an acetone-cleaned 3 mm, 200 mesh uncoated copper grids to the ribbon as they floated on the trough liquid.

Uranyl acetate and lead citrate double staining method for Spurr's Epon was used. Sections were stained with 0.05 percent uranyl acetate in the mixture of 100 percent methanol and 70 percent ethanol (1:3) for 30 minutes at 25°C, rinsed briefly in this methanol and ethanol mixture and dried for 1 minute. Sections were again rinsed in distilled water and stained in lead citrate (Reynolds, 1963) for 5 minutes at 25°C. The sections were then briefly rinsed with 0.02 N sodium hydroxide. After rinsing in distilled water and drying, sections were examined with a Zeiss EM-9S-2 electron microscope and significant observations were recorded photographically.

In the electron microscopic examination 50 grids were examined from three to four blocks of each tissue for a total of 150 to 200 grids. From these grids 25-30 pictures of each tissue were recorded and examined. Because of the irregular occurrence of the spicules no statistical analysis was conducted.

RESULTS AND DISCUSSION

Reaction of Digitonin with Alkaloids

Digitonin reacts with cholesterol and forms an insoluble cholesterol-digitonide complex (Windaus, 1910). The complex appears as crystalline material in light microscopy and as starchy trilaminar spicules and whorls in conventionally prepared tissue for electron microscopy (Albert and Rucker, 1975). Reaction of digitonin is specific for steroids having an unesterified 3β-hydroxyl group. The complex forms regardless of the nature of the side chain in the sterol. Even 3β-hydroxy-17-keto steroids form digitonides (Nes and McKean, 1977). Digitonin (Figure 1) may be used to localize solanidine because of its structural similarity with the chemical structure of cholesterol, especially, the unesterified 3β-hydroxyl group (Figure 2). Solanine and chaconine without this free 3β-OH group (Figure 3) can be thought of as a solanidine glycoside. A computer search of the literature provided no evidence of digitonin reacting with the Solanum alkaloids. Results of this study indicated that digitonin could and did react with solanidine.

Glycoalkaloid mixture extracted from potato sprouts was subjected to TLC, using methanol:1-butanol:acetic acid:water (60:15:15:10) as the developing solvent, which was found best for separating solanine, chaconine, and other extracts (Jeppsen, 1974). The glycoalkaloid mixture separated into three components and was localized with iodine as shown in Figure 4.

According to R_f values, these three components were identified as solanine, chaconine, and solanidine. Based on their relative intensities, both solanine and chaconine were major spots while solanidine was minor. The same results were found in low voltage electrophoretic analysis (Baizoldanov, 1973). Thus, solanine and chaconine were the principal products in the extraction of Solanum. The presence of solanidine has generally been regarded as a product of the hydrolysis of the related glycoalkaloids.

A small quantity of a fine-particled precipitate was formed when the extracted potato glycoalkaloid mixture was reacted with digitonin. This indicated that digitonin was able to react with the components of the potato glycoalkaloid mixture. The supernatant from this test was subjected to TLC, the results are shown in Figure 5, which in comparison to Figure 4, show a striking difference in the components separated. There were only two spots resolved instead of three as shown on the TLC plate in Figure 4. No solanidine was identified. Thus, only solanine and chaconine remained in the supernatant obtained from digitonin precipitation test. From these tests it may be concluded that digitonin reacted with solanidine and formed an insolbule digitonide.

Ultrastructural Studies

Comparisons of different combinations of glutaraldehyde, osmium tetroxide, and digitonin were conducted. The electron micrographs indicated

that fixation in the order of digitonin, glutaraldehyde, and osmium tetroxide would not be applicable.

With the fixation method of Williamson (1969), subtle ultrastructural alternations in many membrane types occurred (Figure 6). These effects indluded enhancement of trilaminar unit membrane and the occurrence of artifacts, as those previously described by Williamson (1969). Figure 6 shows a discontinuous enhancement, exaggerated trilaminar structure, and scalloping plasma membrane. In addition, many mitochondria were swollen with bulging of both the inner and the outer membranes (Figures 6 and 7), occasionally leading to distortion of shape and to disruption. In extreme cases, the membrane changes resulted in myelin-like figures located within mitochondrial bulges, vacuoles and the cytoplasm (Figure 6). Among these effects, the discontinuity of membranes was often difficult to differentiate from those in the unit membrane profiles due to bariations in the plane of sectioning. The artifacts took the form of membranous whorls, spicules, and structures resembling "starched" membranes, which were concerned as the sites of the cholesterol-digitonide complex (Williamson, 1969). However, Napolitano, Saland, Lopez, Sterzing, and Kelley (1972) suggested that spicules and whorls should not be considered the only sites of free cholesterol preservation in tissues, since they were artifacts. Although the Williamson's (1969) method resulted in the most easily recognizable changes, it did not work in our study.

Napolitano, Sterzing, and Scaletti (1969) recommended a new fixation method which resulted in the stabilization of cholesterol in tissues without the occurrence of the artifacts mentioned above. Scharnbeck and Schaffner (1970) stated that spicules appeared when Napolitano, Sterzing, and Scaletti's (1969) procedure was modified by using routine instead of rapid dehydration. Since our interest was in the localization of solanidine in potato tuber, ultrastructural studies were performed on tissues fixed according to Napolitano's modified procedure. This method resulted in an easily recognizable change and also worked best on potato tissue (Figure 8).

Glutaraldehyde alone was used as a control fixative for comparison with the Napolitano's modified procedure. Since there were no electron dense metals in the fixative, glutaraldehyde alone yielded an overall bland image (Figure 9). Osmium tetroxide is widely used in postfixation. According to Griffiths and Beck (1977) osmium reacts with large concentrations of cholesterol precursors or with other steroids synthesized from cholesterol. In order to not interfere with the localization of solanidine, postfixation was not applied to any sample in this study although it was usually desired.

All cells and their constituents appeared normal. The most common feature of glutaraldehyde fixed tissue was the rich and granular micrograph image of the cytoplasm which was due to the preservation of proteinaceous material (Figures 9-12). A number of transparent vacuoles can be seen in Figures 9 and 10. Figure 11 shows two etioplasts in a potato sprout cell. A highly ordered prolamellar body is present in each etioplast. A proplastid

is seen in Figure 12. Clusters of phytoferritin particles and a group of stroma vesicles are also observed. Phytoferritin has been located electron microscopically in the proplastids of meristems and young light- and dark-grown seedlings (Hyde, Hodge, Kahn, and Birnstiel, 1963). However, only cell walls and cellular debris can be seen in the peridermal complex tissue (Figures 13 and 14).

When potato sprouts were incubated with osmium tetroxide and fixed in glutaraldehyde, the ground cytoplasm of the cell appeared granular (Figure 15). In Figure 15, the nucleus also appeared somewhat fibrillar or coarsely granular. Whaley, Mollenhauer, and Leech (1960) reported that in cells well fixed with osmium tetroxide, there appeared to be considerable clumping or agglomeration in the ground substance, as shown in Figure 16, but it was not clear whether this involved the entire ground substance or only portions or components of it. Figure 17 shows a myelin body present in the cell, which may have been derived from the multifunctional plastid after the splitting off of the outer membranes (Fox, Manners, and Myers, 1972).

