The development, physiology, and function of selected plant calcium oxalate crystal idioblasts

Albert Paul Kausch
Iowa State University

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The development, physiology, and function of selected plant calcium oxalate crystal idioblasts

by

Albert Paul Kausch

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Botany
Interdepartmental Major: Botany (Molecular, Cellular and Developmental Biology)

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For the Graduate College

Iowa State University
Ames, Iowa
1983
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INTRODUCTION

Deposits of insoluble calcium oxalate occur as microscopically visible crystals in a wide variety of plant taxa. Their formation in plants is considered to be nonpathological and is typically described as intracellular in origin. The crystallization process often occurs within highly specialized and complex cells called crystal idioblasts and is apparently under strict biological control. Crystal idioblasts often may develop within meristematic tissues and often differ considerably from cells in surrounding tissues in their size and appearance. Although a number of investigators have observed the ultrastructure of crystal idioblasts with light and electron microscopy, the developmental changes associated with crystal formation are still not resolved. Furthermore, the cellular machinery shown to be involved with crystal production in the cells of some plants is not equally obvious in other systems.

Crystal cells have been observed in many different organs and diverse tissues of a legion of different plants. The size, shape, location, and orientation of the idioblast, as well as the shape of the crystal produced within the idioblast, are considered to be characteristic for a given species. Some of these characteristics, the initiation of idioblast development, and the number of idioblasts ultimately formed may be affected by changes in environmental and nutritional conditions (Zindler-Frank, 1975; Franceschi and Horner, 1979). The causal stimuli governing idioblast initiation are unknown.

Oxalate may be produced biochemically in plants through at least five separate pathways in vivo. The specific synthetic pathway of this crystal
precursor has never been correlated with crystal formation in any plant. Similarly, the origin of calcium utilized in plant crystallization has never been elucidated.

The complexly detailed and specific nature of crystal formation and the wide occurrence of this phenomenon in plants has led many authors to speculate upon the function of their production. Despite the fact that crystallization is often observed in meristematic regions, the relationship of crystal formation to surrounding tissue and organ development has only recently been considered (Horner and Wagner, 1980).

The prevalence, biological curiosity, and aesthetics of plant crystals have inspired numerous investigators over the years. However, many aspects regarding their formation and function remain unknown. One of the goals of this project has been to understand what is involved developmentally as an undifferentiated cell becomes specialized for Ca oxalate accumulation. Besides observations of ultrastructural developmental events, attempts have been made to gain insight into metabolic and physiological events linked to idioblast differentiation. Another goal of this project has been to begin elucidation of inductive factors which affect idioblast cytodifferentiation in organ and tissue cultures. As a consequence of this project, some assessments concerning the function of these cells in normal plant growth can be made.

So little is known about the biology of calcium oxalate crystal cells that an investigation on any aspect of their development or physiology at this time seems pertinent. Actually, any fool with a few facts is an expert provided no one else knows any more than the fool. In discussions of eastern philosophy, we say that genius makes apparent complexity
simple to approximate the reality (Tao) of its origin; fools create complexity. Poets repeat, "It ain't why, why, why, why, why, why, . . . it just is." Science, in pursuit of truth (in fact, Aristotle's form of the truth), asks eternal whys, creating complexity. All is change and flow; ask why at this moment and it is something else by the time the answer is in hand. Today's scientific truths are tomorrow's belly laughs . . . it just is, it just is. It is my hope that through all of the whys in this dissertation that some form of the eternal change, however removed, may be appreciated from the still shot frames of nature depicted between these covers.

The so-called alternate dissertation format was chosen for presentation of my doctoral research. This format allows presentation of work that has been, or will be, submitted to various journals for publication. These journal articles are in the form of separate parts of the dissertation and the specific journal to which these papers are submitted is given on the title page of each.
LITERATURE REVIEW

Oxalate Biosynthesis

Oxalic acid is a dicarboxylic acid \([\text{(COOH)}_2]\), produced in plants through at least five biochemical pathways (Hodgkinson, 1977; Franceschi and Horner, 1980a). Oxalate has been reported to originate in some plants via enzymatic cleavage of oxaloacetate (Chang and Beevers, 1968) and/or ascorbate metabolism (Wagner and Loewus, 1973). However, it is generally accepted that the major intermediate precursors of oxalate in higher plants are glycolate and glyoxalate (Kpodar et al., 1978). These precursors may arise from the glycolate pathway (i.e., photorespiration; Tolbert, 1973), the glyoxalate bypass (Kornberg and Krebs, 1957) or purine degradation (Huang, 1982). Certain plant pathological fungi oxidize glyoxalate to oxalic acid in the presence of peroxisomal NAD-glyoxalate dehydrogenase (Armentrout et al., 1978) but this system is not found in any higher plant species (Huang, 1982). Salient features of oxalate synthetic pathways in plant cells provide a necessary background for this dissertation on calcium oxalate crystal idioblast biology.

The glycolate pathway

Glycolate arises as a result of photosynthesis (Tolbert, 1973) and is the principal substrate of photorespiration (Tolbert, 1973; Zelitch, 1975a,b) as the starting point of the glycolate pathway. Its formation is \(O_2\) and light dependent and inhibited in high \(CO_2\) concentration, although the exact route of its production is not entirely clear (Zelitch, 1971). Glycolic acid is one of the first formed products of photosynthesis in
higher plants (Norris et al., 1955; Tolbert, 1973). Gibbs (1971) believes that the production of glycolate is closely associated with photosystems (PS) I and II. He found that under normal light and CO₂ levels, H₂O₂ is formed, presumably by PS II as the result of the interaction of a reduced carrier with molecular oxygen. Subsequently, the peroxidation of a TPP-C₂ complex formed by a transketolase reaction yields glycolate.

Chollet and Orgren (1972a) present the most reasonable hypothesis for glycolate origin to date. They found that the oxygenase activity of ribulose diphosphate (RuDP) carboxylase ultimately produces glycolate from O₂ and RuDP. If O₂ was removed from the reaction medium, no glycolate was synthesized. In such a reaction, O₂ is actually a competitor of CO₂ for the RuDP on the RuDP carboxylase and is therefore an inhibitor of photosynthesis. The effect of O₂ on RuDP carboxylase would cause the oxidation of RuDP to 3-phosphoglyceric acid and 2-phosphoglycolic acid. The dephosphorylation of 2-phosphoglycolate via the action of phosphoglycolate phosphatase (Tolbert et al., 1969) will give rise to glycolic acid. This may normally occur to a slight extent but increases with a decrease in CO₂ or an increase in O₂ concentration (Chollet and Orgren, 1972b). This inhibition has been shown in vivo in Chlamydomonas reinhardtii (Bowes and Berry, 1972). This alga excretes glycolic acid in greater amounts as O₂ concentration is increased, and less CO₂ is fixed. Under the conditions of high light, high O₂ and low CO₂ concentrations, the RuDP oxygenase activity would be favored, explaining the enhanced production of glycolate under these conditions (Chollet and Orgren, 1972a). RuDP carboxylate (oxygenase) has been found by Malnoe et al. (1979) to be exclusively in
the chloroplastic fraction. Thus, the initial reaction of the glycolate pathway must occur in the chloroplast.

The most well-known immediate product of glycolic acid is glyoxylic acid. Actually, it is glyoxylic acid that is capable of undergoing various reactions, depending upon several cellular factors and conditions, which ultimately determine the fate of glycolic acid carbon (Zelitch, 1971). Nord and Vitucci (1947) reported the oxidation of glycolic acid to oxalate, suggesting glyoxylate as an intermediate, by a wood decaying fungus. Richardson and Tolbert (1961) in a study of several plants found that the oxidation of glycolate resulted in various products in different species. The products were a result of subsequent reactions made possible by different enzymes after glyoxylate was produced from glycolate.

Glycolic acid oxidase, an enzyme capable of oxidizing glycolate to glyoxylate, was first reported by Clagett and coworkers in 1949 in the green parts of many plants. Kenten and Mann (1952) isolated a plant hydroxy-acid oxidase which, in the presence of catalase, could oxidize glycolate to glyoxylate and glyoxylate to oxalate. The catalase was necessary to remove hydrogen peroxide produced from glycolate oxidation, which would otherwise nonenzymatically oxidize glyoxylate to formic acid and CO₂ (Bornkamm, 1965). Glycolic acid oxidase was not detected in early stages of plant development by Clagget et al. (1949) and etiolated plants had to be exposed to light before enzyme activity could be detected. Noll and Burris (1954) reported the presence of glycolic acid oxidase in all 16 common plant families which they studied. They also found the enzyme in etiolated seedlings and noted that exposure to light resulted in its in-
crease. The older the green plant material, the more oxidase activity was present.

There are differences in oxidase activity and enzymatic forms among several plant species. Of the plants tested by Richardson and Tolbert (1961), the tobacco enzyme was the most active and the spinach enzyme was the least active, with sugar beet and Swiss chard enzymes intermediate between the extremes. Three different forms of glycolate oxidase have been recognized. Baker and Tolbert (1967) found a nonflavin form of glycolate oxidase in etiolated wheat seedlings that was activated by ferredoxin. Bornkamm (1969) described two forms of the enzyme; one was flavin mononucleotide (FMN) activated and the other was substrate activated (glycolate preferentially to glyoxylate). The enzyme described previously by Baker and Tolbert (1967) was confined to young tissue and they suggested that it may be converted to FMN (or possibly substrate) activated enzyme in older tissue. Tolbert et al. (1968) found that the vast majority of glycolate oxidase, and hence the oxidation reaction of glycolate to glyoxalate, occurs within the peroxisome. Glycolate oxidase is considered as a ubiquitous marker enzyme for plant peroxisomes.

While the enzyme glycolate oxidase has been fully characterized in plants (Bornkamm, 1969), the physical and catalytic properties seem to vary somewhat among species. Zelitch and Ochoa (1953) characterized a highly purified glycolic acid oxidase from spinach leaves which had a flavoprotein as its prosthetic group. The enzyme did not catalyse any further reactions with glyoxylate, but could oxidize other substrates besides glycolic acid (i.e., lactic acid) and utilize electron acceptors
other than $O_2$. This enzyme in barley is somewhat unstable; all activity may be lost after three hours of dialysis (Kolesnikov et al., 1959).

Richardson and Tolbert (1961) found that the activity of glycolate oxidase in spinach is not necessarily restricted to oxidizing glycolate to glyoxylate in vivo, but under specific cellular conditions, glyoxylate is oxidized to oxalate. Furthermore, they showed that the flavin-linked enzyme was competitively inhibited by oxalate. They implied that this provided for the regulation of oxalate synthesis. The Michaelis-Menten plots also show that the enzyme had a greater affinity for glycolate than for glyoxylate.

Millerd et al. (1963d) found that glycolate oxidase from Oxalis was not affected by the presence of oxalate and had no preferential affinity for glycolate or glyoxylate. It may be that in vivo, the activity of glyoxylate oxidation may be high if oxalate is removed from the reaction site.

A number of fates may befall the glyoxylate produced from photosynthetically derived glycolate in the glycolate oxidase reaction. It may return to the chloroplast to be reduced to glycolate by glycolate reductase (Zelitch, 1953) so that a glycolate-glyoxylate shuttle between the peroxisome and chloroplast is established (Carles and Assailly, 1954).

Much of the glyoxylates produced from the oxidation of glycolate is transaminated to glycine in the presence of glutamic acid (Richardson and Tolbert, 1961). Glycine may give rise to serine and $CO_2$ by a hydroxymethyltransferase reaction (Tolbert and Cohan, 1953). Zelitch (1971) found that the serine may become deaminated to hydroxypyruvic acid, which after reduction and phosphorylation enters the sugar synthetic cycle as PGA.

A third possibility for glyoxylate metabolism, previously suggested by
Nord and Vittucci (1947) and Kenten and Mann (1952), was substantiated when Zbinovsky and Burris (1952) found that labelled glycolic acid, infiltrated into tobacco and buckwheat leaves, was converted to oxalate. An oxalic acid cycle based on the activity of glycolate oxidase and glycolate reductase was proposed by Carles and Assailly (1954).

In view of the foregoing information, oxalate appears to arise from glycolate via glyoxylylate in the green tissues of certain plants. Numerous early studies had suggested that oxalate synthesis was related to photo-synthetically derived carbon compounds (Steinmann, 1917; Pucher et al., 1939; Stutz and Burris, 1951; Vickery and Palmer, 1956; Tavant, 1967). Seal and Sen (1970) found a close association between oxalate formation and photosynthesis in *Oxalis corniculata*. Oxalate concentration was found to increase during the hours of peak photosynthesis and decrease during the evening and night. They suggested that the production of oxalate through the glycolate pathway is very much light dependent because its precursor (glycolate) is produced in the light. Photosynthetically derived glycolate is oxidized by glycolate oxidase to glyoxylylate and then further oxidized to oxalic acid by the same enzyme (Chang and Beevers, 1968; Seal and Sen, 1970; Zindler-Frank, 1974).

**The glyoxylate bypass**

Glyoxylylate is also produced through a photosynthetically independent pathway involving Kreb cycle intermediates. Kornberg and Krebs (1957) were the first to elucidate a sequence of glyconeogenic reactions which were to become known as the glyoxylate bypass or the glyoxylate cycle. The glyoxylate cycle is involved in the transformation of stored lipid
material to carbohydrate (Carpenter and Beevers, 1959). Kornberg and Krebs (1957) found that acetyl-CoA derived from the β-oxidation of fatty acids, combined with oxaloacetate to form citrate under the action of citrate synthetase. The citrate is then subsequently converted to isocitrate by aconitase. Isocitric acid, in turn, could be enzymatically cleaved by isocitrate lyase to form succinate and glyoxylate. The succinate becomes involved in the reaction of the TCA cycle and subsequent gluconeogenic reactions. The glyoxylate may combine with acetyl-CoA to produce malate via malate synthetase (Kornberg and Beevers, 1957) and also become involved in gluconeogenesis (Neal and Beevers, 1960). However, the glyoxylate produced in the isocitrate lyase reaction could conceivably be used for oxalate biosynthesis (Miller and Beevers, 1959).

A number of studies have demonstrated an association between TCA cycle intermediates and oxalic acid production. Miller and Beevers (1963a,b,c,d) investigated the formation of oxalic acid in shoots of Oxalis pes caprae. The formation of glyoxylate by the action of isocitrate lyase was traced using 14C labelled isocitrate. Carbon from labelled glyoxylate was then rapidly and efficiently incorporated into oxalic acid. Osmond and Avandhani (1968) showed that Kreb cycle intermediates become labelled following short term incorporation of 14CO2 in Atriplex leaves in the dark. Label presumably from some Kreb cycle compound was shown to slowly accumulate in glyoxylate and oxalic acid.

Furthermore, they identified the presence of isocitrate lyase and glycolate oxidase and found that glyoxylate from isocitrate could be converted to oxalate in the dark via this pathway.
Isocitrate lyase is the key enzyme of the glyoxylate cycle which is involved in lipid–carbohydrate transformations (Stumpf, 1965). The enzyme was not considered to be widespread in green tissues of plants (Carpenter and Beevers, 1959) but generally confined to those tissues in which active breakdown of lipids occurs, like castor bean seeds (Kornberg and Beevers, 1957) and pine seeds (Ching, 1970). However, as the studies of Miller et al. (1963a,b,c,d), Morton and Wells (1964) and Osmond and Awandhani (1968) indicate, an association between isocitric acid lyase activity and oxalate synthesis exists in the leaves and shoots of some plants.