Potato sprout tissue preserved with osmium tetroxide (Figure 15), in comparison to the same tissue preserved with glutaraldehyde only (Figure 9), presented a striking difference in the appearance of the vacuole and cytoplasm. Figure 15 shows electron dense particles dispersed throughout the vacuoles, while there is almost nothing shown in the vacuoles in Figure 9. Furthermore, it seemed that more electron dense particles appeared in the cytoplasm of cells fixed with osmium tetroxide than those cells fixed with

glutaraldehyde only. Comparisons of general relations of osmium tetroxide and glutaraldehyde with cell constituents show that both fixatives do not react with polysaccharide, but partially fix nucleic acids. Osmium tetroxide partially fixes protein while glutaraldehyde readily fixes protein. The striking difference between these two fixatives is that phospholipids and unsaturated fats are easily fixed with osmium tetroxide but not glutaraldehyde (Dawes, 1971). The double bond of cholesterol reacts with osmium tetroxide to form a cyclic osmate ester (Dreher, Schulman, Anderson, and Roels, 1967), which has sufficient electron density to be visible as small particles (Florendo and Barrnett, 1973).

Friend and Brassil (1970) reported that mammalian cells known to synthesize steroids show distinct patterns of osmium deposition that differ from the patterns in nonsterol synthesizing cells. Prolonged staining (48 hours) with unbuffered osmium tetroxide was suggested as a cytochemical marker, based on a direct relationship between the pattern of osmium deposition and the synthesis of steroids. However, this technique has not been applied in this study because over-fixation was not a desirable result.

Figures 18, 19, 20, and 21 show cells of potato peridermal complex tissue preserved with osmium tetroxide and glutaraldehyde. Cytomembranes are reasonably sharp. Not much information could be derived from these cells when compared with those preserved with glutaraldehyde only (Figures 13 and 14).

The penetration of glutareldehyde, osmium tetroxide and digitonin into the specimen was occasionally irregular. Some sections had the appearance of tissue fixed by osmium tetroxide alone, while the majority of sections showed typical glutaraldehyde effects. According to Scharnbeck and Schaffner (1970), wherever the osmium tetroxide effect was prevalent, no spicules could be found.

The digitonin-solanidine complex was recognized as darkly stained needles or spicules. On cross sections, the spicules were presented as electron-dense circles (Figure 22) or spiral-like lamellar structures around an electron-lucent central core. Spirals consisted of one and one-half turns (Figures 23, 26). These findings were similar to those previously described by Scharnbeck and Schaffner (1970). The diameter of such cross sections was approximately 700-900 Å with a few 300-400 Å. The light inner core was 200-400 Å in diameter. Longitudinal sections showed them to be cyclindrical shape with slightly tapering ends. Thus, the variation of measurements of cross sections could be explained. The spicules were approximately 4,000-8,000 Å in length. Many of them appeared to be shorter due to the plane of sectioning.

This complex was frequently very electron-dense (Figures 24, 28). Several spicules were observed (Figures 25-34), and these were more numerous in the vacuole (Figures 32, 33). In addition, spicules were also found in the cytoplasm (Figure 27). They were frequently noted near membranes, either tonoplast or plasmalemma (Figures 29, 34), but were not membrane

bound. In the electron micrographs approximately 0-8 spicules per vacuole were observed and 0-4 spicules were observed in the cutoplasm.

There appeared to be no relationship to the rough ER (Figure 30), smooth ER (Figure 31), proplastid (Figures 24, 31) or to other organelles.

Observations from tissues preserved in glutaraldehyde alone were considered as the control group for all, since glutaraldehyde resulted in good fixation of cells and did not react with solanidine. Osmium tetroxide may react with solanidine since cholesterol reacts with osmium tetroxide (Higgins, Florendo, and Barrnett, 1973). Observations of tissues preserved with osmium tetroxide and glutaraldehyde may be considered as supporting evidence, that solanidine was present in vacuoles and cytoplasm.

Figure 35 shows typical cells from the peridermal complex tissue of potato tuber which had been light treated. The digitonin-solanidine complex shown in Figure 36 was the only one found in the cytoplasmic strand of green potato peridermal complex in this study. A tuber peridermal complex cell from the control group is shown in Figure 37. Relatively little information was obtained from these cells. Kuo (1973) reported that fresly peeled healthy tubers contain little or no glycoalkaloids though they are normally found in peel.

Because all free 3β-hydroxy-sterols combine with digitonin, this reaction is nonspecific (Nes and McKean, 1977). Cholesterol, sitosterol, stigmasterol, campesterol and brassicasterol may also be precipitated by digitonin. Stigmasterol, campesterol and brassicasterol occur in trace

amounts in potato tuber (Duperon, Brillard, and Duperon, 1972), and in sprouting tuber (Duperon, Duperon, and Thiersault, 1971). β-Sitosterol and cholesterol are the more prevalent sterols but are still found in minute amounts (Duperon, Brillard, and Duperon, 1972). Furthermore, it is well known that in higher plants, free sterols and sterol esters commonly occur together with sterol glucosides and their acylated derivatives (Hartman, Fonteneau, and Benveniste, 1977). Neither sterol glycosides nor esterified sterols react with digitonin (Nes and McKean, 1977). During storage (with ensuing germination) the total sterol content of the tuber remained stationary, but the proportion of acylesterol glycoside decreased while that of the esterified sterols increased (Duperon and Duperon, 1973). Duperon, Duperon, and Thiersault (1971) reported an increase in the quantity of both steroid glucosides and esterified steroid glucosides in the sprouting potato tuber. In addition, the greatest proportion of free sterols occurred in the starch granules (Duperon, Brillard, and Duperon, 1972). No spicules were found in starch granules in our study. Free sterols are found in the sprout and peel in such minute amounts, if at all, that they can be ignored.

A pathway was suggested by Ramaswaym, Behere, and Nair (1976) for the formation of solanidine in chloroplasts in green potato peels. However, no solanidine-digitonide complex has been seen in the chloroplast in this study.

Although the synthesis of glycoalkaloids in potatoes is activated by light, regions of high metabolic activity such as meristematic tissues, either

eye region or tip of sprout, are also active sites of glycoalkaloid biosynthesis whether they are light or dark grown. Kaneko, Watanabe, Taira, and Nitsuhashi (1972) reported when Veratrum plants were fed with acetate-1-^{14}C, the radioactivity of solanidine increased continuously in the dark. Moreover, α-solanine, α-chaconine and solanidine rapidly accumulated in injured tubers at or near the sites of injury (Kuo, 1973). Ishizaka and Tomiyama (1972) also reported that most of the solanine seemed to be distributed in tissue adjacent to the newly formed meristematic tissue zone. There may be another route for the synthesis of solanidine, which is different from the one suggested by Ramaswamy .

Results of this study indicate that no spicules were present in ER. The enzymes involved in the synthesis of squalene precursors are soluble (Benveniste, Oürisson, and Hirth), 1970). The conversion from squalene to sterols requires approximately 30 steps which are catalysed by membrane-associated enzymes (Hartmann, Fonteneau, and Benveniste, 1977). Chesterton (1968) reported that during conversion of mevalonic acid to cholesterol ester in vivo, squalene, lanosterol, cholesterol and cholesterol ester are primarily bound to the ER, indicating this to be the major site of cholesterol and cholesterol ester synthesis from squalene. In mammals, the multienzyme complex of cholesterol biosynthesis is bound to membranes of SER and RER. In higher plants, little is known about the precise cellular sites of sterol biosynthesis characterized by specific steps.

It is already clarified that amino acids are involved in the formation of the nitrogen-containing skeleton of solanidine. Solanidine biosynthesis may be connected with protein metabolism.

Glycoalkaloid synthesis is greatly increased after wounding. DNA synthesis is induced in potato tuber by wounding and starch degradation is also observed (Borchert and McChesney, 1973). Barckhausen and Rosenstock (1973a) stated that the formation of ER would be increased within a short time after wounding. The protein content in the tissues at the wound shows a localized increase and the RNA content is greater near the surface of the wound (Zimmermann and Rosenstock, 1977). Apparently the physiological activity after wounding is enhanced in the cells nearest the wound.

Alkaloid may be derived from different precursors such as terpenes and/or some compounds of the Krebs Cycle (Gizella, 1970). Accumulation of starch and exogenic amino acids by the tuber tissues was different in different tissues (Grigoryuk, 1973). The most active accumulation of amino acids ^{14}C was observed at the stage of intensive growth in the cortical parenchyma, then in the zone of the fibrovascular bundle location and, finally, in the pulp. Different degree of absorption of amino acid-^{14}C by the tissues was due to their age peculiarities and physiological state (Grigoryuk, 1973). It is interesting that solanine is also formed in parenchyma cells of the periderm and cortex of the tubers and in areas of high metabolic activity (Reeve, Hautala, and Weaver, 1969). The concentration of glycoalkaloid is also shown in a descending gradient from the outside inward (Jeppsen, 1974). This seems

to be supporting evidence that starch and amino acids may be involved in the biosynthesis of glycoalkaloids.

In the electron micrographs the solanidine-digitonin complex was recognized as darkly stained needles or spicules. These spicules were observed mostly in the vacuoles in the sprouted tips. However, a few spicules were also noted in the cytoplasm. Relatively few spicules were observed in the peridermal complex.

Roddick (1977) had found that solanine and chaconine accumulated mainly in the soluble fraction of potato plants obtained after differential centrifugation (105,000 g). The soluble fraction contains the vacuolar contents and basic plasma free of organelles. Another investigation was performed on other kinds of plant by Gizella (1970). Results also showed that alkaloids are accumulated in the vacuole or in the plasmatic basic substance and not in any cell organelles. Their results all agree with those presented in this paper.

The difficulties of localizing solanidine in potato tubers may be explained by the small quantities present, as exemplified by the few spicules seen in the electron micrographs. Relatively small quantities of solanidine may only be present because of the speed by which this compound is converted to solanine or chaconine. Another alternative is that the digitonin reaction forming the insoluble solanidine-digitonide complex may not be as sensitive as the reaction with cholesterol in animal tissues.

Although solanidine has been reported in actively growing young sprouts of potato varieties (Clemo, Morgan, and Raper, 1936; Lampitt et al., 1943). It has not been observed in mature potato tubers (Zitnak, 1961). However, solanidine has been found in trace amounts during glycoalkaloid analysis of potato tuber tissue (Zacharius, Kalan, Osman, and Herb, 1975). Its presence has generally been regarded as a product of the hydrolysis of the related glycoalkaloids. Kaneko et al. (1972) reported that solanidine was present in Solanum tuberosum at the budding period. Solanidine has not been generally observed in sprouts or tubers (Zacharius et al., 1975).

Zitnak (1961) stated that under conditions where glycoalkaloids synthesis was readily induced, solanidine was rapidly produced in excess of amounts that could be bound as solanine or chaconine. The formation of the glycoalkaloids would likely be limited by the availability of the glycosidic sugar components (Zitnak, 1961). The aglycone, solanidine, has been attributed to the result of an exhausted sugar supply required for incorporation to form glycoalkaloids (Zacharius et al., 1975). Since potato tubers are rich in starch, periderm and cortex in comparison to sprouts, have more carbohydrates available. Thus, sprouts are more likely to exhaust the sugar required for incorporation of glycoalkaloids. This possibility may explain why more spicules have been found in the sprout tissue.

The use of digitonin in electron microscopic fixative was first introduced to localize solanidine in the potato tuber. Although digitonin has been widely used for cholesterol localization, it may not be sensitive enough to

mark the location of solanidine. Since digitonin reacted quantitatively with free cholesterol in a 1:1 ratio, and since its molecular weight was approximately three times that of cholesterol, it was anticipated that treatment of tissues with digitonin might produce dimensional and/or configurational alterations in cellular organelles containing free cholesterol (Williamson, 1969). Napolitano et al. (1972) stated that the presence of "artifacts" in tissues prepared with digitonin in the fixative was highly variable.

Autoradiographic analysis might be a more accurate method of localizing solanidine. Digitonin can stabilize solanidine. By utilizing tritiated digitonin of high specific activity for autoradiography, solanidine loss could be minimized during preparation and the digitonin-stabilized solanidine might possibly be visualized after relatively short periods (7-14 days) of exposure. Generally, it took 6 weeks to several months before suitable autoradiographs could be developed for electron microscopy.

CONCLUSIONS

Solanidine, a direct precursor of solanine was reacted with digitonin to from a solanidine-digitonide complex. Our rationale for forming this complex is that solanidine has a chemical structure similar to cholesterol and numerous reports in the literature indicate cholesterol reacts with digitonin to form a cholesterol-digitonide complex, which can then be localized with the electron microscope. Moreover, this was an indirect method of lacalizing the site(s) of solanine synthesis in potato tubers.

Application of TLC techniques before and after incubating potato sprout extract with digitonin indicated that a solanidine-digitonide complex was formed. However, the ultrastructural study provided only limited evidence as to the exact location of solanidine in the cells of the sprout or peridermal complex. This may reflect small quantities of solanidine present, rapid conversion of solanidine to solanine or chaconine or insensitivity of the method.

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APPENDIXES

Appendix I:

Abbreviations, Legends, and Figures

Explanation of figures

Abbreviations are as follows:

CA:	Chloroamyloplast	PF:	Phytoferritin
CW:	Cell Wall	Pl:	Plasmalemma
CY:	Cytoplasm	PP:	Proplastic
D:	Dictyosome	Pr:	Prolamellar Body
EP:	Etioplast	R:	Ribosome
G:	Grana	PER:	Rough Endoplasmic Reticulum
IS:	Intercellular Space	S:	Starch
L:	Lipid Droplets	SD:	Solanidine-digitonide Complex
M:	Mitochondrion	SER:	Smooth Endoplasmic Reticulum
MB:	Microbody	SG:	Starch Granule
MF:	Myelin Figure	SV:	Stroma Vesicle
MG:	Melanin Granule	T:	Tonoplast
ML:	Middle Lamella	V:	Vacuole
N:	Nucleus		
NE:	Nuclear Envelope		
Nu:	Nucleolus		
PB:	Protein Body		
Pd:	Plasmadesma		

Appendix II: Figures 1-37

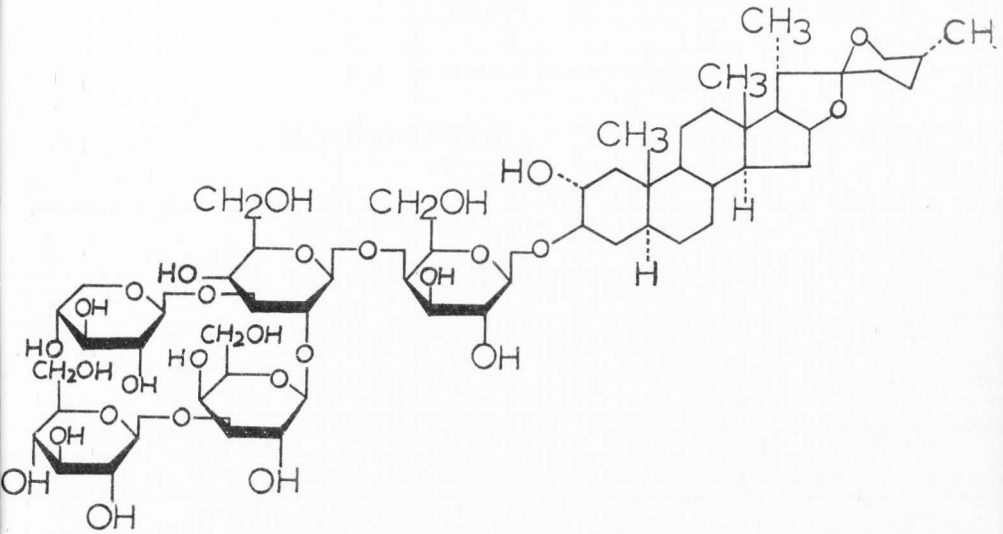
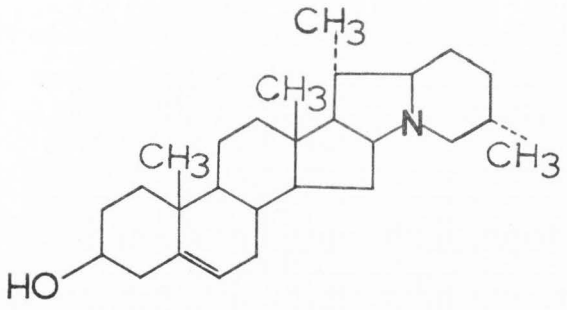
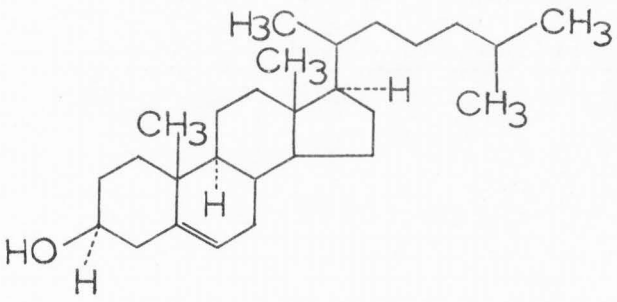