The enzymes for the glyoxylate cycle are present in microbodies called glyoxysomes (Breidenbach and Beevers, 1967). More than 80% of the particulate activity of the two enzymes unique to the glyoxylate cycle, isocitrate lyase and malate synthetase, were found sedimented in the glyoxysomal fraction, while citrate synthetase and malate dehydrogenase, which occur in both the TCA and glyoxylate cycles were present in both the mitochondrial and glyoxysomal fractions. Cooper and Beevers (1969a,b) have shown the glyoxysomes of higher plant cells to be the site of both β-oxidation and of the enzymes of the glyoxylate cycle. Glyoxysomes are generally considered to be confined to nongreen tissues. Other microbodies, which are related to glyoxylate metabolism, are referred to as peroxisomes (Tolbert et al., 1968). The peroxisomes are present only in green tissues and contain a high percentage of the glycolate oxidase (Tolbert et al., 1968), as well as catalase (Frederick and Newcomb, 1969) in the cell. These are the principal enzymes involved in the glycolate pathway.
Some studies in plant development have shown that the glyoxysomes present in high numbers in fatty seeds eventually transform into peroxisomes as the tissues green (Trelease et al., 1971). Cucumber cotyledons have glyoxysomes with isocitrate lyase, malate synthetase, and catalase. Usually, catalase is considered to be associated only with peroxisomes (Gruber et al., 1970). In cucumber cotyledons, the glyoxysomes are dispersed among spherosomes. As these tissues become green and the lipid stores are depleted, the glyoxysomes are no longer present as such; they become peroxisomes (with catalase) physically associated with the chloroplasts (Trelease et al., 1971). It is not known whether these two types of microbodies are indeed separate entities or merely the same structure with different enzymatic constituents (Beevers, 1971). Longo and Longo (1970) observed an increase in the activities of the glyoxylate cycle enzymes in the first four days of maize seed germination and then a subsequent decline. In Triticum leaves, even in early development, there are no glyoxysome-specific enzymes present (Feierabend and Beevers, 1972). All microbodies in this tissue at all developmental stages have catalase and glycolate oxidase which classify them as peroxisomes. Peroxisomes contain the principal enzymes of the glycolate pathway (Tolbert et al., 1969).

**Oxaloacetate cleavage**

Apparently, some plants are capable of producing oxalic acid from oxaloacetic acid, another TCA cycle intermediate. In 1956, Hayashi et al. showed that the enzyme oxaloacetic acid hydrolase catalyzed the cleavage of oxaloacetate to acetate and oxalic acid in the fungus
Aspergillus. Chang and Beevers (1968) showed that aspartate, succinate, oxaloacetate, and malate were all converted to oxalic acid in beet and spinach leaves, presumably through the oxaloacetic acid hydrolase reaction. Furthermore, TCA cycle inhibitors stopped any accumulation of oxalate. They implied that oxaloacetate was giving rise to oxalate in normally functioning plant cells, but the role of such a pathway is not understood. They speculated that this system may provide a necessary pathway for oxalate synthesis in nongreen tissues which lack glycolate oxidase. This pathway might also operate in tissues where lipid breakdown is not extremely active through the glyoxylate bypass. However, this assumes a necessary function for oxalate in all plant cells. Furthermore, Chang and Beevers (1968) found this reaction occurring in green spinach leaves. Clearly, further work is still necessary to establish this mechanism unequivocally.

Ascorbic acid metabolism

Crude extracts from plant tissues typically oxidize L-ascorbate rapidly to dehydroascorbate, which is sometimes further degraded to oxalic and tartaric acids (Loewus et al., 1975). Ascorbate oxidase catalyzes the oxidation of ascorbate by molecular oxygen to yield dehydroascorbate and water (Butt, 1980). A wide range of copper-complexing agents inhibits the enzyme, including: cyanide, diethylthiocarbamate, H₂S, potassium ethyl xanthate, 8-hydroxyquinaline, thiourea, salicylaldoxime, azide, and cupferron (Butt, 1980). A role for copper in the catalytic activity of the enzyme is indicated by the inhibitory action of these complexing reagents.
The enzyme is frequently described as soluble; however, appreciable proportions of the enzyme in a tissue homogenate may be sedimented at very low speeds, in fractions in which only cell wall fragments are demonstrable (Honda, 1955). Seventy percent of the total activity of cabbage leaves (Hallaway et al., 1970), maize root tips (Mertz, 1961) and orange fruit (Vines and Oberbacher, 1963) is sedimented with cell walls, and this proportion increases further in root tips of older barley seedlings and in maturing cells in the maize root tip. The properties of the wall-bound enzyme are not different from those of the soluble enzyme (Hallaway et al., 1970). This association between the cell wall and ascorbate oxidase appears not to arise during extraction (Hallaway et al., 1970; Butt, 1980), and should be taken into account when considering the physiological role of this enzyme.

The oxidation product of the ascorbate oxidase reaction, dehydroascorbate is further metabolized to tartaric acid and oxalate in some plants (Wagner and Loewus, 1973) in a series of redox reactions. The most likely reducing agent for dehydroascorbate is glutathione (GSH) in the presence of GSH-dehydroascorbate reductase (Foyer and Halliwell, 1977). The maintenance of reduced glutathione in plant tissues under oxidizing conditions suggests the operation of some further reduction system. The rapid reduction of oxidized to reduced glutathione was demonstrated to be due to NADPH-glutathione reductase. This enzyme has been purified to homogeneity from spinach leaves (Halliwell and Foyer, 1978) and contains FAD. The enzyme is cosedimented with NADP⁺:glyceraldehyde-3-phosphate dehydrogenase in chloroplasts. In contrast, dehydroascorbate reductase
appears to be restricted to the cytosol. Therefore, metabolized ascorbate is oxidized first in cell walls, moved to a cytosol reaction site and further metabolized in plastids, where a redox system is apparently established.

The role of these enzymes in plant tissues has to be considered in relation, on the one hand, to their possible coordinated action to give a sequence of electron carriers from NADPH to molecular oxygen and, on the other, to the possible functions of ascorbate and glutathione in regulating or maintaining processes within the cell during growth, maturity, or senescence. The earliest and most obvious suggestion was that of a redox system, in plastids, in which NADPH is oxidized by successive transfer of electrons and hydrogen through glutathione and ascorbate to molecular oxygen (Butt, 1980).

The role of ascorbate metabolism to other organic acids or hexose sugar is less apparent because of the unknown functions of these products. Wagner and Loewus (1973) reported that oxalate formation may result from ascorbic acid metabolism in Pelargonium crispum. Ascorbic acid metabolism, however, does not yield oxalate in all plants, but may give rise to tartarate and hexose sugars (Wagner and Loewus, 1974). In labelling experiments, Yang and Loewus (1975) found that plants that develop large amounts of oxalate will metabolize ascorbate to oxalic acid, whereas non-oxalate accumulators would not convert ascorbate to oxalic acid. Nuss and Loewus (1978) further supported this notion, and also reported that carbons one and two from the ascorbate molecule are lost to oxalate in that reaction.
Purine degradation

Purine metabolism in both plants and animals results in production of xanthine (Thomas and Schrader, 1981). In the presence of molecular oxygen and water, xanthine is converted to uric acid in a reaction mediated by xanthine oxidase. Uric acid is a common excretory production of humans, primates generally, and Dalmation dogs. However, in many organisms, uric acid is oxidized to allantoin by urate oxidase in peroxisomes. This reaction requires oxygen and water and yields carbon dioxide and hydrogen peroxide. Subsequently, allantoin may be hydrolyzed to allantoic acid in the presence of allantoinase.

Allantoin and allantoic acid, ureides, are important metabolites for nitrogen transport in the phloem of some plant species. In species of the genera Persea, Acer, Platanus, and Aesculus, the xylem saps contain allantoin and allantoic acid as the major forms of nitrogen, representing up to 99 percent of the total transport nitrogen. The transport of organic nitrogen from the nodules to the shoots of symbiotic nitrogen-fixing legumes, some species (Vicia and Pisum) translocate asparagine whereas most other species, including Glycine and Vigna, transport ureides as the major nitrogen carriers (Rawsthorne et al., 1980).

Only low activities of ureide pathway enzymes are found in plant peroxisomes. Low activities of urate oxidase are present in various types of plant peroxisomes, including glyoxysomes (Theimer and Beevers, 1971), leaf peroxisomes, and unspecialized peroxisomes (Huang and Beevers, 1971). Allantoinase is demonstrable in some plant peroxisomes, but with a very low activity (Theimer and Beevers, 1971). Content of ureide metabolic
enzymes have only been studied recently in the tissues of those plant species that utilize ureides as nitrogen transport metabolites (Huang, 1982).

In nodule extracts of soybean and cowpea, substantial activities of xanthine oxidase, allantoinase and urate oxidase are present (Rawsthorne et al., 1980). In soybean nodules, large and proliferous peroxisomes are present in the uninfected root cells, adjacent to infected cortical cells which contain small or degenerated peroxisomes (Newcomb and Tandon, 1981). In nodules, catalase and urate oxidase are localized in peroxisomes whereas xanthine oxidase and allantoinase are restricted to the cytosol and microsomes, respectively (Hanks et al., 1981). These peroxisomes contain a high activity of urate oxidase which is at least several fold higher than that in other plant peroxisomes (Hanks et al., 1981). This high activity has recently enabled ultrastructural localization of urate oxidase in several legume nodules (Vaughn et al., 1982) with the CeCl₃ technique. Apparently, unlike the peroxisomes in ureotelic animals, soybean peroxisomes contain only a small part of the ureid metabolic pathway. Perhaps they do not function in the totality of this pathway, per se.

Allantoic acid may be metabolized to ureidoglycolic acid by allantoinase, and then to glyoxylate and urea by ureidoglycolase (Thomas and Schrader, 1981). It is conceivable, but has not been demonstrated, that this glyoxylate could be oxidized in the peroxisome by glycolate oxidase to oxalic acid. Oxalic acid produced by this sequence of reactions could contribute to calcium oxalate crystal synthesis, but requires further investigation.
Oxalic acid

Once oxalate is produced, it may accumulate in small amounts as the free acid (Soderstrom, 1962), it may form ionic soluble salts (i.e., Na⁺, K⁺) or become precipitated as an insoluble salt (Bornkamm, 1965), or it may be further oxidized to CO₂ (Finkle and Arnon, 1954). Ranson (1965) suggests that the difference may be accounted for by the pathway through which the oxalate was produced; i.e., from photosynthetically derived glycolate or from ascorbate, oxaloacetate, or isocitrate. Crombie (1960) suggested that the metabolic role of oxalate may be manifold and vary from plant to plant. Richardson and Tolbert (1961) hypothesize that oxalate production may occur because the enzyme glycolate oxidase has reached a limit in evolutionary development, allowing an undesirable product. However, this hardly explains the varied modes of oxalate synthesis. Few authors discuss reasons for oxalate formation; most often the question is abated by referring to oxalate as an undesirable end product or a toxic agent against predation. In none of the described conditions of oxalate accumulation was there any suggestions that oxalic acid contributes to the metabolism of the plant (Franceschi and Horner, 1980a).

Apparently then, the higher plant diverts part of its assimilated carbon into a presumable waste product. The notion that such an uneconomical pathway has survived evolutionary processes is difficult to accept. Zindler-Frank (1976) notes that the apparent uselessness of Ca oxalate (the salt) is even less understandable since it does not precipitate indiscriminately anywhere in the plant tissue, but rather crystallization occurs in many cases, within highly specialized structures
In an attempt to explain a physiological role of calcium oxalate and free oxalic acid, early investigators Amar (1904), Stuhl (1920), Muller (as cited by Zindler-Frank, 1975) thought of excess calcium uptake as harmful and the oxalate necessary to tie it up. Later studies (Olsen, 1939; Scharrer and Jung, 1954; Ackermann, 1958) showed that increasing the calcium concentration in the substrate would result in a concomitant increase in the oxalate concentration in the analyzed tissues. Rasmussen and Smith (1961) substantiated these reports and showed that increased potassium concentration would also result in increased oxalate concentration. In 1965, Bornkamm showed that calcium deficiency caused an increase in soluble oxalate together with a decrease in insoluble oxalate and suggested that ionic calcium was in equilibrium with insoluble calcium oxalate. These studies seem to suggest an interrelationship between oxalate synthesis and ion regulation.

Several investigations have been conducted to determine if a causal relationship exists between calcium level, stages of oxalate biosynthesis, and the differentiation of idioblast containing calcium oxalate crystals. Osmond (1967) found that in Atriplex leaves there was no correlation between oxalate synthesis and calcium absorption. He suggested that oxalate was synthesized in response to the total cation complement and not merely the calcium level. Zindler-Frank (1975) obtained results in a study on young plants of Canavalia ensiformis that depicted calcium as responsible
for the induction of oxalate synthesis. In earlier studies, she found that the number of cells which differentiate into idioblasts is dependent on the amount of nutritional calcium (Frank, 1972) and that inhibition of glycolate oxidase decreases the number of idioblasts (Zindler-Frank, 1974).

Franceschi (1978) and Franceshi and Horner (1979, 1980b) used callus cultures of *Psychotria punctata*, a more controlled system, and found that calcium levels did influence the number of crystal idioblasts formed. They also report that induced inhibition of glycolate oxidase results in a reduction in the number of crystal idioblasts; however, inconclusive results were obtained for ascorbate synthetase inhibition and no experiments were conducted to block isocitrate lyase. Furthermore, they conclude that calcium absorption and oxalate synthesis are probably dependent upon each other and both appear to be important in idioblast differentiation. These studies suggest that any parenchymatous cell, which normally does not differentiate into an idioblast, has the capability to become a crystal containing cell if the proper inductive stimulus is provided. The reverse may be true as well. It is still unknown, however, how increased levels of calcium or oxalate cause idioblast initiation. Idioblast initiation by calcium or oxalate strongly suggests a physiological function for both beyond sequestration of an undesirable waste product.

Rasmussen and Smith (1961) suggest that the apparent relationship between calcium levels and oxalate synthesis (as well as idioblast differentiation) is the result of a maintenance of ionic balance by the plant. Excess calcium may be removed by causing oxalate synthesis, resulting in
idioblast initiation and crystal formation (Franceschi, 1978). In several cases, breakdown or reabsorption of calcium oxalate crystals have been reported (Scott, 1941; Calmés, 1969; Bornkamm, 1965; Arnott and Pautard, 1970; Calmés and Fiquemal, 1977; Tilton and Horner, 1980). Calcium oxalate reabsorption and crystal idioblast initiation caused by calcium indicate that the crystals may serve as a storage source for calcium (Franceschi and Horner, 1979). These observations in themselves imply a physiological role for oxalate (and calcium) which so far has been so difficult to understand or so subtle as to evade elucidation.

Calcium Oxalate Deposits

Of all of the oxalates, the insoluble salts have been known the longest and studied the most thoroughly (Arnott and Pautard, 1970). Furthermore, of all of the insoluble calcium salts, such as sulfate, phosphate, silicate, carbonate, oxalate, citrate, tartarate, and malate which occur as microscopic, crystalline cellular inclusions, calcium oxalate is the most common in plants (Arnott, 1973). Intracellular crystals of calcium oxalate have been studied more than any other insoluble calcium salt in plants (Arnott, 1966). While the biological curiosity and aesthetics of plant crystals have attracted so much attention over the years, many aspects regarding their formation and function remain unclear.

Calcium oxalate deposits occur as various types of crystals in the vacuoles of highly specialized plant cells (sometimes called idioblasts) and are considered to be of nonpathological origin (Arnott, 1966, 1973, 1976; Eilert, 1974; Franceschi and Horner, 1980a). Various calcium
oxalate crystal shapes and aggregates have been described (Franceschi and Horner, 1980a). The most commonly encountered forms are: (1) raphides, needle-shaped crystals occurring in bundles of numerous crystals per cell; (2) styloids, an elongated, needle-shaped crystal occurring singly per cell; (3) prisms, regularly shaped pyrimidal or bipyramidal crystals; (4) crystal sand, aggregates of many small free crystals per cell; (5) druses, a spherical star-like crystal aggregate occurring one to many per cell; and (6) conglomerate crystals composed of two or more of the above types.

Various investigations have published results which demonstrate that gross crystal morphology may be related to the amount of water of hydration of the component oxalate (Franceschi and Horner, 1980a). McNair (1932) first suggested this notion and described the two principal chemical forms of plant calcium oxalate. The monohydrate (CaC₂O₄·H₂O), also known as whewellite, forms crystals belonging to the monoclinic system of crystallization. The dihydrate (CaC₂O₄·2·2.5 H₂O), also known as weddellite, develops crystals belonging to the tetragonal system. Raphides have been identified as whewellite (Al-Rais et al., 1971), druses (Rivera and Smith, 1979) and prisms have been reported as weddellite (Al-Rais et al., 1971; Rivera, 1973). Collectively, past investigations seem to indicate that all raphides are whewellite (Franceschi and Horner, 1980a); whereas, several discrepancies in the X-ray diffraction data between other crystal types prevented definitive correlations (Al-Rais et al., 1971; Franceschi and Horner, 1980a).
The shape of calcium oxalate crystals within a given taxonomic group is often very specific (Arnott, 1966, 1973; Arnott and Pautard, 1970). This property has induced some workers to try to correlate phylogeny with crystal type production (Gulliver, 1864; Jacard and Frey, 1928; Chart-schenko, 1932; Heintzelman and Howard, 1948). Studies within crystal producing families like the Leguminoseae have shown that crystal shapes (or types) do not show discernible patterns of phylogeny. Tapetal cells, which would be expected to share physiological similarities among plants, of 167 legume species were examined by Buss and Lersten (1972) and no correlation of crystal shape could be established in the various taxa. Furthermore, not even a consistency of crystal deposition was observed; some taxa did not have crystals. The taxonomic value of crystal shape must rely on a general genetic control. Scurfield et al. (1973), Horner and Wagner (1980) and McNair (1932) all have observed crystals of different shapes occurring within adjacent cells. Horner and Wagner (1980) have observed three different types of crystals in the anthers of Capsicum annuum. Oxalate crystal production appears to be a more fundamental physiological process at the tissue level rather than a characteristic of genera or families of plants. The only justifiable comparisons which can be made are between families which produce insoluble oxalate and those that do not accumulate oxalate (Bornkamm, 1965, 1969). These differences may reside in the system of oxalate synthesis (Bornkamm, 1969) or specific calcium requirements (Horak and Kinzel, 1971; Zindler-Frank, 1976). It may be that the calcium metabolism rather than the oxalate, which is
the more important determining factor in oxalate deposits (Franceschi and Horner, 1979).

The distribution of calcium oxalate crystal idioblasts throughout any plant is characteristic of that species and, therefore, predictable (Arnott and Pautard, 1970; Eilert, 1974). The number of idioblasts present in any one plant is not fixed, however. Various external factors may determine the number of crystal idioblasts present at any one time in any plant, some of which have been previously discussed (Zindler-Frank, 1974, 1975, 1976; Franceschi and Horner, 1979).

Crystals have been reported in a large number of cell types and diverse tissues; Stebbins et al. (1972) report crystals in many different tissues of a single plant. Calcium oxalate crystals have been reported in common cell types (nonidioblastic) such as tapetal cells (Buss and Lersten, 1972; Horner, 1977), xylem (Czaninski, 1968), collenchyma (Thoday and Evans, 1932), stomium cells (Horner and Wagner, 1980) as well as other tissues in the anther (Schmid, 1976, 1980) in cells containing protein bodies in cotyledons (Buttrose and Lott, 1978a, b; Lott and Buttrose, 1978) and in sclerids (Gaudet, 1960; Arnott and Pautard, 1970).

Crystal idioblasts have been reported in nearly all major tissue types, either confined to specific tissues or organs or found throughout the plant (for reviews, see Arnott, 1973; Franceschi and Horner, 1980a). Crystals of calcium oxalate have been found in every vegetative plant part including roots (Arnott, 1966, 1976; Mollenhauer and Larson, 1966; Stebbins et al., 1972; Eilert, 1974; Horner and Franceschi, 1978), stems (Sakai and Hanson, 1974; Wattendorff and Schmid, 1973), corms and rhizomes (Sakai and
Hanson, 1974; Sunell and Healey, 1979; this study) and most commonly in petioles and leaves (Arnott, 1966, 1976; Frank and Jensen, 1970; Horner and Whitmoyer, 1972; Sakai and Hanson, 1974; and others). Crystal cells have been reported in all major regions of the plant reproductive system (Horner, 1977; Franceschi and Horner, 1980b; Tilton and Horner, 1980) in woody tissues (Chattaway, 1953, 1955, 1956) as well as in cork and bark tissues (Wattendorff and Schmid, 1973).

It is interesting to note that cells containing crystals of calcium oxalate have been frequently reported in association with the aerenchyma and air space tissue in several aquatic plants. Cells bearing clustered crystals of calcium oxalate have been noted in the aerenchymatous petioles of *Nelumbo* (Solereder, 1908), leaves of *Pandanus*, *Typha* and *Sparganium* (Solereder and Meyer, 1933) and stems and leaves of *Myriophyllum* (Frey-Wyssling, 1935; Hasman and Ilanc, 1957; Horner and Franceschi, 1978). Sclerids, common in the aerenchyma or in the walls of lacunae of *Euryale*, *Nuphar*, *Nymphaea*, *Nymphoides* and *Victoria* bear crystals of calcium oxalate (Metcalfe and Chalk, 1950; Gaudet, 1960). The thalli of *Spirodela* and *Lemna* are only a few cells thick with large intercellular spaces and lacunae (Sculthorpe, 1967) and calcium oxalate crystals are reportedly accumulated in the thallus, root cortex and epidermis of these plants (Sculthorpe, 1967; Arnott, 1976). Kaul (1972) notes crystal idioblasts filled with druses located in the diaphragms between air spaces in *Sparganium americana*. Dudinskii (1976) reports that the formation of raphides coincides with the development of the air-filled cavities in leaves of *Typha latifolia*. Although not mentioned in his reports, raphide
crystal idioblasts are distinguishable in micrographs in Kaul's subsequent studies on *Sparganium eurycarpum* (Kaul, 1973) and *Typha latifolia* (Kaul, 1974). Sakai and Hanson (1974) often found crystal idioblasts traversing the aerenchyma and jutting into the air canals in *Colocasia*, *Alocasia* and *Xanthosoma*. In rhizomes and leaves of *Colocasia*, crystal cells also appeared adjacent to small intercellular spaces. Horner and Franceschi (1978) describe crystal idioblasts found protruding from the septa into the air spaces in *Myriophyllum*, *Pistia*, and *Eichhornia*. Mikesell and Schroeder (1979) report that raphide crystals form in diaphragm cells during the development of chambered pith in stems of *Phytolacca americana* while aerenchyma provides an air conducting system between aerial and submerged plant parts as well as structural rigidity (Williams and Barber, 1961). Perhaps the often attending crystals aid in the developmental formation of these tissues and/or mechanical support.

Numerous authors have noted that crystal idioblast formation often occurs in very young tissues during differentiation. Arnott (1966) and Eilert (1974) have reported that crystal cells develop from the meristematic cells in the root tip of *Yucca torreyi*. Both reports indicate that the idioblasts mature in sequence in files up to 38 crystal cells within the cortical region of the root. Stebbins et al. (1972) found crystals in the region of elongation below the apical meristem of apple shoots. Many investigators have noted that idioblast formation and leaf differentiation occur concomitantly (Frank and Jensen, 1970; Horner and Whitmoyer, 1972; Sakai and Hanson, 1974; Franceschi, 1978; and others). Recently, Horner and Wagner (1980) correlated the formation of druse
crystals in stomium cells with anther development during microsporogenesis in *Capsicum annuum*. Franceschi (1978) showed that crystal formation was not correlated to the age of the callus line, i.e., idioblast differentiation did not occur synchronously throughout the culture.

Numerous authors have speculated upon the function of calcium oxalate deposits in plants and these suggestions are for the most part unsubstantiated. Surprisingly, despite numerous developmental studies concerned with idioblast differentiation, until recently (Horner and Wagner, 1980) no correlations have been made between the formation of calcium oxalate crystals, development of idioblasts, and the differentiation of the surrounding tissue. Probably, the difficulty with trying to assign or elu­ci­date a function of calcium oxalate crystals is that many authors presuppose merely one function, where in fact, there may reside a whole array of reasons for their production. Considering the great diversity among tissues and cells in which crystals are formed, it is likely that in different plants and different tissues, the crystals may have different functions. Therefore, it may be necessary, in a consideration of functionality of calcium oxalate crystals in any particular system, to entertain a number of proposals.

The most commonly encountered proposals of calcium oxalate crystal functions in plants are: (1) an accumulation of a metabolic waste product; (2) a method of maintaining ion balance or calcium storage; (3) a mechanism of predatory defense; (4) donation of mechanical support; (5) involvement in photosynthetic efficiency; and (6) a mechanism of gravity perception as statoliths. The functions of plant oxalate biosynthesis
have already been considered; however, the functional significance of the formation of specifically shaped Ca oxalate crystals also deserves attention.

Since in most cases, accumulated oxalate is either not re-used or metabolized very slowly (Ranson, 1965; Bornkamm, 1969), many believe that calcium oxalate crystals are a means of isolating this so-called waste product from the system (Foster, 1956). However, the presence of both the free acid and the oxalate salt in some plants (Crombie, 1960), such as *Oxalis sp.* (Millerd et al., 1963a; Baker, 1952) and *Haloegeton glomeratus* (Williams, 1960) is not explained by the undesirable waste product concept. Why is not all of the oxalic acid precipitated out? Furthermore, many plants demonstrate the capability of removing or reabsorbing crystals after their deposition (Bornkamm, 1969; Arnott and Pautard, 1970; Tilton and Horner, 1980). The removal of oxalate as an undesirable waste product into crystals would not be expected to be a reversible procedure.

The evidence that calcium oxalate crystals can be reabsorbed suggests that their deposition may be a method of maintaining ion balance or of calcium ion storage or sequestration. It has been noted that crystal idioblast initiation usually occurs in very young tissues (Arnott, 1962; Eilert, 1974). Meristematic cells are usually not highly vacuolate and would not therefore be able to compartmentalize ion influxes as readily as mature plant cells. Franceschi (1978) suggests that the formation of cells specialized for this task would be advantageous since the regulation of ionic balance is important to proper tissue or organ development. Furthermore, a number of investigations has shown a correlation between
oxalate biosynthesis and calcium uptake (Dunne, 1932; DeKock et al., 1973; Rasmussen and Smith, 1961).

A number of reports suggests that the crystals may have a role as a storage site for calcium. Zindler-Frank (1975) and Franceschi and Horner (1979) have demonstrated the calcium dependency of both anabolic and catabolic phases of crystal development. In physiological activity then, the plant or callus retains the capability to adjust to changing conditions by depositing or removing calcium oxalate. Scott (1941) reported the reabsorption of druse crystals during leaf differentiation in Ricinus communis. Calmés (1969) and Calmés and Carles (1970) reported that as the young leaves of Parthenocissus tricuspidata develop, raphide crystals in the older stems are reabsorbed and that the calcium was found to be transported to the young stems. Sunell and Healey (1979) show that calcium oxalate is resorbed from idioblasts in older taro corms (Colocasia esculenta). Tilton (1978) observed that raphide crystals in the peripheral region of the carpellar cortex in Ornithogalum were reabsorbed during later stages of carpel maturation. Tilton and Horner (1980) suggest that this crystal catabolism during carpel maturation may represent mobilization of calcium from the carpel to the developing seeds. The mechanism for the removal of precipitated calcium oxalate in plants that have this capability is not known and the prevalence of this capability throughout the plant kingdom is uncertain.

There is evidence which indicates that the formation of calcium oxalate crystals may be a response to a physiological gradient established for the function of sequestering calcium ions. Horner and Wagner (1980)
observed that stomium cell wall degradation in anthers of *Capsicum annuum* is commensurate with the formation of calcium oxalate druse crystals in those cells. The walls surrounding each stomium cell are continually degraded by removal of wall material prior to and during crystal formation. They suggest the wall material (including wall-bound calcium and other ions) are moved into the cell, facilitating crystal production and resulting in stomium cell wall disintegration at the time of pollen release. Thus, they believe that by internalizing and sequestering wall-bound calcium during microsporogenesis (see Horner and Wagner, 1980), the formation of crystals aids in wall degradation between the locules and contributes to the pollen release mechanism. A similar mechanism may operate in the development of other types of tissues as well.

It has been noted that crystal cells are often found in association with aerenchyma and air space tissues in aquatic plants. In a developmental study of the crystal idioblasts in the aerenchyma of *Colocasia*, Sakai and Hanson (1974) note that dissolution of the middle lamella immediately surrounding the idioblast caused the release of the idioblast from the surrounding tissue. They suggest that solubilization of the middle lamellar calcium pectate is apparently caused by excess oxalate during raphide crystal formation. Currier (1956, as cited by Sakai and Hanson, 1974) reported that competing substances act in solubilization of the middle lamella by the formation of insoluble calcium oxalates. Some breakdown of middle lamella was also observed during leaf development in *Xanthosoma*, but not in *Alocasia*. Induced middle lamellar breakdown could
conceivably lead to tissue aerenchymatization or air space formation via schizogeny.

Dudinskii (1976) observed that raphide crystals develop in an orderly arrangement in conjunction with the formation of air canals during leaf morphogenesis in *Typha latifolia*. He reports that the idioblasts are dead cells at maturity and concludes that the formation of the air-filled cavities can be regarded as a process of local isolation of regions of parenchyma situated within the forming cavity. Isolated from living tissue by a layer of dead raphide idioblasts, the interior cells collapse and disintegrate to leave an air-filled cavity. This explanation may be an oversimplification; however, a process similar to that found in *Capsicum* anthers (Homer and Wagner, 1980) and in *Colocasia* aerenchyma (Sakai and Hanson, 1974) may be in operation here.

Calcium oxalate crystals have been recognized to attribute the plant protection against predation. In his book *Poisonous Plants of the United States and Canada*, Kingsbury (1964, and references cited therein) states that it is generally believed that the intense burning sensation and irritation which accompany ingestion of plants laden with calcium oxalate crystals occur mechanically by penetration of the crystals into the mucous membranes and to a lesser degree chemically by the salt itself. Pohl (1965) found that contact dermatitis from *Ornithogalum caudatum* resulted from the penetration of the skin by raphide crystals and the subsequent introduction of the sap. Thurston (1976) has described the calcium oxalate crystals in stinging nettle (*Tragia ramosa*) as miniature poison darts. The large, grooved crystals are barbed at the ends and reside
within the toxin filled lumen of stinging cells. On contact, the end wall of the stinging cell is pushed back allowing for penetration of the skin by the crystal tip. The groove carries toxin from the stinging cell into the wound causing an allergic reaction. Grooved and H-shaped crystals are not uncommon (Sakai and Hanson, 1974; Sakai et al., 1972; Arnott, 1966).

Other proposed functions for the presence of calcium oxalate crystals in plants are reasonable explanations, although they have not received much attention. Schneider (1901) suggests that the calcium oxalate crystals aid in the mechanical support of tissues as microarchitectural compounds. Schürhoff (1908) observed the association of druse crystals with the thin photosynthetic layer of tissue in *Peperomia* leaves. Horner and Hill (unpublished data via personal communication) found the chloroplasts in that layer are situated nearly around the druses and that the crystal morphology was affected by the light intensity at which the plants are grown. Thus, they feel that the crystals may be involved with photosynthetic efficiency, at the light levels where this plant naturally occurs, by acting as miniature prisms, collecting and directing light to the chloroplasts. Sunell and Healey (personal communication, University of California, Irvine) feel from observations on the formation of druses in *Colocasia* leaves that a similar mechanism may be involved there as well. Finally, Audus (1962) considered crystals as possible statoliths involved in the perception of gravity by some plants, but this suggestion is not well substantiated.
The Development and Ultrastructure of Crystal Idioblasts

A comparison of the results from previous investigations on the differentiation of crystal idioblasts shows a series of ultrastructural events which appear common to most of the systems examined so far. These events occur chronologically in an orderly sequence as summarized below:

1) Meristematic cells differentiate early with respect to the surrounding tissue, into precrystal cells which are often characterized by a dense protoplasm (Frank and Jensen, 1970; Schütz et al., 1970; Horner and Whitmoyer, 1972; Horner and Wagner, 1980), enlarged nuclei and nucleoli (Hurel-Py, 1938, 1942; Schlichtinger, 1956; Horner and Whitmoyer, 1972; Franceschi, 1978; Franceschi and Horner, 1980a), and plastids that lack starch (Mollenhauer and Larson, 1966; Eilert, 1974; Arnott, 1976).