Figure 1. The chemical structure of digitonin.

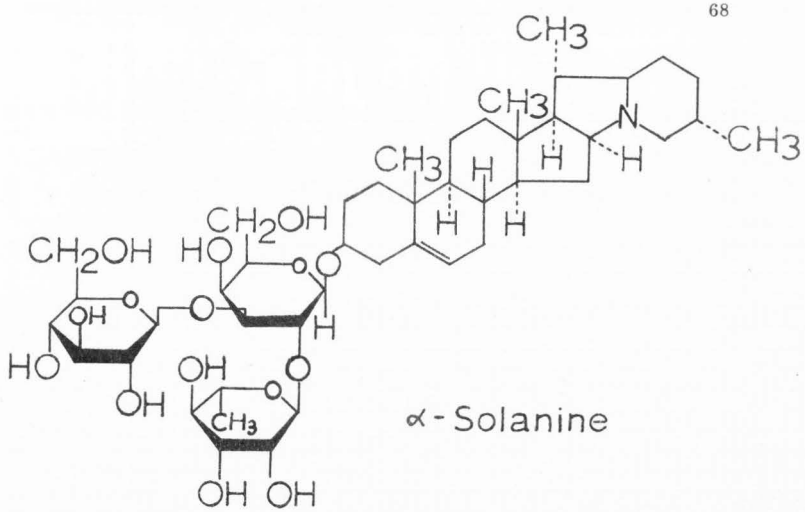


Solanidine



Cholesterol

Figure 2. Comparison of the chemical structure of solanidine and cholesterol.



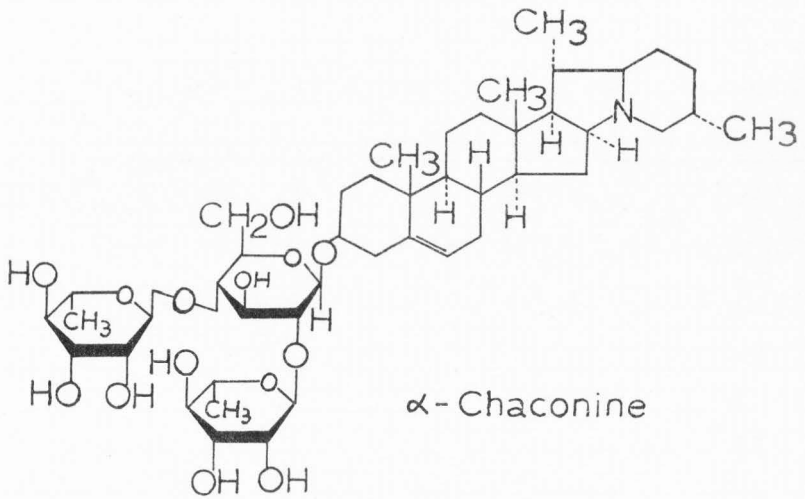


Figure 3. Comparison of the chemical structure of α-solanine and α-chaconine.

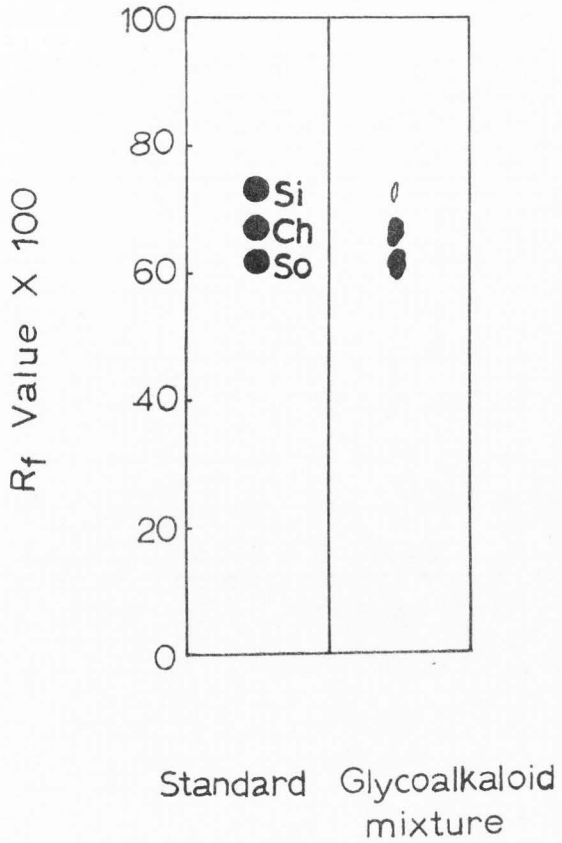


Figure 4. Thin-layer chromatogram of the glycoalkaloid mixture extracted from potato sprouts.
(Si = solanidine, Ch = chaconine, and So = Solanine)
● major spots O minor spot

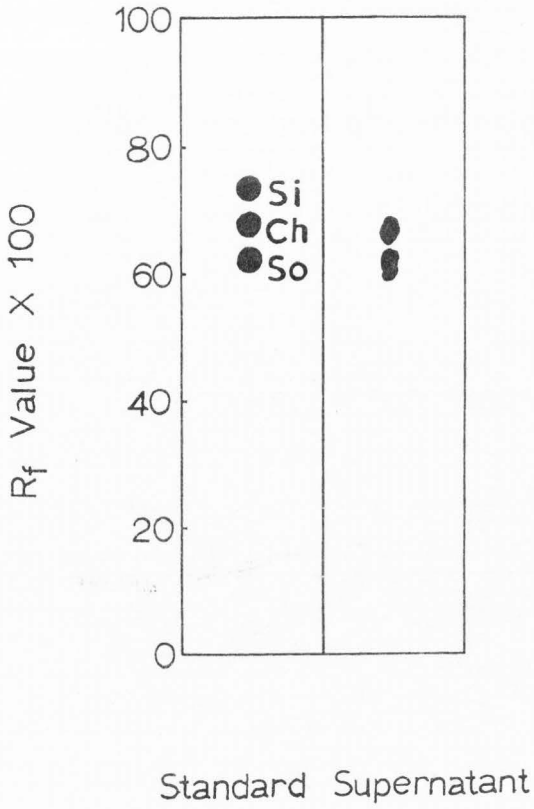


Figure 5. Thin-layer chromatogram of the supernatant from precipitation of digitonin and glycoalkaloid mixture extracted from potato sprouts. (Ch = chaconine, So = solanine)

Figure 6. Potato sprouts, control. Digitonin-induced alternations and scalloping (arrow) of plasma membrane. Williamson procedure. X 23,550.

Figure 7. Potato sprouts, light treated. Digitonin-induced alternation with swelling of mitochondria by bulging of both inner and outer membranes. Williamson procedure. X 22,765.

Figure 8. Potato sprouts, control. Spicules (arrows) are seen within the cytoplasm, which are sites of solanidine-digitonide complex. Modified Napolitano procedure. X 16,975.

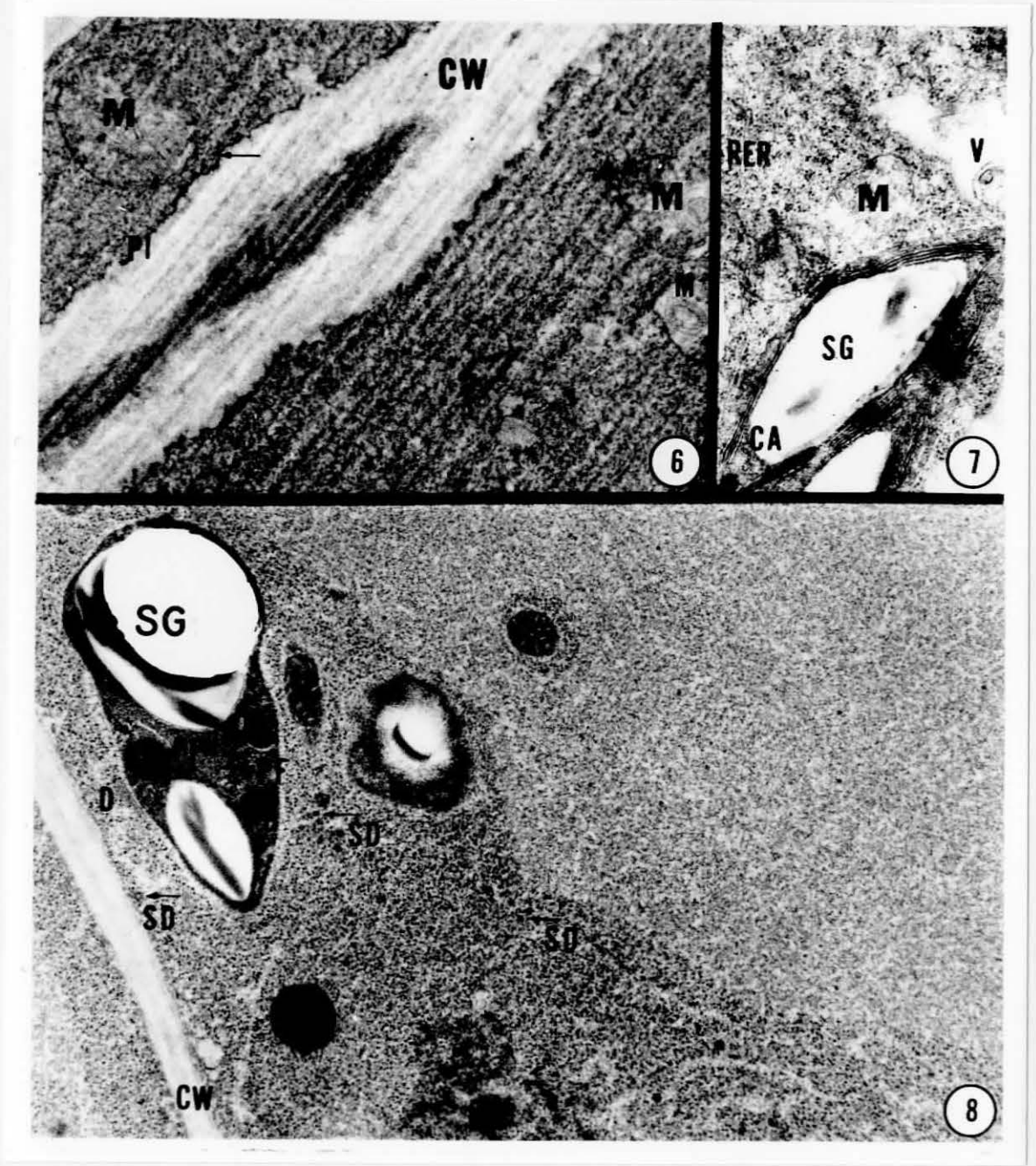

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Figure 9. Potato sprouts. Glutaraldehyde fixation. X 13,580.

Figure 10. Potato sprouts. Glutaraldehyde fixation. X 13,580.

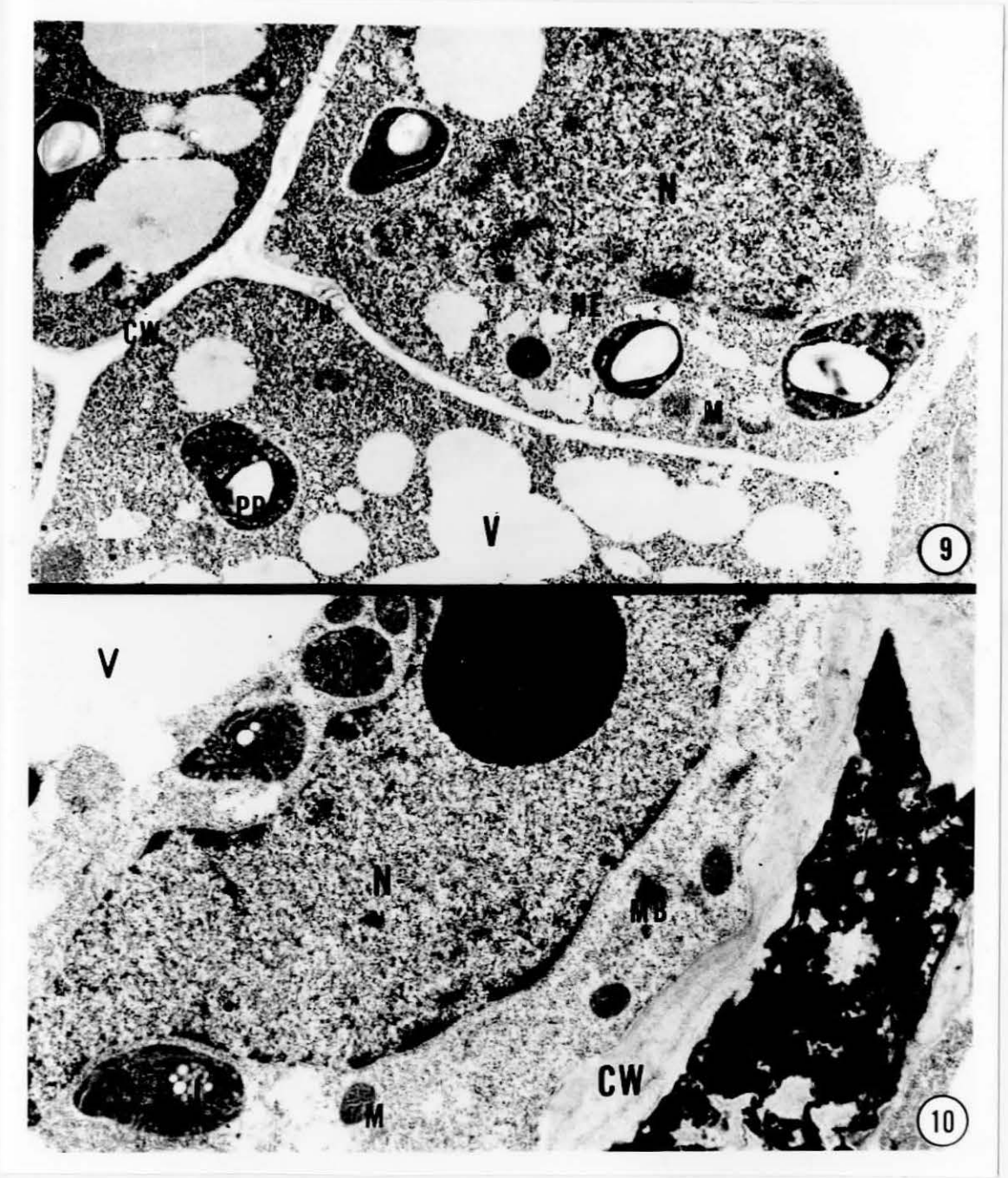
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Figure 11. Potato sprouts. Glutaraldehyde fixation. X 14,550.