2) Small vacuoles coalesce to form a single large central vacuole, and preraphide cells often become slightly elongate (Horner and Whitmoyer, 1972; Eilert, 1974). Vesicles, tubules, membrane complexes and crystal chambers may form within the vacuole (Arnott and Pautard, 1965, 1970; Ledbetter and Porter, 1970; Horner and Whitmoyer, 1972; Eilert, 1974; Chiu and Falk, 1975; Horner and Wagner, 1980). In the cytoplasm, the plastids become greatly modified (Arnott, 1966; Eilert, 1974) and the ER may assume secretory activity (Mollenhauer and Larson, 1966). Plasmalemmasomes (Marchant and Robards, 1968) and the appearance of endocytic activity have been reported (Horner and Whitmoyer, 1972; Eilert, 1974; Horner and Wagner, 1980).

3) Crystal formation usually occurs within the membrane-bound crystal chambers in the vacuole (Eilert, 1974). The size and number of raphide
crystals increases as the cell matures (Horner and Whitmoyer, 1972) and cell elongation is often pronounced.

4) The vacuole continues to enlarge with cell maturity. Flocculent material, numerous vesicles, tubules, free membranes and plasmalemmasomes all have been reported in the vacuole as crystals enlarge or develop (Horner and Whitmoyer, 1972). Modified plastids undergo reversion or disintegration (Mollenhauer and Larson, 1966; Eilert, 1974). Crystal chambers of raphide crystals may attain secondary lamellations (Eilert, 1974; Wattendorff, 1976a,b; Tilton and Horner, 1980) and flocculent material may accumulate to form a dense matrix or mucilage.

5) At maturity, most crystal idioblasts are reportedly living cells. Raphide crystals in bundles generally remain within the central vacuole often surrounded by a dense muscilage (Strasburger, 1898; Esau, 1953). A thin peripheral cytoplasm with only a sparse organelle complement remains.

All previous studies on plant crystal formation describe the complex ultrastructure of crystal idioblasts and the many developmental changes that occur throughout differentiation. The above generalized outline only typifies the events involved in crystal formation. Many aspects of individual reports cannot be correlated in other studies on different plants or crystal types, and therefore, many questions regarding the differentiation of crystal idioblasts remain to be resolved. The current evidence suggests that calcium oxalate formation in plant cells is under strict biological control and not merely simple precipitation. Attempts to crystallize calcium oxalate *in vitro* (Bouereau, 1954) have shown that it is not possible to reproduce the shapes of biologically occurring plant
crystals experimentally. Therefore, a review of past studies regarding the ultrastructure of the crystal cell system, through the involvement of each organelle, might provide much insight into the processes of idioblast differentiation and crystal formation.

Much of the literature involves investigations on the development of raphide crystal idioblasts. Since certain aspects of their development may be unique to the formation of raphide crystals and the subject of this study also involves raphide crystals, much of this discussion will be concerned with these cells in particular. The development of other crystal cell types, ones that form druses (Price, 1970; Horner and Wagner, 1980), styloids (Arnott, 1966; Wattendorff, 1976a) and prisms (Frank and Jensen, 1970), will be used for comparative and supplementary purposes.

The nucleus

One of the first observable changes associated with raphide crystal idioblast development is an enlargement of the nucleus and nucleolus (Franceschi and Horner, 1980a). Hurel-Py (1938, 1942) found that the nuclei of raphide forming cells in *Vanilla* exhibit obvious enlargement relative to preceding stages and to adjacent nonraphide cells. Rakován et al. (1973) reported that the nuclei in raphide crystal cells in *Monstera deliciosa* appeared enlarged and modified in comparison to the surrounding tissue. The developing idioblasts of *Psychotria* leaves (Horner and Whitmoyer, 1972) and in callus culture (Franceschi and Horner, 1979) show larger nuclei and denser nucleoli than neighboring noncrystal cells. Horner and Whitmoyer state that the earliest indications that a parenchyma cell will become a crystal cell are that the cell becomes
larger concomitantly with an increase in nuclear diameter. They suggest that this structural modification may indicate that crystal cell nuclei experience endopolyploidy. Mollenhauer and Larson (1966) observed that the enlarged nuclei of some crystal cells become lobate through development, another indication of endopolyploidy (Nagl, 1978). Indeed, based on nuclear volume, Schlichtinger (1956) reported that the ploidy level in raphide crystal cells in Gibbaeum heathii ranged from 2n to 64n. However, as Nagl (1979) points out, increased nuclear volume is not proof of increased DNA content and may be a result of chromatin dispersion. Foster (1956) states that crystal-containing cells were distinct in size and shape and often derived from unequal divisions. In young Canavalia leaves, the crystal idioblasts occur in pairs, as products of a single mother cell (Frank and Jensen, 1970). Kowalewicz (1956) reported that in raphide crystal cells in Epilobium, the nucleus undergoes a mitosis but is not followed by cytokinesis. Since some form of nuclear restitution or endo-cycle is highly suspect in crystal cell differentiation, a definition of terms regarding these phenomena would be helpful.

Nagl (1978) defines the term "endo-cycle" to designate a DNA replication cycle within the nuclear envelope and without spindle formation. He reviews and outlines five possible mechanisms through which DNA synthesis in endo-cycles occur (see Figures 1a-f) and where they occur in the cell cycle (Figure 2). The endomitotic cycle (Geitler, 1939a; see Figure 1b) results in a nucleus with multiple entire genomes which increase in definite geometric periodicity. The endoreduplication cycle (Levan and Hauschka, 1953; see Figure 1c) or polytenization also results in multiple
genomes but the endochromosomes do not separate from each other and polyteny results. DNA underreplication (van Oostveldt and van Parijs, 1972, 1976; see Figure 1d) occurs when small portions of the genome are not replicated, or replicated less often than the remainder of the DNA during an endo-cycle. DNA amplification (Lima-de-Faria, 1974; see Figure 1e) is characterized by the extrareplication of only certain sequences, resulting in local polytenization (Nagl, 1978). The amplified DNA becomes detached from the chromosome and degraded in the nucleus or in the cytoplasm. Endoreduplication and amplification cycles may occur at the same time in development (see Figure 1f) where the endopolyploid chromosome is stable and the amplified sequences are degradative.

Instances of endopolyploidy, underreplication and amplification are well documented in many eukaryotes (see Nagl, 1978, for a complete review) and a discussion of their characteristics and mechanisms would be voluminous. Moreover, the functional significance of endo-cycles in cell differentiation has been discussed ever since their discovery (Nagl and Rücker, 1972; Evans and van't Hof, 1975; Magakyan, 1976; and many others). However, the role of endo-cycles in the development of somatic plant cells is probably related and best understood by their observed consequences.

One consequence of endo-cycles is that continuous and increasing RNA synthesis may occur (Nagl, 1973) and therefore increase the potentiality for protein synthesis in the cell throughout differentiation (Bennett, 1973; Clutter et al., 1974). Walbot et al. (1972) showed that the rate of RNA synthesis to the amount of DNA per cell was higher in endomitotic suspensor cells than meristematic cells in the embryo proper of Phaseolus;
the rate of RNA synthesis is commensurate with the amount of DNA per cell. Nagl (1973) demonstrated that RNA synthesis continues unabated during endomitosis in Allium root tips, but ceases during mitosis. Scharpe and van Parijs (1973) found a correlation between the high degree of polyplody in parenchyma of Pisum sativum and the high rate of RNA and protein synthesis. Besides RNA synthesis, cell growth and differentiation can proceed in endocycling cells, uninterrupted by nuclear division and cytokinesis (van Parijs and Vandendriessche, 1966; Nagl, 1972; Capesius and Stöhr, 1974; Pearson, 1974). Nagl (1978) comments that this phenomenon may be because endochromosomes could possess a different transcriptional potential compared to mitotic cells. He also states that cells which demonstrate high degrees of endopolyploidy show obviously high overall levels of metabolism.

The number of plastids per cell may be directly related to the level of endopolyploidy (Butterfass, 1963; 1973) which points out another consequence and possible advantage of endocycling cells. Although Butterfass (1967) found that the basic number of chloroplasts per cell is controlled by nuclear genes, the number of plastids ultimately produced is a function of the level of endopolyploidy (Butterfass, 1973). Nagl (1978) points out that this implies that the extent of chloroplast replication is limited by the amount of nuclear DNA. Butterfass (1973) further suggests that nuclear DNA replication may be regulated by the chloroplast genome, as seems to be the case in Chlamydomonas (Blamire et al., 1974). The relationship of endopolyploidy and plastid condition suggests some metabolic
advantage for the endo-cycling cell (Butterfass, 1968; Nagl, 1978; Guern et al., 1975).

A further consequence of endo-cycling cells is related to the fact that cell division is inhibited. Nagl (1978) states that proliferating cells are involved largely in autosynthetic activities and suggests that endo-cycles might liberate a cell from this involvement for the development of heterosynthetic cell and tissue specific functions without abolishing growth. Walbot et al. (1972) found that endopolyploid tissues function at significantly higher RNA synthetic and overall metabolic rates than in diploid tissues of the same individual. Mitotic activity may also inhibit the accumulation of RNA needed for a specific process in differentiation, as discussed by Stebbins (1965). In fact, a relationship between endopolyploidy and cell elongation (endopolyploid cells are generally larger than diploid cells) has been suggested (Barlow, 1972; Barlow and Sargent, 1975; Davies, 1976). Therefore, endo-cycles might serve to allow specific cell (and ultimately tissue and organ) differentiation through inhibition of mitotic stages.

While this is not an exhaustive summary of the purported consequences of endo-cycling cells, crystal idioblasts demonstrate many of these previously mentioned characteristics. Numerous authors have noted from ultrastructural studies that the cytoplasm of young crystal cells is often dense and organelle rich (Mollenhauer and Larson, 1966; Eilert, 1974; Arnott, 1976; Franceschi, 1978) and thus appears to be metabolically more active than surrounding cells (Horner and Whitmoyer, 1972). Furthermore, enlarged nucleoli observed in crystal idioblasts (Horner and Whitmoyer,
1972; Rakován et al., 1973) reflect the increased physiological activity of the cell (Avers, 1976). However, no studies have been conducted yet to show an increase in RNA or protein synthesis during idioblast differentiation. Plastid modification (size, number, and morphology) also often accompanies crystal cell development (Mollenhauer and Larson, 1966) and will be discussed later. Raphide crystal cells are often larger (but not always, see Mollenhauer and Larson, 1966) and often experience tremendous cell elongation during development (Franceschi and Horner, 1980b). These observations, among others, of crystal idioblast development may be the consequences of differentiation within an endo-cycling cell.

Plastids

Many investigators have observed plastids early in crystal cell differentiation which are dissimilar from those in surrounding cells. The distinctive plastids found in many crystal idioblasts have been called crystalloplastids (Arnott, 1966). Arnott and Pautard (1970), Horner and Whitmoyer (1972), and Eilert (1974) all describe the plastids in young idioblasts as small, rather electron-dense, undifferentiated and containing only a few short thylakoids. They are similar to the proplastids found in meristematic tissues (Kirk and Tilney-Bassett, 1967) and are distinctive from surrounding cells because they contain no starch (Eilert, 1974). All reports of these plastids to date find that a characteristic feature that distinguishes them from the plastids in surrounding non-crystal cells is that they do not develop visible starch deposits anytime during cell development. Before crystal formation, the crystalloplastids may develop several morphologically distinct regions. Arnott (1966) and
Eilert (1974) described three zones in *Yucca* idioblast plastids: a relatively electron-dense zone, a translucent zone, and a lamellar zone. Furthermore, both reports suggest that these areas may represent specialized regions of activity within the plastid, analogous to pyrenoids of certain algal chloroplasts (Bouck, 1965) in this regard. Mollenhauer and Larson (1966) found osmiophilic droplets in the dense region (which they called a lobe or appendage) of the plastid. The crystalloplastids of orchids (Mollenhauer and Larson, 1966) and *Psychotria* (Horner and Whitmoyer, 1972) are smaller, those of *Yucca* (Arnott, 1966; Eilert, 1974) are longer, and those of *Lemna* (Arnott, 1973) are about the same size as the plastids in the surrounding noncrystal cells. Morphologically, they appear similar to the specialized plastids found in myrosin idioblasts (Jorgensen, personal communication, University Denmark) and the plastolysosomes described by Nagl (1977).

The fate of crystalloplastids in mature idioblasts is unclear. Eilert (1974) reports that the crystalloplastids in *Yucca* increase in size subsequent to crystal formation. Mollenhauer and Larson (1966) suggest that the enlarged modified plastids revert to a proplastid-like condition following crystal deposition. Eilert (1974) observed a large scale degeneration of the mature plastids within the central vacuole; the contents of the plastid became incorporated into the vacuole. However, she also reports that a few survived and revert to the proplastid-like condition.

The developmental role of these plastids is not known; however, numerous authors have speculated about them since their discovery. Crystalloplastids may be involved with the production of an oxalate pre-
cursor. Oxalic acid oxidase has been found to be associated with normal Chenopodium chloroplasts (Nagahisa and Hatori, 1964). This information led Arnott (1973) to speculate that perhaps these modified plastids lack the oxidase, allowing for oxalate production and hence crystal formation. The presence of oxalic acid oxidase in normal chloroplasts would inhibit crystal formation according to Arnott. However, this is inconsistent with reports on oxalate metabolism (see Hodgkinson, 1977; and Franceschi and Horner, 1980a for reviews). These plastids may be the site, however, of enzyme complements essential to oxalate biosynthesis.

Many raphide idioblasts produce a muscilage material during development (Esau, 1953) which has been shown to contain carbohydrate and protein (Mollenhauer and Larson, 1966; Sakai and Hanson, 1974; Tilton and Horner, 1980). Eilert (1974) reports that the accumulation of the muscilage in the vacuole of Yucca root idioblasts coincides with the degeneration of the crystalloplastids. She suggests the role of the idioblast may be completed after the crystals are formed, and the disposal of the unneeded organelles in the vacuole results in muscilage formation.

Crystalloplastids may represent a key and multifunctional organelle in the development of crystal idioblasts. The plastids may influence or stimulate endopolyploidy (Butterfass, 1973), regulate the production of oxalate precursors, and possibly assume lysosomal activity as plastolyosomes (Nagl, 1977).

The vacuole

Most often crystals have been found to form in the central vacuole of the idioblast (Franceschi and Horner, 1980a) and numerous changes in the
contents of the vacuole have been observed before, during, and after crystal formation. Eilert (1974) reports that numerous small vacuoles in young crystal cells coalesce to form the central vacuole prior to any crystal formation. Shortly after this coalescence, many investigators have observed the formation of crystal chambers (membranous structures which may serve as molds in which the crystals develop, see Arnott and Pautard, 1965, 1970; Arnott, 1966; Frank and Jensen, 1970; Schütz et al., 1970; Horner and Whitmoyer, 1972; Eilert, 1974), membranous complexes (Horner and Whitmoyer, 1972), paracrystalline bodies (Horner and Wagner, 1980), tubules and vesicles (Arnott and Pautard, 1965; Franceschi, 1978) within the central vacuole.