Figure 12. Potato sprouts. Glutaraldehyde fixation. X 14,550.

Figure 13. Potato peels, control. Glutaraldehyde fixation. X 14,550.

Figure 14. Potato peels, light treated. Glutaraldehyde fixation. X 16,975.

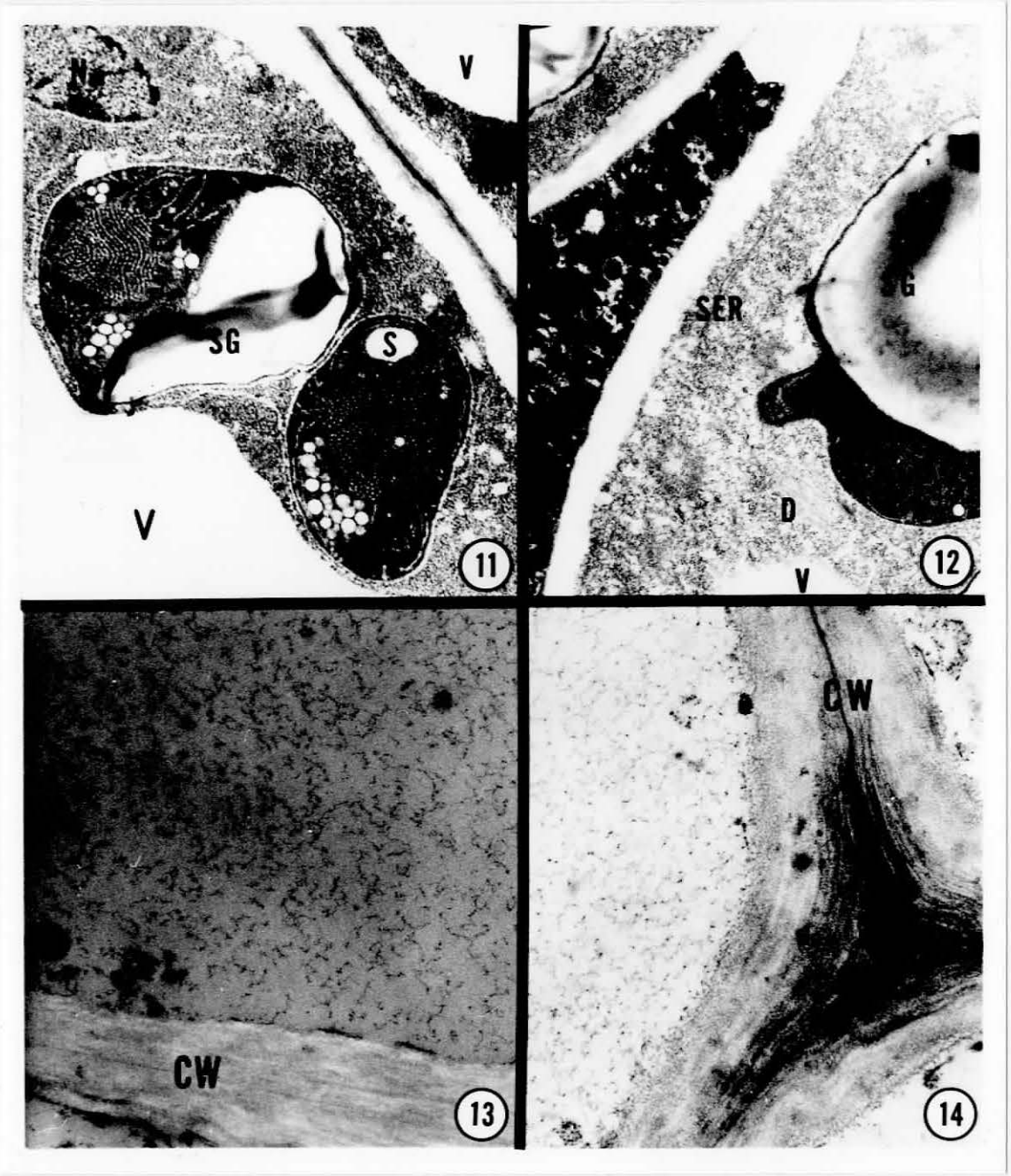
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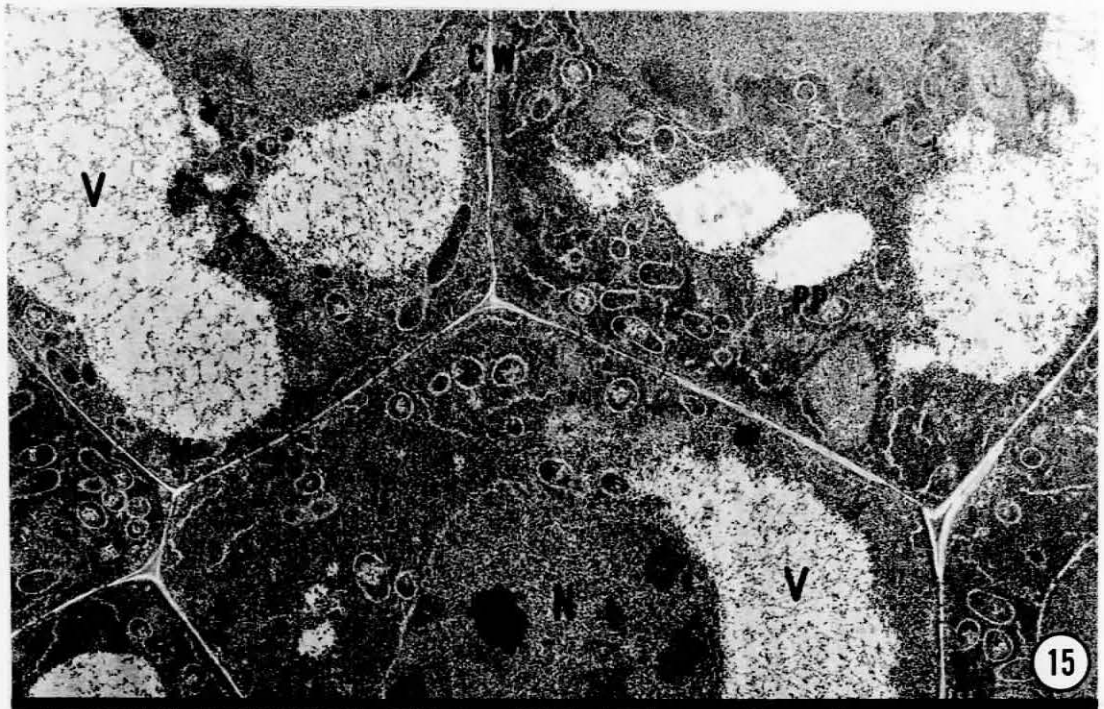
Figure 15. Potato sprouts. Osmium tetroxide and glutaraldehyde fixation. X 5,580.