The origin of crystal chambers is not known, although Horner and Whitmoyer (1972), Eilert (1974) and Horner and Wagner (1980) suggest that they may be derived from plasmalemmosomes. Three different types of chambers associated with raphide formation have been reported. In Yucca, cross sections show individual rectangular chambers which correspond in size and shape to the crystals found within them (Arnott, 1973; Eilert, 1974). The second type of raphide crystal chamber has been described in Lemna (Arnott, 1966; Arnott and Pautard, 1970) and Spirodea (Ledbetter and Porter, 1970). The crystals in both of these species are H-shaped. These chambers are formed within parallel lamellae which have angular bends which bridge the two membranes. The crystals form in these contact points. Schütz et al. (1970) described the third type of chamber from cross sections of Oenothera idioblasts. These chambers consist of a single membrane bent to form several squares in which the crystals form.
Crystal chambers such as those described from raphide crystal idioblasts where the membranes conform to the shape of the crystal are reported also in the formation of styloids (Arnott, 1966) and prisms (Frank and Jensen, 1970; Parameswaran and Schultze, 1974). Horner and Wagner (1980) show a somewhat different arrangement in the development of druse crystals in stomium cells of Capsicum. They report membrane complexes associated with paracrystalline bodies, which they suggest serve as nucleation sites for druse crystal formation. Prior to the appearance of these structures, they observed numerous plasmalemmasomes near the cell wall which may be the precursor structures to the membrane complexes. Rivera (1973) could not find any structures (membrane complexes of crystal chambers) in the vacuoles of Echinomastus druse crystal cells prior to crystal formation. A crystal sheath may exist between the crystal and the crystal chamber in some idioblasts (Price, 1970).

Membranous tubules are common features in vacuoles of developing raphide idioblasts (Horner and Whitmoyer, 1972). Arnott and Pautard (1970) suggest that these tubules may serve as transport channels for crystal precursors to the chambers. Similarly, the numerous vesicles observed in the central vacuole during crystal formation may carry calcium oxalate precursors (Franceschi, 1978). The function of such supposed transport systems may be to keep crystal precursors compartmentalized and prevent crystallization outside the crystal chambers or growing crystals.

Various other structures are sometimes found in the vacuoles of crystal idioblasts during their development. Chiu and Falk (1975) reported ribosome-like particles associated with the crystal chamber mem-
branes, and suggested an in situ production of proteins required for crystal formation. Mollenhauer and Larson (1966) report the absence of membrane bound crystal chambers in Vanilla and Monstera; the formation of crystals was reported to occur in a carbohydrate matrix. Later, however, Rakován et al. (1973) working with the same species of Monstera reported that the crystals may form in dictyosome-derived vesicles.

As previously mentioned, a carbohydrate/protein mucilage, slime or packet has been frequently reported in the vacuole of raphide idioblasts during later stages of development after crystal formation (Robyns, 1928; Esau, 1953; Sakai et al., 1972; Eilert, 1974; Wattendorff, 1976a,b; Wheeler, 1979; Tilton and Horner, 1980). It has been suggested that this mucilage may cause crystal release when it swells under osmotic changes (Sakai and Hanson, 1974; Wheeler, 1979). This mucilage may also give rise to secondary lamellate structures that form around some raphide crystals (Eilert, 1974; Tilton, 1978; Wattendorff, 1976a,b). Lamellate crystal chambers surrounded by mucilage have been described in Agave leaves (Wattendorff, 1976a,b), Yucca roots (Eilert, 1974), and Ornithogalum ovules (Tilton and Horner, 1980). All of these taxa are monocotyledons. Eilert (1974) describes the presence of numerous membranes (or "half-membranes") 50 Å thick, with free ends within the vacuole during mucilage formation. The membranes accumulated around crystals to form blankets of secondary lamellae. Tilton and Horner (1980) report that the lamellations in Ornithogalum are composed of numerous small tubules.
Cellulosic sheaths of wall-like material may enclose some crystals during later stages of idioblast development (Scott, 1941; Frank and Jensen, 1970; Price, 1970; Thurston, 1976; Wattendorff, 1976b). The function(s) of the mucilage, secondary lamellated crystal chambers or the cellulosic sheaths is not known.

The endoplasmic reticulum and ribosomes

The endoplasmic reticulum (ER) is a prominent feature of some crystal idioblasts and may play a significant role in crystal formation throughout development. Mollenhauer and Larson (1966) witnessed a distinct increase in the amount of ER per unit raphide cell cytoplasm during cell enlargement in Vanilla. They suggest that this proliferated ER is involved in internal secretion of material which accumulates in the vacuole. Schötz et al. (1970) felt that the ER in Oenothera raphide idioblasts was responsible for the vesicles, crystal chambers and eventual formation of crystals. Ledbetter and Porter (1970) suggested a similar ER function in the crystal cells of Spirodella. In Yucca (Eilert, 1974) and in Psychotria (Horner and Whitmoyer, 1972), prominent ER profiles were evident; however, they did not appear to produce vesicles. Arnott and Pautard (1965) report that Lemma raphide idioblasts are also exceptionally well-endowed with ER throughout development. Interconnected tubular and sheetlike forms of ER are commonly distributed throughout the cytoplasm (Mollenhauer and Larson, 1966; Eilert, 1974) and generally do not show association with any particular organelle (Eilert, 1974). While the implications of an internal secretory function of raphide cell ER are not
clearly elucidated, the form and quantity of ER suggests that it must play a role in the special activities of these cells.

All investigators note that rough ER prevails although cisternae of smooth ER are also seen. The numbers of ribosomes per unit of ER are similar to that found in adjacent noncrystal cells (Mollenhauer and Larson, 1966); however, the number of free cytoplasmic ribosomes and polysomes appears to be much greater, giving these cells a characteristically dense cytoplasm (Eilert, 1974; Horner and Whitmoyer, 1972).

**Dictyosomes**

Numerous dictyosomes (Golgi bodies) have been observed in crystal cells throughout all stages of development (Arnott and Pautard, 1965; Eilert, 1974; Frank and Jensen, 1970; Horner and Whitmoyer, 1972; Horner and Wagner, 1980; Ledbetter and Porter, 1970; Price, 1970; Schötz et al., 1970). These reports all indicate that there are more dictyosomes present per unit area in the cytoplasm of crystal idioblasts than in adjacent non-crystal cells. Eilert (1974) reported that some dictyosome derived vesicles in *Yucca* formed small vacuoles which would later coalesce with the central vacuole. She also implicated the dictyosome in mucilage accumulation during late crystal cell development, but could not correlate this with the Thiery reaction of carbohydrates (Seligman et al., 1965). In *Monstera*, dictyosome derived vesicles may serve as crystal chambers in some species as suggested by Rakován et al. (1973). Horner and Whitmoyer (1972) and Mollenhauer and Larson (1966) both report that the dictyosomes show no conspicuous activity. The developmental role of dictyosomes during crystal and mucilage formation is unclear.
Microbodies, mitochondria and other cytoplasmic structures

As previously mentioned, microbodies, glyoxysomes and peroxisomes contain enzyme complements key in the glyoxylate cycle and the glycolate pathway, respectively. Therefore, one would expect these organelles to be well represented in the cytoplasm of developing crystal idioblasts because of the role of microbodies in the production of oxalic acid. However, most investigators have found only sparse numbers of microbodies (Amott, 1973). Mollenhauer and Larson (1966) found microbodies in crystal cells to be similar in number, distribution and appearance to those in surrounding cells. They describe these organelles as single membrane-bound bodies about 0.25 µm in diameter with a uniform internal matrix; these bodies lacked the dense core described in many plant microbodies (e.g., Frederick and Newcomb, 1969). Sakai and Hanson (1974) note a negative reaction for peroxidase activity in Colocasia crystal idioblasts. Horner and Whitmoyer (1972) report that microbodies were no more frequent in crystal cells than in adjacent mesophyll cells and were sometimes partially encircled by ER. Frank and Jensen (1970) found no evidence whatever for high numbers of microbodies in crystal cells. They report, however, a buildup of protein bodies during crystal cell development. This is interesting in that high oxalate production has been strongly correlated with high protein synthesis (Bornkamm, 1965). Horner and Wagner (1980) report that microbodies appear in the cytoplasm just prior to crystal formation and are absent in nearly mature crystal cells in the stomium of Capsicum. Eilert (1974) observed a few scattered, lattice containing microbodies present at all stages of crystal cell development, yet she doubted if they played a
significant role in oxalate production. Price (1970), Parameswaran and Schultze (1974), and Sakai and Hanson (1974) did not mention microbodies in their reports and there is no evidence in their micrographs of these organelles.

The fact that only sparse populations of microbodies, or none at all, are found in developing crystal cells raises some important questions concerning the origin of oxalate used in crystal formation. The oxalate, if produced within the idioblast by either the glyoxalate cycle or the glycolate pathway, may be synthesized in other organelles, perhaps crystalloplastids, with microbody-like enzyme complements. The oxalate may also be produced within the idioblast via oxaloacetate cleavage (Chang and Beevers, 1968) or by ascorbate metabolism (Wagner and Loewus, 1973). The oxalate may originate in surrounding or distant cells and be mobilized into the idioblast. The origin of oxalate used in crystal formation has not been determined for any system.

Most authors report that mitochondria in crystal cells resemble those in nonidioblastic cells in size, frequency in the cytoplasm and appearance (Arnott, 1973). Frank and Jensen (1970) pointed out, however, that although similar numbers of mitochondria (and dictyosomes) are present in crystal and noncrystal cells, the amount of cytoplasm in enlarged idioblasts is much greater; therefore, perhaps a quantitative difference exists between the two kinds of cells in regard to the total number of organelles present. Eilert (1974) reported that mitochondria were more numerous in Yucca idioblasts than other cells in the root. She also noted frequent associations between the mitochondria and the tonoplast.
It is well-accepted that mitochondria in animal systems can accumulate calcium against a gradient. Crang et al. (1968) and Neff (1971) found that the mitochondria were responsible for calcium accumulation during calcite production in the calciferous glands of earthworms. Others have noted the ability of animal mitochondria to accumulate calcium (Greenwalt et al., 1964) and to transfer ions (Rasmussen, 1966). Lehninger (1970) has proposed a mechanism for both calcification that centers on the ability of mitochondria to accumulate calcium. He suggests that mitochondria have calcium carriers in their membranes which respond to an electrochemical gradient which has been generated by the electron transport system.

Plant mitochondria may be capable of mechanisms for calcium accumulation similar to those found in animals. Isolated plant mitochondria can accumulate and discharge $\text{Ca}^{++}$, but they do not do so in preference to ATP generation (Johnson and Wilson, 1973) as do some animal mitochondria. It has been reported (Hodges and Hanson, 1965) that calcium phosphate is precipitated in mitochondria from etiolated maize. Johnson and Wilson (1972) found that in divalent cation uptake, plant mitochondria accumulate phosphate but never oxalate. Arnott (1966) speculated that the mitochondria may serve as calcium pumps in the calcification of plant tissues.

As previously discussed, an attractive pathway for oxalate production in nongreen tissues of higher plants is through the enzymatic cleavage of oxaloacetate, a TCA intermediate (Chang and Beevers, 1968). Thus, it seems reasonable that such a reaction may occur within the mitochondria. It is possible, therefore, that the mitochondria may play a role in the
accumulation of calcium and the production of oxalate within crystal cells.

Various other structures have been found in the cytoplasm of crystal cells, but no more frequently than in adjacent noncrystal cells. Numerous investigators have reported the presence of spherosomes (membrane-bound lipid bodies) in the crystal cell cytoplasm throughout development (Mollenhauer and Larson, 1966; Eilert, 1974; Horner and Wagner, 1980). Horner and Wagner (1980) observed lipid bodies seemingly becoming deposited in the vacuole just prior to druse crystal formation. Price (1970) noted an absence of lipid bodies and tannin inclusions in druse crystal cells in Cercidium, both of which were common in surrounding non-crystal cells. Microtubules have been found near the plasmalemma, similar to noncrystal cells (Eilert, 1974; Horner and Wagner, 1980).

The cell wall

A number of reports on the development of crystal idioblasts have mentioned that the walls of these cells appear different from those of adjacent cells. Mollenhauer and Larson (1966) and Sakai and Hanson (1974) distinctly show a sparsity of plasmodesmata between raphide cells or between raphide cells and adjacent noncrystal cells. However, Eilert (1974) observed that intercellular connections were more common in the walls of crystal cells than other cells. Changes in cell wall structure have been observed during crystal cell ontogeny. Sakai and Hanson (1974) note a distinct layering of the cell wall and a loosely structured or dissolving middle lamella during the development of raphide crystal cells in Colocasia and Alocasia. Also, they noted that the walls become thinner at
the ends of these cells and thickened towards the middle; the cell wall appeared modified for forceful raphide release. Parameswaran and Schultze (1974) found that crystal-containing cells had thickened walls which showed a lamellation identical to a sclerid wall. They also report the presence of lignin in their crystal cell walls. Mollenhauer and Larson (1966) reported that the appearance of raphide cell walls in *Vanilla* does not start to change until the latter developmental stages, when the transverse end walls rupture and allow direct protoplast continuity. As previously discussed, Horner and Wagner (1980) show a direct correlation between crystal cell wall degeneration, crystal development, and anther dehiscense. Perhaps, the observed changes in crystal cell walls through development is reflective of the overall function of the idioblast in the differentiation of a given tissue.
Figures 1a-f, 2. Summary of mechanisms through which DNA synthesis occurs in endo-cycles (redrawn with permission from Nagl, 1978).

Figure 1a. Normal mitotic cycle followed by karyokinesis.
Figure 1b. Endomitosis.
Figure 1c. Endoreduplication.
Figure 1d. Underreplication.
Figure 1e. Amplification.
Figure 1f. Endoreduplication-amplification.
Figure 2. Schematic of cell cycles for mechanisms of extrareplication of DNA. EM, endomitosis; ER, endoreduplication; UR, underreplication; A, amplification; R, restitution.

The flow that can be followed is not the eternal flow.

Lao tzu
PART I: DEVELOPMENT OF SYNCYTIAL RAPHIDE CRYSTAL IDIOBLASTS IN THE CORTEX OF ADVENTITIOUS ROOTS OF VANILLA PLANIFOLIA L. (ORCHIDACEAE)
Development of syncytial raphide crystal idioblasts in the cortex of adventitious roots of *Vanilla planifolia* L. (Orchidaceae)

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ABSTRACT

The initiation and formation of calcium oxalate raphide crystals and developmental aspects of crystal idioblasts were studied in adventitious roots of *Vanilla planifolia* L. (Orchidaceae). Root crystal idioblast initials are first recognized by intense fluorescence of the cytoplasm with the acridine orange method; nuclear and nucleolar enlargement gradually ensues. Crystal chambers form in association with an electron-dense vacuolar amorphous material and calcium oxalate deposition occurs within these chambers. Vacuolar cables of tubules become associated with the crystals. They apparently are involved with anchorage of the crystals and the orientation of the raphide crystal bundle. Crystalloplastids differentiate lobed regions similar to those seen in other mucilaginous raphide idioblasts, and their development precedes mucilage accumulation. At this time, raphides change from being four-sided in cross section to six- and eight-sided. Idioblast maturation is completed as idioblast transverse walls in a file break down to form a syncytium. This study also introduces bas-relief photographic processing for image enhancement of conventional light and transmission electron micrographs.
INTRODUCTION

Bundles of needle-shaped calcium oxalate crystals, called raphides (Esau, 1965), have been reported in diverse tissues and organs of a large number of plant species (for reviews see Arnott and Pautard, 1970; Hodgkinson, 1977; Franceschi and Horner, 1980a; Pentecost, 1980). Microscopic investigations have shown that the cells which produce these crystals, termed raphide crystal idioblasts (Foster, 1956), are complex ultrastructurally and contain cytological modifications involved with the crystallization process (Franceschi and Horner, 1980a). Calcium oxalate precipitation is not indiscriminate in plant cells, but is the controlled result of cell differentiation (Kausch and Horner, 1982, 1983a). Despite much speculation the function of plant calcium oxalate crystals, and the cells which produce them, is not understood.

Arnott (1962) observed longitudinal files of raphide-forming cells in the developing root cortex of certain Yucca species. Each file of cells was found to represent an ontogenetic series, and he recognized the value of such a system for study of specialized cell differentiation. Mollenhauer and Larson (1966) utilized a similar system to study developmental changes in raphide forming cells in adventitious root cortex of Vanilla planifolia and Monstera deliciosa. This latter investigation was part of an ultrastructural survey of angiosperm root tips, and consequently lacks many developmental details of raphide crystal cell differentiation.

The portion of our investigation reported here is part of a larger study involving the relationship between the nuclear condition of Vanilla planifolia raphide crystal idioblasts and the cytological differentiation
of these cells. The purposes of this study are to report our observations on the initiation of crystal formation and to establish a developmental sequence of events for the raphide idioblasts in *Vanilla* aerial roots using various microscopic techniques.

In this study, we also introduce the use of bas-relief photographic processing for image enhancement of conventional light and electron micrographs. Bas-relief processing is a printing technique presented in many darkroom procedural manuals (Langford, 1981). The technique produces photographs in which structures appear as side-lit, low-relief images. This type of contrast enhancement is advantageous for illustration of certain microscopic structures.
MATERIALS AND METHODS

Samples of adventitious roots were taken from *Vanilla planifolia* L. (Orchidaceae) plants grown in the departmental greenhouse, and processed similarly for scanning (SEM) and transmission (TEM) electron microscopy. Root apices, 2 mm in length, were cut longitudinally, aspirated, and fixed in 3% glutaraldehyde in a 50 mM phosphate buffer (pH 7.2) for 16h at 4 C. Following three buffer rinses, specimens were post-fixed in 1% osmium tetroxide (same buffer and pH) for 2h, rinsed three times with the buffer, then dehydrated in an ascending ethanol series. After three rinses in 100% ethanol, samples were subdivided for SEM and TEM processing.

Root apices for SEM observation were then either infiltrated with Freon 113 TF and critical point dried or air-dried directly. Dried specimens were mounted on brass discs with silver paste and coated with gold palladium in a Polaron E5100 sputter coating unit. All SEM observations were made on a JEOL JSM-35 microscope at an accelerating voltage of 18 kV.

Root apices for TEM observations were infiltrated with propylene oxide and then with Spurr's medium (Spurr, 1969). Survey sections for light microscopy (LM) were cut 1-2 μm thick and stained with methylene blue-azure II-basic fuchsin (Humphrey and Pittman, 1974). Thin sections were cut with a diamond knife on a Reichert Om-U2 ultramicrotome, mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-11C TEM.

Some root samples were also fixed in formalin-acetic acid-alcohol for 24h, and processed by standard paraffin procedures (Berlyn and Miksche, 1976). Sections were cut 10 μm thick and stained with acridine orange
(Price, 1969) for fluorescence light microscopy (FLM). Histochemical determination of crystals as calcium oxalate was done on similar sections, using the procedure of Yasue (1969) and viewed with brightfield LM.

Bas-relief photographic processing was accomplished with conventional TEM micrographs on 3 1/4 x 4 inch Dupont Cronar Ortho S Litho sheet film. The negatives were contacted onto the same film and processed with Kodak D-19 developer. Various exposure and development times were examined. Positive contacts and negatives were matched point for point on a light table, shifted only slightly out of register and taped together. This sandwiched "double negative" was then enlarged. Bas-relief imaging was accomplished for light micrographs by combining a black and white negative with a color positive in a similar fashion.
RESULTS

Raphide crystal idioblasts form in contiguous longitudinal files within the cortex of Vanilla planifolia aerial roots. A file of idioblasts is never interrupted by nonidioblastic cells and, hence, there is a complete ontogenetic series in each file. Acropetal cells are chronologically and developmentally youngest. Intense fluorescence of the cytoplasm, seen with acridine orange treatment and FLM (Figure 1), is the first characteristic to distinguish idioblasts from surrounding root cortical cells very early in their development. Intense cytoplasmic fluorescence is maintained during idioblast maturation. Nuclear and nucleolar enlargement occur gradually during idioblast development (Figure 1), but youngest idioblast initials have nuclei and nucleoli that are the same size as those in the cortical parenchyma. Fluorescence of the cytoplasm masks any differential chromatin staining in crystal idioblasts (Figure 1): cytoplasm fluoresces orange, nuclei uniformly yellow-orange (heterochromatin in cortical parenchyma nuclei are yellow-green), and nucleoli bright yellow.

Idioblast initials have numerous small vacuoles (Figures 2, 3) in a ribosome-dense cytoplasm with closely packed organelles (Figure 3). Plastids are simple, lack starch inclusions, and resemble eoplasts (Possingham, 1980). In contrast, plastids of the surrounding cortical parenchyma are chloroplasts containing a dense stroma, thylakoid and grana systems and, often starch deposits. The small vacuoles contain an electron-dense amorphous material which is attached to the tonoplast in numerous places (Figures 3, 4, 5) and appears to originate from its sur-
face. These vacuoles coalesce and a larger, or central, vacuole (Figures 4, 5) forms which contains large amounts of the amorphous material. The extent to which this material accumulates in the central vacuole of a crystal idioblast is seen best with SEM (Figure 5).

Crystal deposition follows accumulation of this amorphous material and formation of larger vacuoles. This development occurs within a three cell region of an idioblast file (Figure 2). Crystal initiation is an ephemeral event and relevant observations are made only by viewing many specimens.

Crystal chambers form in close association with the amorphous material in larger vacuoles (Figure 6). These are membrane-bound chambers without crystals and are not directly connected to the tonoplast. Crystal deposition occurs after crystal chamber formation and within the chambers. One to five small raphides appear in larger vacuoles (Figures 7, 8) together with empty crystal chambers and amorphous material (Figure 8). Raphides of various sizes are observed in the same vacuole (Figures 9, 10, 11). The amorphous material remains in association with the crystals (Figures 10, 11) and attached to the tonoplast (Figure 11). Small vacuoles contain and contribute this material (Figures 11, 12) during central vacuole formation and enlargement.

Plastids become distinctive (Figure 12) and, during central vacuole formation, correspond to descriptions of undifferentiated crystalloplastids observed in crystal idioblasts of some other plants (Kausch and Horner, 1983a). Both mitochondria and crystalloplastids are often observed
Figures 1-8. Early stages of idioblast development and initiation of raphide crystal formation. Line scales: Figure 1 = 25 μm; Figure 2 = 10 μm; Figures 3, 4, 6, 8 = 1 μm; Figures 5, 7 = 5 μm. Key to labelling: C - crystal; CW - cell wall; D - dictyosome; ER - endoplasmic reticulum; M - mitochondria; N - nucleus; Nu - nucleolus; P - plastid; V - vacuole.

Figure 1. Acridine orange FLM shows intensive cytoplasmic fluorescence of an idioblast file. Crystal production commences in cells midway in the length of file shown. Idioblast nucleus (arrow) is enlarged in comparison to cortical parenchyma nuclei.

Figure 2. Region of crystal formation in an idioblast file.

Figure 3. Dense cytoplasm of idioblast initial contains many small vacuoles with amorphous material.

Figure 4. Large vacuole with dense amorphous material, attached to tonoplast (arrows).

Figure 5. Idioblast initial with central vacuole containing amorphous material.

Figure 6. Empty crystal chamber in association with amorphous material.

Figure 7. Idioblast initial with three small raphides in central vacuole.

Figure 8. Higher magnification in cell in Figure 7 shows crystals and empty chambers in association with amorphous vacuolar material.
in dumbbell configurations (Figure 12), suggestive of their replication. Crystallo-plastids are also often observed as cup-shaped.

As idioblast development continues, nuclei and nucleoli continue to enlarge and more crystals are produced within the central vacuole (Figures 13, 14, 15). Crystals always are parallel to each other within an idioblast after a bundle is produced (Figures 14, 15). Raphide bundles usually become oriented in the longest axis of the cell, at a diagonal (Figure 15), but eventually re-orient perpendicular to the longitudinal direction of file growth as idioblast size increases (Figure 16). Raphide bundles may be perpendicular to each other in adjacent idioblasts (Figure 16), but they never orient parallel to the direction of cell file growth. Figure 16 illustrates crystals that appear curved; this may be an artifact of critical point drying since this was not observed in fixed, air-dried specimens or in thick sections viewed with LM.

Positive reaction with the Yasue technique (Yasue, 1969) distinguishes the crystals as calcium oxalate. From their inception, crystals are four-sided in cross section (Figures 11, 17) and have a shape indicative of the monoclinic system of crystallization (calcium oxalate monohydrate, CaC₂O₄·H₂O). The amorphous material remains associated with crystals as they increase in size and number in the central vacuole (Figure 17).

Transverse cell walls between crystal idioblasts are thin (Figure 17) and attenuate progressively during idioblast maturation. Plasmadesmata are never observed to connect idioblasts through these walls. Intracellular connections to surrounding cortical cells are extremely rare, and are
Figures 9-15. Raphide crystal bundle production. Line scales: Figures 9, 13, 14, 15 = 5 μm; Figures 10, 11 = 2 μm; Figure 12 = 1 μm. Key to labelling: Cp - crystalloplastid; M - mitochondria; N - nucleus; Nu - nucleolus; V - vacuole.

Figures 9. Young idioblast with raphides of various sizes.

Figure 10. Raphides associated with amorphous material.

Figure 11. Bas-relief TEM, positive and negative are of equal density, showing raphide bundle and amorphous material attached to tonoplast. Note small vacuoles containing this material and holes left by crystals which fall out during sectioning.

Figure 12. Bas-relief TEM of idioblast cytoplasm containing small vacuoles and crystalloplastids.

Figures 13-15. Developmental sequence shows raphide bundle formation. Raphide bundle is oriented diagonally within the cell in Figure 15.
observed only during early stages of idioblast development. Crystal idioblasts exist as isolated cells within the root cortex.

The cytoplasm retains a metabolically active appearance (Figure 17), dense with ribosomes and generally organelle-rich in comparison to adjacent cortical cells. Developing crystal idioblasts contain numerous active dictyosomes (Figure 17) and many polysomes are associated with a prevalent ER system. Spherosomes and microbodies are observed only infrequently, their profiles not as common as in cortical parenchyma. Pleiomorphic or elongated profiles of crystalloplastids are occasionally observed (Figure 17). Mitochondria remain unchanged and dumbbell configurations are observed (Figure 17) throughout the stages of crystal production.

As crystal initiation ceases, no new crystals are added to a bundle, even though crystal growth continues. The average number of crystals per cell after cessation of crystal initiation is 128±22 (N=32). At this stage, a number of cytological modifications occurs in the idioblasts. Crystalloplastids become enlarged and a layer, dense with plastid ribosomes, forms at their periphery (Figure 18). The ER lumen widens irregularly and accumulates a material slightly more dense than the cytoplasmic ground substance (Figures 18, 19, 22). Cables of tubules form within the vacuole in association with the amorphous vacuolar material (Figures 19, 20). These cables are appressed to crystal chamber membranes and extend from the crystals (Figure 19). Observations with SEM indicate that cables may connect to the tonoplast (Figure 21); this is not substan-
Figures 16-23. Idioblast development after raphide bundle formation. Line scales: Figure 16 - 10 μm; Figures 17, 18, 19 = 1 μm; Figure 20 = 0.1 μm; Figures 21, 22, 23 = 2 μm. Key to labelling: C - crystal; Cp - crystalloplastid; CW - cell wall; D - dictyosome; ER - endoplasmic reticulum; M - mitochondria; Nu - nucleolus; V - vacuole.

Figure 16. Raphide bundles perpendicular to each other in adjacent idioblasts.

Figure 17. Dense positive/light negative bas-relief TEM produces high contrast, high relief image. Note elongate crystalloplastid profile and thin transverse cell wall.

Figure 18. Enlarged, differentiating crystalloplastids with dense periphery.

Figure 19. Cables of vacuolar tubules associated with amorphous material and a crystal.

Figure 20. Bas-relief TEM of tubules in oblique and cross section (arrow). Associated amorphous material is less electron dense and hence shows less relief than the tubules.

Figure 21. Cables of tubules appear to be connected to tonoplast (arrow).

Figures 22, 23. Cables of tubules in vacuolar channels (arrows).
tiated in sectioned material. The cables extend toward opposite corners of an idioblast through vacuolar channels (Figure 22, 23).

Crystalloplastids develop one to several lobed regions (Figure 24). The lobes are compartmentalized from the larger remaining portion of the plastid by invaginations of the inner plastid envelope (Figure 24). These lobes are dense with plastid ribosomes and contain a number of dense osmiophilic plastoglobuli. The dense peripheral layer is gone and is interpreted as an intermediate to the lobed crystalloplastids. Bas-relief image enhancement shows variation in electron density within a lobe (Figure 24). The simple thylakoids of the larger portion of the plastid are tubular invaginations of the inner plastid membrane.

Methylene blue-azure II staining differentiates lobed crystalloplastids from other cytoplasmic features in thick sections viewed with LM (Figure 25). Bas-relief imaging further enhances this effect (Figure 25). The nuclei and nucleoli are quite enlarged by this stage in comparison with nuclei and nucleoli of adjacent cortical cells (Figure 25).

Mucilage accumulates in the central vacuole and, with SEM, appears as a reticular matrix (Figure 26). At the onset of mucilage accumulation, crystals undergo a change in shape, becoming six-sided at their ends and eight-sided toward their middle regions (Figures 27, 28). Crystals at this stage are ensheathed with secondary lamellated crystal chambers composed of similar tubular material that constitutes the cables.

As vacuolar mucilage surrounds the crystals (Figure 29), the transverse cell walls between older idioblasts in a file attenuate progressively. These thin cell walls eventually rupture, releasing the crystal
idioblast protoplasts and forming a syncytium. The separate protoplasts do not appear to fuse, but eventually rupture. As root diameter increases away from the root apex from growth and division of the cortical cells, the syncytia widen (Figures 30, 31). At maturity, *Vanilla planifolia* aerial roots contain many hollow syncytial tubes running longitudinally through the root cortex.
Figures 24–31. Idioblast maturation and syncytium formation. Line scales: Figure 24 = 1 µm; Figures 25, 27 = 10 µm; Figures 16, 28 = 5 µm; Figures 29, 30, 31 = 50 µm. Key to labelling: V = vacuole.

Figure 24. Bas-relief TEM of mature lobed crystalloplastid.
Figure 25. Bas-relief LM of idioblast file. Dense cytoplasmic structures are lobed crystalloplastids. Idioblast nuclear diameter is enlarged in comparison to cortical parenchyma nuclei.
Figure 26. Reticular mucilage fills vacuole surrounding crystals; note cable of tubules attached to crystal (arrow).
Figure 27. Fractured crystals are six-sided toward their ends and eight-sided in their central portion.
Figure 28. Ensheathed crystals fractured through their eight-sided region.
Figure 29. Mucilage-filled mature idioblasts.
Figures 20, 21. Transverse walls break down and syncytium is formed and widens.
DISCUSSION

Many aspects of calcium oxalate raphide crystal idioblast development in aerial roots of *Vanilla planifolia* are common to differentiation of similar cells observed in other plants. Meristematic cells differentiate precociously, in comparison to surrounding tissues, into idioblast initials which have been characterized in a number of plants by a dense protoplasm (Frank and Jensen, 1970; Schütz et al., 1970; Horner and Whitmoyer, 1972; Horner and Wagner, 1980; Kausch and Horner, 1983a) and/or enlarged nuclei and nucleoli (Hurel-Py, 1938, 1942; Schlichtinger, 1956; Horner and Whitmoyer, 1972; Franceschi and Horner, 1980a; Kausch and Horner, 1983a). Intense fluorescence of *Vanilla* idioblast initials with the acridine orange method indicates high RNA content and a metabolically active system (Price, 1969). Observations with TEM show that these cells have a ribosome-dense cytoplasm. This characteristic distinguishes crystal idioblast initials before any other observable cytological change. Nuclear and nucleolar enlargement in *Vanilla* idioblasts are subsequent developmental events.