Figure 16. Potato sprouts. Osmium tetroxide and glutaraldehyde fixation. X 5,580.

Figure 17. Potato sprouts. Osmium tetroxide and glutaraldehyde fixation. X 14,550.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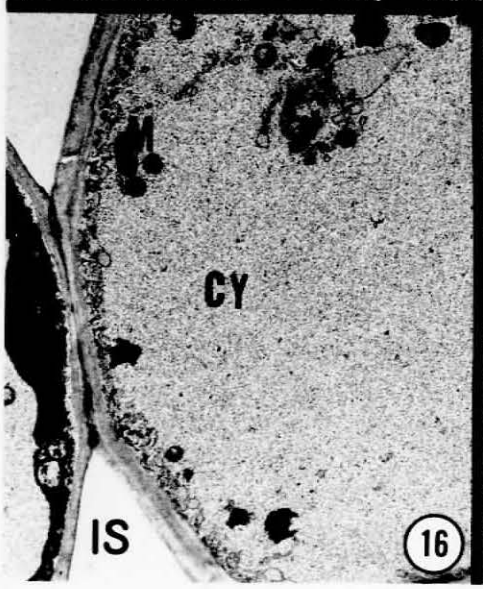
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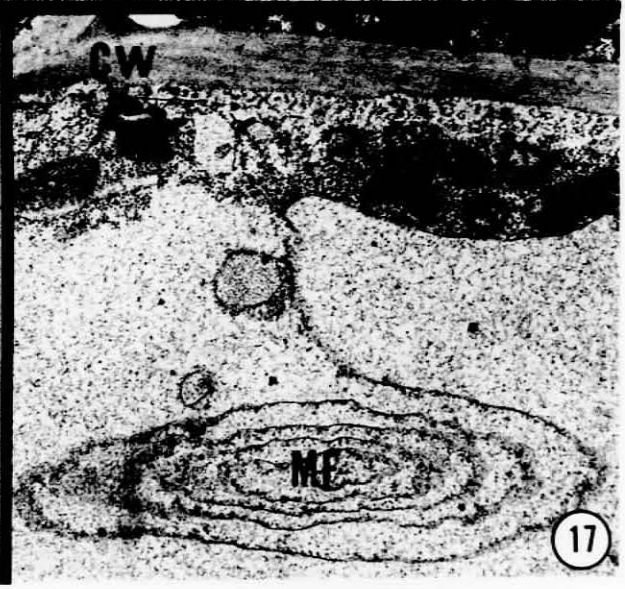
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Figure 18. Potato peels, light treated. Osmium tetroxide and glutaraldehyde fixation. X 11,155.

Figure 19. Potato peels, light treated. Osmium tetroxide and glutaraldehyde fixation. X 7,760.

Figure 20. Potato peels, control. Osmium tetroxide and glutaraldehyde fixation. X 11, 155.

Figure 21. Potato peels, control. Osmium tetroxide and glutaraldehyde fixation. X 14,550.

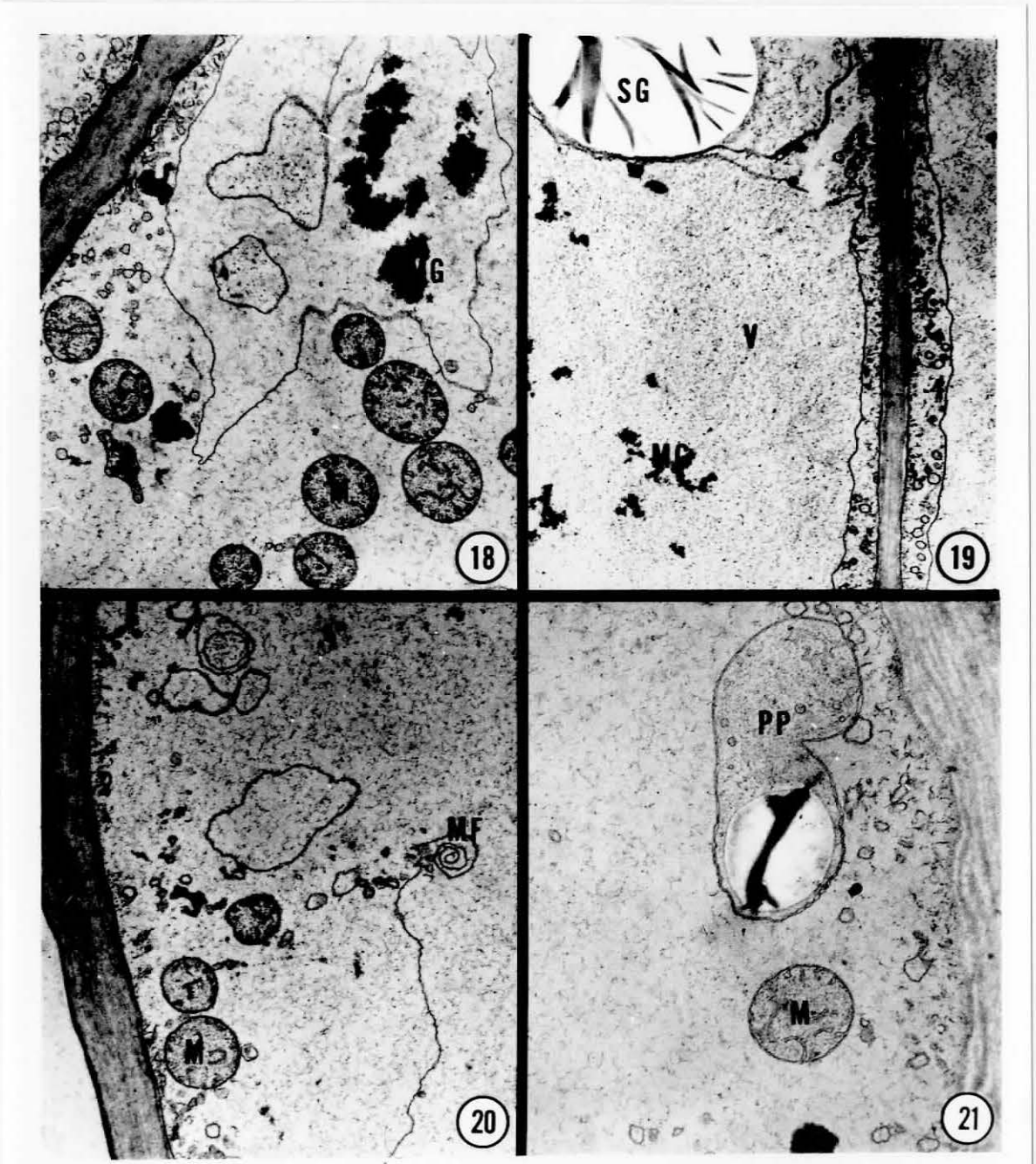
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Figure 22. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 23. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 24. Potato sprouts. Modified Napolitano procedure. X 5,580.

Figure 25. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 26. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 27. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 28. Potato sprouts. Modified Napolitano procedure. X 12,610.

Figure 29. Potato sprouts. Modified Napolitano procedure. X 12,610.

Figure 30. Potato sprouts. Modified Napolitano procedure. X 14,550.

Figure 31. Potato sprouts. Modified Napolitano procedure. X 14,550.