Within crystal idioblast initials, small vacuoles have been reported to coalesce to form a larger or central vacuole (Arnott and Pautard, 1970; Horner and Whitmoyer, 1972; Eilert, 1974; Chiu and Falk, 1975; Horner and Wagner, 1980; Kausch and Horner, 1983a) in which vesicles, tubules, membrane complexes, paracrystalline nucleation sites, and/or crystal chambers may form or accumulate. Formation of a large vacuole always precedes crystal formation. Coalescence of small vacuoles which contain electron-dense amorphous material occurs in *Vanilla* idioblast initials prior to
crystal formation. Crystal chambers form in association with, and possibly from, this material. Even though a sparse flocculent substance has been observed in vacuoles of some raphide crystal idioblasts (Eilert, 1974; Kausch and Horner, 1983a), an association of crystal chambers with this type of material has not been observed previously. We believe this material functions analogously to vacuolar paracrystalline structures reported in some crystal idioblasts (Horner et al., 1981; Wagner, 1983) to support or facilitate formation of crystal chambers and/or crystallization. Mollenhauer and Larson (1966) reported that crystallization in Vanilla idioblasts occurred within a carbohydrate matrix. We do not believe that their data warrant such a conclusion.

Cytoplasmic origin of calcium oxalate crystals has been suggested to occur in a number of plants (Netolitzky, 1929; Scott, 1941; Küster, 1956; Wattendorff, 1969). Crystal formation is thought to occur inside of cytoplasmic strand in such a way that the crystals appear to be within central vacuoles. However, a number of investigators (Arnott and Pautard, 1970; Horner and Whitmoyer, 1972; Eilert, 1974; Horner and Wagner, 1980; Horner, Kausch, and Wagner, 1981; Kausch and Horner, 1981, 1983a) interpret crystal formation as an intravacuolar process facilitated by specialized membranes, called crystal chambers (Arnott and Pautard, 1970), that form prior to crystallization (Franceschi and Horner, 1980a; Horner and Wagner, 1980). Our investigation shows that crystallization occurs in crystal chambers and that these chambers are in the vacuole proper and not within transvacuolar cytoplasmic strands.
Crystalloplastids (Arnott, 1966; Eilert, 1974) have been reported in many differentiating and mature crystal idioblasts (Mollenhauer and Larsen, 1966; Arnott, 1966, 1973, 1976; Eilert, 1974; Kausch and Horner, 1983a) and are distinguished from plastids in surrounding cells by their morphology and absence of starch inclusions. Kausch and Horner (1983a) examined the origin and differentiation of crystalloplastids in *Typha* raphide crystal idioblasts and suggested that these plastids may be multifunctional organelles during idioblast differentiation. Crystalloplastids in *Vanilla* appear and differentiate similarly.

Crystalloplastids which differentiate lobed regions have been observed only in raphide crystal idioblasts which accumulate vacuolar mucilage (Arnott, 1966; Mollenhauer and Larson, 1966; Eilert, 1974; Kausch and Horner, 1983a). Kausch and Horner (1983a) suggested that these lobed crystalloplastids may be involved in mucilage formation. The lobed region in *Typha* was observed in close association with ER during mucilage accumulation. In *Vanilla* idioblasts, the crystalloplastids differentiate a lobed region concomitant with the appearance of material which accumulates in dilated ER lumen. We concur with Mollenhauer and Larson (1966) that this material probably is the vacuolar mucilage and that the ER at this stage is involved with an internal secretory activity. We believe also that the differentiated lobed region may be involved with this process because of its concurrent developmental formation.

The production and accumulation of vacuolar mucilage is coincident with formation of lamellated crystal sheaths (Eilert, 1974; Tilton and Horner, 1980; Kausch and Horner, 1983a,b) and a change in crystal shape
(Horner et al., 1981). This situation is reiterated in Vanilla and results in mature raphides that are six- and eight-sided.

The vacuolar cables of tubules which become associated with crystal bundles were observed with light microscopy in mature raphide idioblasts of a number of monocotyledonous species by Kohl (1899). In Vanilla, we observed the formation of these structures only after formation of a raphide bundle. Hence, we do not believe that they function in crystal initiation or growth. We think that perhaps these tubules anchor and maintain raphide bundle orientation; however, their formation and function remain enigmatic and deserve further investigation.

Information from the investigators indicates calcium oxalate crystal formation may be involved with tissue morphogenesis in some plants (Sakai and Hanson, 1974; Dudinskii, 1976; Horner and Wagner, 1980; Kausch and Horner, 1981). Specifically, middle lamella and/or cell wall degradation has been noted to occur in proximity of developing crystal idioblasts in a number of plants. Kausch and Horner (1981) suggested the role of crystal idioblasts during such tissue development might be understood as one of localized calcium ion regulation; i.e., as a calcium sink. Indeed, formation of cortical syncytia in Vanilla roots via schizo-lysigenous breakdown of transverse idioblast cell walls may be another example of such involvement. Tilton and Horner (1980) reported formation of syncytial raphide crystal liferous idioblasts in carpels of Ornithogalum caudatum, presumably by a similar mechanism. Another possibility in the case of the aerial roots of Vanilla, which are photosynthetic, is that the syncytia may function in gas exchange and transport in mature portions of the roots.
ACKNOWLEDGMENTS

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H. J. Arnott: The change from four-sided to six- and eight-sided crystals is very interesting. What do you think causes this? How are the lamellated crystal chambers involved in this process?

Authors: We believe that the change in crystal shape is affected by poisoning of existing crystal faces. Components of the mucilage and/or lamellated crystal chambers may be responsible for such a poisoning event. The lamellated crystal chambers may also be involved in stabilization of the crystals. Neither formation of lamellated chambers nor change in crystal shape occur in Psychotria punctata idioblasts that do not produce mucilage (Horner and Whitmoyer, 1972).

H. J. Arnott: Could you expand on what you believe the role of the crystalloplastids is in differentiation of these crystal cells?

Authors: Designation of any specific biochemical function to crystalloplastids during crystal idioblast development is mostly speculative until more information is gained. However, we have observed in Vanilla that these organelles assume a distinctive appearance, compared to plastids in surrounding nonidioblastic cells, prior to and during crystal production. After cessation of crystal initiation, but before mucilage accumulation, crystalloplastids enlarge and differentiate lobed regions. This pattern of crystalloplastid development has been seen in Yucca (Arnott, 1966; Eilert, 1974) and Typha (Kausch and Horner, 1983a). We now have preliminary evidence from experiments with Yucca crystal idioblasts that radioactive precursors of oxalic acid may be concentrated in crystalloplastids.
Kausch and Horner (1983a) show that a PA-TCH-SP positive (carbohydrate) material accumulates in the lobe region of crystalloplastids during mucilage production in *Typha*. Also, only mucilaginous crystal idioblasts have been reported to contain lobed crystalloplastids (Kausch and Horner, 1983a). *Psychotria punctata* raphide crystal idioblasts do not produce mucilage and do not differentiate lobed crystalloplastids (Horner and Whitmoyer, 1972). We believe, therefore, that crystalloplastids may be involved in oxalate synthesis during crystal formation, and produce mucilage precursors later in idioblast development; however, conformation of this hypothesis awaits further investigation.

H. J. Arnott: I am hard pressed to understand that the role of the crystal idioblasts is to be a "calcium sink." Why would an aerial root require a calcium sink? It seems that calcium would be vital to the developing apex for many physiological functions, not the least of which is the continued formation of crystal idioblasts. Does *Vanilla* grow in areas where calcium is so abundant that files of crystal cells must be formed to take up the "excess" calcium?

Authors: A localized, cellular calcium sink may be necessary within an aerial root, or other meristematic tissues where crystal idioblasts are often found, for involvement in programmed tissue morphogenesis of the organ, and not necessarily to take up environmentally "excess" calcium. The distribution of crystal idioblasts throughout any plant is characteristic of its species (Arnott and Pautard, 1970) not its environment, and therefore predictable and probably genetically determined. Most crystal idioblasts are formed within meristematic tissue, precociously with re-
spect to surrounding cells. Therefore, the speculation that crystal idioblasts are involved in tissue morphogenesis is not unrealistic. We hypothesize that calcium could be selectively deprived from cell walls near differentiating crystal idioblasts, and used instead in crystal production. Selective wall calcium deprivation may be hormonally achieved and facilitate increased cell wall plasticity in tissues surrounding developing crystal cells. It may be more than coincidental that crystal cells are found in aerenchymatous tissues of many plants (Kausch and Horner, 1981). Furthermore, Horner and Wagner (1980) reported that druse crystal formation occurs concomitantly with adjacent stomium cell wall breakdown in Capsicum anthers. Conceivably, the formation of calcium oxalate crystals may occur in response to a physiological gradient, established for controlled and regulated sequestration of calcium ions during development. This notion is further supported by the fact that calcium oxalate does not merely precipitate indiscriminately in plants, but requires differentiation of ultrastructurally, and no doubt physiologically, complex cells. Of course, calcium is vital to many functions in a developing root apex, but its cell and tissue specific regulation is important also. Perhaps, therefore, crystal idioblasts play a role in tissue morphogenesis and functioning is localized calcium regulatory units rather than merely passive participation as a dump site for "excess" calcium.

V. R. Tilton: What is your speculation as to the ontogeny of the vacuolar calbes of tubules?

Authors: The vacuolar cables of tubules appear to arise de novo and are always associated with flocculent, electron-dense amorphous material.
This material may be a precursor of the tubules. Free ends of these tubules have been observed.

M. A. Webb: The authors note that nuclei are enlarged and the cytoplasm is organelle-rich in idioblasts as compared to neighboring cortical parenchyma cells. To what extent are these features a function of cell enlargement? Do you have any information on the volumes of these components relative to cell size?

Authors: The cytoplasm of *Vanilla* crystal idioblasts is more dense with ER, polysomes, and free ribosomes at all stages of development despite an obvious large increase in cell volume. Plastids and mitochondria are observed in dumbbell configurations, indicative of their replication, and their increase in numbers must result. These events do not necessarily have to occur just because the cell is enlarging, but do reflect a metabolically active system. We have not done any morphometric analysis at this time and therefore do not have any quantitative information concerning the volumes of these components relative to cell size.

J. Wattendorff: Would not the raphide-continuing slime be an excellent tool to make biting roots disagreeable to animals like worms or slugs? When a fresh *Hyacinthus* root tip is cut off, an interminable flow of slime and raphides leaves the root for many minutes via the slime tubes which for leaves were described by Hanstein in 1859.

Authors: Certainly, a predatory defense mechanism of raphides and mucilage would substantiate evolutionary survival value of both crystal idioblasts and the plants that produce them. A predatory defense function for
raphides is very likely in such plants as *Tragia*, stinging nettle; however, we do not believe that proposed functions for crystal idioblasts need be mutually exclusive, or common for these cells in all plants.

E. Zindler-Frank: It would be interesting to know the time of development. For instance: once started is crystal precipitation completed in a comparatively short time? This might give some information as to how far this physico-chemical event is controlled by the plant.

Authors: This type of information would be difficult to obtain in *Vanilla* roots. However, we have conducted experiments with crystal initiation and idioblast differentiation in isolated root cultures of *Yucca torreyi* and information concerning timing of these events has been determined. Full development from idioblast initials requires about 24h in *Yucca*, depending on culture conditions. These results have been submitted for publication in another journal.

E. Zindler-Frank: Could you tell whether raphides in similar amounts are formed in the earth roots of *Vanilla*, too? I wonder whether there is a connection between oxalate biosynthesis and the photosynthetic metabolism of the aerial roots.

Authors: Qualitative assessments suggest that raphide number is similar in earth roots of *Vanilla*.

E. Zindler-Frank: Do the large nuclei of the idioblasts correspond to cell size?
Authors: A specific correlation has not been measured, but nuclei gradually increase in diameter throughout idioblast development and concomitant with increasing cell size and DNA content.
APPENDIX
Figures 31-36. Ultrastructural characteristics of crystal idioblast initials. Key to labelling: AM - amorphous material; CI - crystal idioblast initial; M - mitochondria; P - plastic; V - vacuole.

Figure 31. File of young crystal idioblasts; vacuole formation, and crystal initiation and production are ephemeral events that occur within three cells of a file. 1250X

Figures 32-25. TEM of idioblast initials showing replicating plastids and mitochondria and vacuoles containing electron-dense amorphous material. 7237X, 17,260X, 16,500X, 5,650X, respectively.

Figure 36. SEM of idioblast initial with amorphous material in the central vacuole. 3000X

"The energy of nature is displayed in the smallest things and in the smallest things lies excellence of art."

Algarotti
Figures 37-41. Calcium oxalate crystal initiation. Key to labelling: C - crystal; N - nucleus.

Figures 37-40. SEM of crystal initiation showing small newly formed crystals and empty crystal chambers (arrows). 4,200X, 10,800X, 5,000X, 9,340X, respectively

Figure 41. TEM of idioblast during crystal initiation. 11,220X

"[There is] no dependence upon words and letters;
Direct pointing to the mind of man;
Seeing into one's own nature.

Tan-Hsia

"No gate stand on public roads;  
There are paths of various kinds;  
Those who pass this barrier  
Walk freely throughout the universe."

Mumon-Kan
Figures 48-51. Vacuolar cables of tubules in crystal idioblasts of *Vanilla*. 8,000X, 14,000X, 28,000X, 21,000X, 15,000X, 9,200X

"Talk of mysteries! Think of our life in nature - daily to be shown matter, to come in contact with it - rocks, trees, wind on our cheeks! The solid earth! The actual world! The common sense! Contact! Contact! Who are we? Where are we?"

Henry David Thoreau
Figures 54-57. Fluorescence microscopy of crystal idioblasts stained with acridine orange. Key to labelling: N - nucleus.

Figure 54. Cytoplasm of idioblast fluoresces more intensely than surrounding parenchyma cells. 470X
Figure 55. Enlarged nuclei fluoresce very intensely. 670X
Figure 56. Crystal chambers fluoresce slightly. 670X
Figure 57. Idioblast at syctial formation, note broken wall, nuclei are liberated, but do not fuse with each other. 670X

"The great Tao flows everywhere, to the left and to the right, All things depend upon it for existence, and it does not abandon them. To its accomplishments it lays no claim. It loves and nourishes all things, but does not hold it over them."

Yiin-men

Figure 58. Comparison of bas-relief and convention TEM of an idioblast initial with a crystal chamber in the central vacuole (arrows). 6,734X

We shall see but little way if we require to understand what we see. How few things can a man measure with the tape of his understanding! How many greater things might he be seeing in the meanwhile!

Henry David Thoreau
Figure 59. Raphide bundle formation in association with electron-dense material. 9,230X. Key to labelling: M = mitochondria, V = vacuole.

"God, the Great Giver, can open the whole universe to our gaze in the narrowest of spaces."

Rabindranath Tagore

In this light my spirit soon saw through all things and in all creatures, in herb and grass knew God, who he is, how he is, and what is his will.

Jakob Boehme
"Whatever aid is to be derived from the use of a scientific term, we can never begin to see anything as it is so long as we remember the scientific term which always our ignorance has imposed on it. Natural objects and phenomena are in this sense forever wild and unnamed by us."

Loren Eisely

"... there is nothing inorganic. This earth is not, then a mere fragment of dead history, strata upon strata, but living poetry, like leaves of a tree - not a fossil earth but a living specimen."

Henry David Thoreau
"Nature will bear the closest expection. She invites us to lay our eye level with her smallest leaf and root, and to take an insect view of its plain."

Henry David Thoreau

What is laid upon us is to accomplish the negative; the positive is already given.