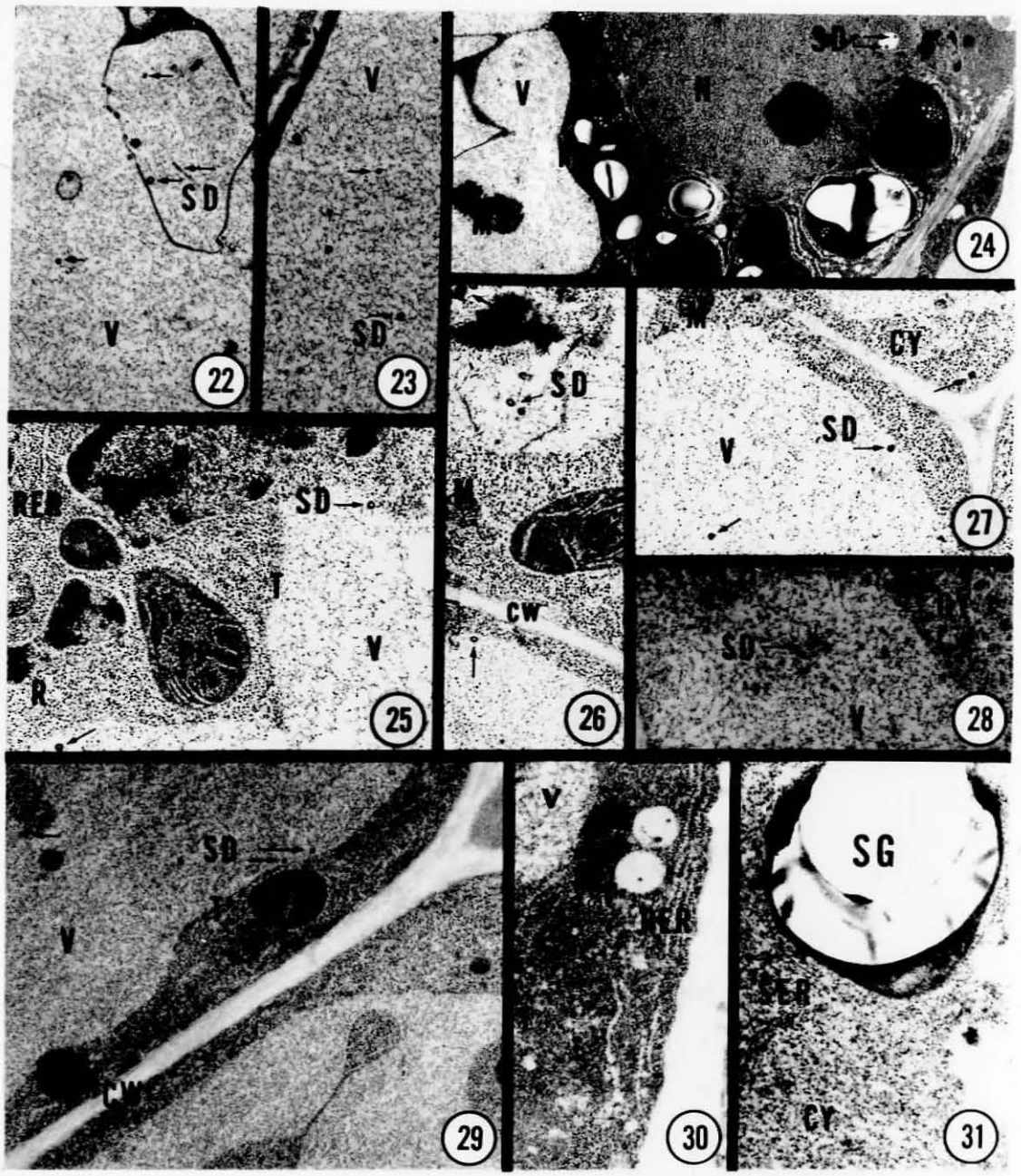
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Figure 32. Potato sprouts. Modified Napolitano procedure. X 14,550.

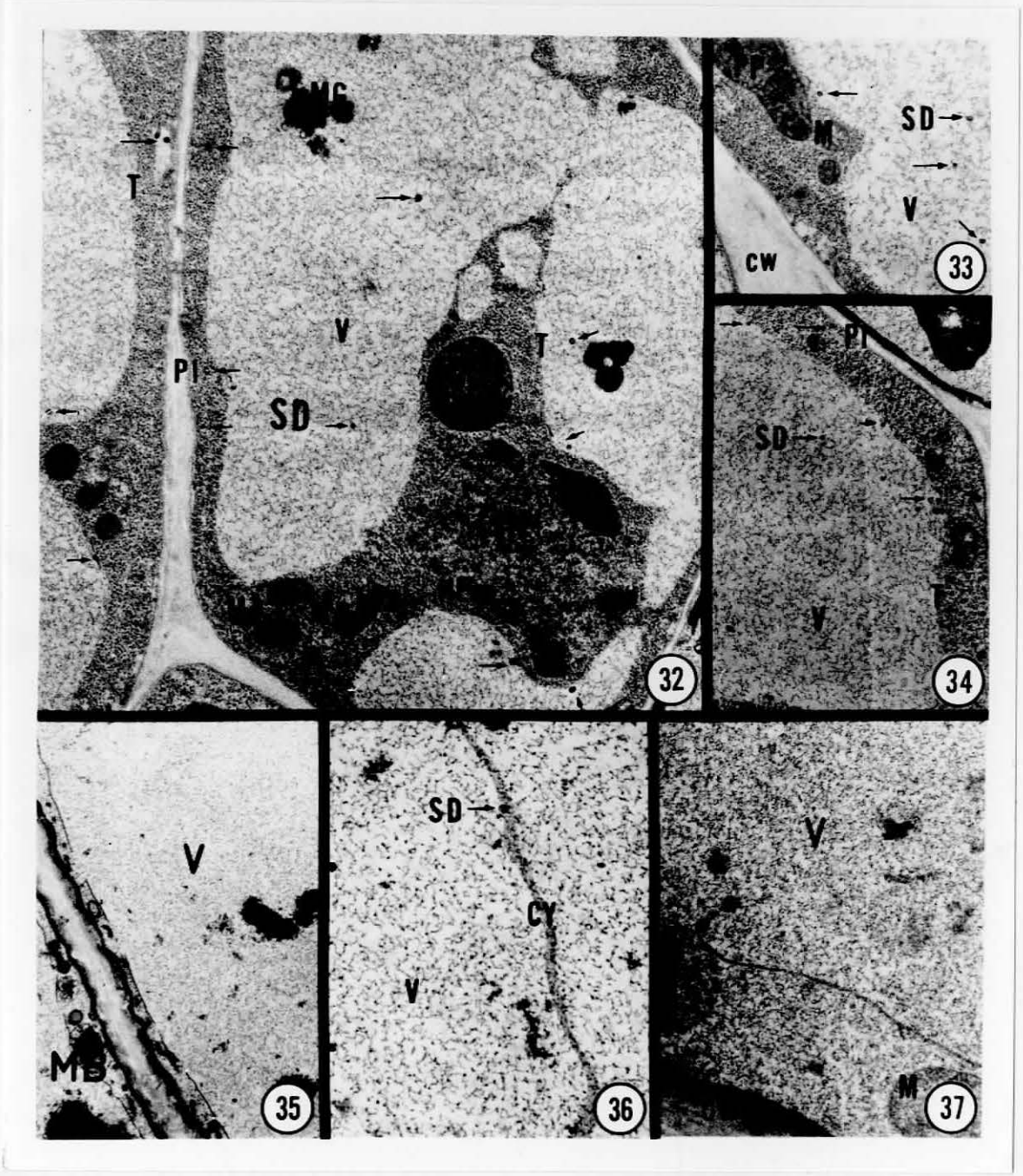
Figure 33. Potato sprouts. Modified Napolitano procedure. X 12,610.

Figure 34. Potato sprouts. Modified Napolitano procedure. X 12,610.

Figure 35. Potato peels, light treated. Modified Napolitano procedure. X 5,580.

Figure 36. Potato peels, light treated. Modified Napolitano procedure. X 14,550.

Figure 37. Potato peels, control. Modified Napolitano procedure. X 14,220.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