Franz Kafka
Figure 62. Bas-relief TEM processed for maximum contrast and relief enhancement, positive is more dense than negative to produce this effect. 22,300X

"Wu-wei"

unknown origin
PART II: INCREASED NUCLEAR DNA CONTENT IN RAPHIDE CRYSTAL IDIOBLASTS DURING DEVELOPMENT IN VANILLA PLANIFOLIA L. (ORCHIDACEAE)
Increased nuclear DNA content in raphide crystal idioblasts during development in *Vanilla planifolia* L. (Orchidaceae)

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SUMMARY

Longitudinal files of raphide crystal idioblasts form within the cortical meristematic region of *Vanilla planifolia* aerial roots. Cell and nuclear enlargement occur gradually throughout idioblast development and nuclear diameter approximates idioblast maturity. Cytophotometric determination of nuclear DNA (Feulgen) contents, measured by the two-wavelength method, revealed that all cortical parenchyma cells are diploid (2C= 6.3 pg), whereas all crystal idioblast nuclei are endopolyploid. Idioblast nuclear DNA content ranged from 4C to 32C (106 pg) and averaged 5.9 times that of parenchyma telophase nuclei. Frequency distribution of individual DNA content measurements depicts multiple genomes (increasing with geometric periodicity) to the 8C level, followed by less strict DNA replication within the crystal idioblast genome. The largest nuclei had the highest DNA content. Endomitotic stages of preprophasic heterochromatic dispersion (Z phase) and partial prophasic chromosomal coiling are observed with light and electron microscopy. DNA content values above the 8C level do not fit the geometrical order which is found if the total genome is replicated during each endo-cycle, indicating differential DNA replication. Chromocenter counts substantiate the occurrence of endomitosis to the 8C level and suggest heterochromatin underreplication in higher endopolyploid idioblast nuclei. Possible relationships between observed cytological events of idioblast development and nuclear condition are discussed.
INTRODUCTION

Cells which form intravacuolar crystals of calcium oxalate, sometimes called crystal idioblasts, have been observed in diverse tissues and organs of a large number of plants (Arnott and Pautard, 1970; Franceschi and Horner, 1980a; Pentecost, 1980). Microscopic studies concerning cell development have shown crystal idioblasts to be ultrastructurally complex and differentiated to involve the crystallization process (Horner and Whitmoyer, 1972; Kausch and Horner, 1983a,b). Calcium oxalate crystal shape is considered to be genetically determined (Kausch and Horner, 1983b), and precipitation is apparently controlled by idioblasts during differentiation (Arnott and Pautard, 1970; Kausch and Horner, 1983b). The role of these cells in normal plant tissue growth and functioning is not known.

Crystal idioblast initials are usually formed in meristematic tissues and differentiate precociously in comparison to surrounding cells (Arnott and Pautard, 1970; Franceschi and Horner, 1980a; Kausch and Horner, 1983a). Nuclear and nucleolar enlargement are among the first observable cytological modifications associated with crystal idioblast initiation in many plants (Franceschi and Horner, 1980a,b; Kausch, 1980; Kohl, 1899). Kohl, in 1899, observed increased nuclear diameters in raphide crystal idioblasts of a number of monocotyledonous species. The nuclei of raphide-forming cells of Vanilla (Hurel-Py, 1938, 1942; Mollenhauer and Larson, 1966), Monstera (Mollenhauer and Larson, 1966; Rakován et al., 1973) and Typha (Kausch, 1980) become progressively enlarged and modified throughout idioblast development. Differentiating raphide crystal idioblasts in
Psychotria leaves (Horner and Whitmoyer, 1972) and callus culture (Franceschi and Horner, 1979) have larger nuclei and denser nucleoli than neighboring noncrystal containing parenchyma.

Horner and Whitmoyer (1972) suggested that nuclear enlargement and structural modification may indicate that crystal idioblast nuclei become endopolyploid during differentiation. Enlarged idioblast nuclei in Vanilla reportedly become lobate through development (Mollenhauer and Larson, 1966), another indication of endo-cycling cells (Nagl, 1978). Indeed, based on nuclear volume analysis, the ploidy levels of crystal idioblasts in Gibbaeum heathii were calculated to range from 2n to 64n (Schlichtinger, 1956). However, increased nuclear volume is not proof of increased DNA content (Nagl, 1978, 1979) and is affected by variable amounts of nuclear proteins and extent of chromatin hydration (Nagl, 1978). A preliminary cytophotometric investigation of enlarged crystal idioblast nuclei in Typha angustifolia, however, did reveal elevated DNA contents compared to nuclei from surrounding nonidioblastic cells (Kausch, 1980).

The occurrence of a nuclear endo-cycle is highly suspect in crystal idioblast differentiation and may represent an important developmental feature of this cell type. In the present study, increased amounts of nuclear DNA were observed with Feulgen microspectrophotometry in developing raphide crystal idioblasts of Vanilla planifolia. The purposes of investigation, therefore, were to determine the extent of extra DNA replication and gain an insight into the type of endo-cycle functioning during idioblast differentiation.
This study is a portion of a larger program concerning raphide crystal idioblast development in several plants and tissue cultures (Kausch, 1980; Kausch and Horner, 1982, 1983a,b), including Vanilla planifolia. Also, previous investigators have examined various developmental aspects of these cells in Vanilla (Hurel-Py, 1938, 1942; Mollenhauer and Larson, 1966). These studies provide background information that render Vanilla crystal idioblasts attractive for investigation of the nuclear condition in this plant cell type. Longitudinal files of raphide crystal idioblasts form within the apical meristem of the root cortex (Kausch and Horner, 1983b; Mollenhauer and Larson, 1966). A file of idioblasts is never interrupted by nonidioblastic cells, and once initiated, an idioblast differentiates and does not undergo mitosis or nuclear fusion (Kausch and Horner, 1983b). Nuclear and nucleolar enlargement occur just prior to vacuolar crystal formation and continue to gradually increase in size throughout idioblast maturation. Therefore, nuclear diameter approximates crystal idioblast maturity. Crystal idioblasts are the only cell type in Vanilla root cortex that contain enlarged nuclei. The possible relationships between reported cytological events of development and nuclear condition will be discussed.
MATERIALS AND METHODS

Actively growing adventitious roots were taken from *Vanilla planifolia* L. plants grown in the departmental greenhouse. Roots were submerged, dissected, and fixed in 3:1 ethanol:glacial acetic acid at room temperature. The epidermis was removed by gently scraping the root surfaces with a razor blade. Root apices, 4 mm in length, were excised and the central vascular cylinder was bored out with a 0.35 mm (I.D.) needle. The resultant hollow cone of tissue consisted entirely of root cortex.

Fresh chicken blood was obtained from hens at the Iowa State University Poultry Farm, and collected in sample tubes containing sodium EDTA. Blood smears were made on clean glass slides, allowed to air dry, and stored frozen in a desiccator (Berlyn and Miksche, 1976).

Freshly dissected root cortical preparations and blood smear slides were placed in fresh 3:1 fixative for 4 h at room temperature, then hydrated to distilled water. Hydrolysis was conducted in 5 N HCl at 23°C for 5 to 60 min, stopped at 5 min intervals with ice water. Root material was then teased apart on blood smear slides, squash preparations were made, and coverslips removed with a liquid stream of CO₂ (Doerschug et al., 1978). The slides were then stained in freshly prepared Schiff's reagent for 60 min (23°C) in darkness. Stained slides were bleached in two changes of 5% sodium metabisulfite for 10 min each and rinsed in distilled water (Miksche, 1976). Coverslips were applied following dehydration to 100% ethanol, using diaphane as a mounting medium.

A Leitz MPV microscope photometer, a Leeds and Northrup 2430 DC galvanometer, and a transmitted light illuminating system with a Leitz in-
line mirror monochromator and stabilized 150 watt pressure Xenon lamp were used for all cytophotometric measurements.

In order to ascertain which hydrolysis time allowed maximum DNA-Feulgen staining, and which wavelengths were to be used for cytophotometry, spectral absorption curves were generated for each hydrolysis time. Measurements of ten cortical parenchyma interphase nuclei were taken from 490 nm to 610 nm at 10 nm intervals. These data indicated that 30 min hydrolysis gave maximum DNA-Schiff's dye binding. Cytophotometry was done using the two-wavelength method (Ornstein, 1952; Patau, 1952) with Tb (maximum absorption) at 550 nm and Ta (50% maximum absorption) at 508 nm as determined from the 30 min hydrolysis spectral absorption curve.

Mitotic root cortical parenchyma cells were used as internal standards for relative nuclear DNA contents in Vanilla; telophase nuclei (measured individually) and anaphase figures (measured collectively) supply theoretical 2C:4C ratio. Chicken erythrocytes contain a stable, consistent, and known mass of DNA, 2.62 pg per nucleus (Berlyn and Miksche, 1976) and are routinely used as cytophotometric standards (Nagl, 1978; 1979).

The measuring diaphragm used to compare mitotic figures and chicken erythrocytes was too small for accurate measurement of enlarged crystal idioblast nuclei. Consequently, the diaphragm was adjusted to a larger diameter. Chicken erythrocytes were remeasured using 3, 4, 5, and 6 nuclei per diaphragm and an average DNA mass per nucleus was calculated. A proportionality constant for the two measuring diaphragms was determined.
and used as a correction factor for comparison of DNA contents between
mitotic root cortical parenchyma cells and crystal idioblasts.

Nuclear structure has been studied with brightfield light and trans­
mission electron microscopy (TEM). Preparation procedures for TEM have
been described elsewhere (Kausch and Horner, 1983b).
RESULTS

DNA content (pg) of mitotic root cortical parenchyma cells is calculated from comparison to the mean chicken erythrocyte DNA M value and provides C ratio values (Table I.) for Vanilla. The standard deviation for chicken erythrocyte DNA M values reflects constancy of DNA content and verifies their use as standards in our preparations. The 2C DNA content of telophase nuclei is determined to be 6.3 pg (Table I.). The slightly lower than theoretical 4C value for anaphase figures is attributable to overlapping condensed chromosomes. All cortical parenchyma interphase nuclei have a DNA content of 2C-4C (Table I.), i.e., they all are diploid. The varying DNA content is the result of passage through the mitotic cycle.

Nuclear diameter increases gradually during Vanilla crystal idioblast differentiation (Hurel-Py, 1938; Mollenhauer and Larson, 1966; Kausch and Horner, 1983b), hence, the most mature idioblasts contain the largest nuclei. Since crystal idioblasts are the only cell type containing enlarged nuclei in Vanilla root cortex, they are easily distinguished from parenchyma cells in our squash preparations of isolated cortical tissue. The mean DNA mass of idioblast nuclei is 37.1 pg (Table I.), 5.9 times more DNA than 2C telophase nuclei. All measured idioblast nuclei showed DNA M values higher than the 4C level; they all are polyploid. The standard deviation indicates a wide range of DNA content values for nuclei at various developmental stages.

DNA content (M) values of individual idioblast nuclei are plotted in a frequency histogram to show their relative distribution (Fig. 1). Class
widths are chosen to achieve adequate separation of population peaks at lower DNA content levels. DNA content of idioblast nuclei ranges from 4C to 32C. The largest idioblast nuclei recorded the highest DNA contents, up to 106 pg. The first population peak in the histogram is from diploid interphase nuclei measurements, the presumed precursors of crystal idioblast initials, and represents the 2C level. Two peaks of idioblast nuclei are clearly discernible at 4C and 8C levels, but there are no separable populations at levels above 8C (Fig. 1).

Diploid cortical parenchyma interphase nuclei measure less than 10 μm in diameter (Fig. 2). Their structure is characterized by an average of seven discernible heterochromatic regions (chromocenters) and a moderately Feulgen-stained euchromatic background (Table II, Fig. 2). The smallest cytophotometrically measured idioblast nuclei have a diameter between 10 and 20 μm and typically contain 12 chromocenters (Table II). The number of chromocenters in these nuclei ranges from 9-14, some of which are slightly enlarged compared to those in diploid cells (Fig. 3). Evidence of endomitotic stages is seen in idioblast nuclei of similar diameter (Figs. 4-8). Stages of preprophasic dispersion of heterochromatin, or Z phase (Figs. 4-6), are observed. Examples representing partial prophasic chromosomal coiling also are found (Figs. 7, 8). Mitotic figures of enlarged nuclei do not occur. Crystal idioblast nuclei with diameters between 20 and 30 μm contain 19-25 chromocenters, averaging 20 (Table II). Although their number is higher, chromocenter size is comparable to those in smaller idioblast nuclei (Fig. 9). Nuclear diameter continues to in-
crease considerably (Fig. 10); however, neither the apparent size (Fig. 10) or number of chromocenters changes appreciably (Table II).

Electron microscopy of cortical parenchyma interphase cells (Fig. 11) shows nuclei with heterochromatin mostly associated with the nuclear envelope. The enlarged idioblast nuclei contain numerous heterochromatic regions, some of which appear to be fused (Fig. 12). Euchromatic areas of idioblast nuclei appear more dense than euchromatin of parenchyma cells. Sections through idioblast nuclei apparently lacking heterochromatin are observed and may represent Z phase cells (Fig. 13). Condensed idioblast chromosomes have not been found in sectional material, and may represent an ephemeral event. The nuclear envelope is always intact and nuclear pore size does not change with development. Idioblast nuclei become slightly irregular in shape (Fig. 13), compared to parenchyma cell nuclei (Fig. 11), but do not become excessively lobed or invaginated at any stage during idioblast differentiation. There is no structural evidence with either light or electron microscopy for even weakly polytene chromosomes.
Figure 1. Frequency histogram of DNA content (M) values of individual idioblast nuclei. The first population peak, darkened, is from diploid interphase nuclei, the presumed precursors of idioblast initials. Cross-hatching indicates idioblast nuclei measurements.
DNA Content (M value) of Individual Nuclei
Figures 2-10. Light micrographs of Feulgen stained cortical cell nuclei depicting a developmental sequence of crystal idioblast nuclear enlargement.

Figure 2. Diploid cortical parenchyma cell nucleus showing eight heterochromatic regions.

Figure 3. Enlarged idioblast nucleus with seventeen chromocenters.

Figures 4, 5. Idioblast nuclei showing dispersion of heterochromatin.

Figure 6. Z-phase idioblast nuclei with all heterochromatin dispersed.

Figures 7, 8. Partial prophase chromosomal coiling of endomitotic idioblast nuclei.

Figure 9. Enlarged idioblast nucleus with 23 discernible heterochromatic regions.

Figure 10. Greatly enlarged idioblast nucleus with 22 chromocenters. Note diploid cortical parenchyma nucleus at right.

Figures 11-13. Transmission electron micrographs of cortical cell nuclei.

Figure 11. Cortical parenchyma nucleus, with heterochromatin associated with nuclear envelope.

Figure 12. Enlarged idioblast nucleus showing fused chromocenters.

Figure 13. Presumed Z-phase idioblast nucleus, note the absence of all heterochromatin and condensed nucleolus.
Table I. DNA content of *Vanilla planifolia* L. root cortical parenchyma and crystal idioblast nuclei, and chicken erythrocytes

<table>
<thead>
<tr>
<th>Category</th>
<th>Telophase figures</th>
<th>Anaphase figures</th>
<th>Interphase nuclei</th>
<th>Crystal cell nuclei</th>
<th>Chicken erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>125.0</td>
<td>31.0</td>
<td>75</td>
<td>272.0</td>
<td>80.0</td>
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<tr>
<td>DNA M value $\bar{x}$</td>
<td>58.5</td>
<td>109.9</td>
<td>81.3</td>
<td>343.0</td>
<td>24.2</td>
</tr>
<tr>
<td>$s$</td>
<td>8.7</td>
<td>8.9</td>
<td>19.1</td>
<td>195.6</td>
<td>3.1</td>
</tr>
<tr>
<td>$\frac{s}{\bar{x}}$</td>
<td>0.7</td>
<td>1.6</td>
<td>2.3</td>
<td>12.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Cov</td>
<td>4.8%</td>
<td>8.1%</td>
<td>14.2%</td>
<td>57.0%</td>
<td>12.9%</td>
</tr>
<tr>
<td>Mean DNA Mass (pg)</td>
<td>6.3</td>
<td>11.9</td>
<td>9.5</td>
<td>37.1</td>
<td>2.62$^b$</td>
</tr>
<tr>
<td>Mean DNA [C] ratio</td>
<td>2.0</td>
<td>3.8</td>
<td>3.0</td>
<td>11.7</td>
<td>--</td>
</tr>
</tbody>
</table>

$^a$All raw data corrected for largest spectrophotometer measuring diaphragm.

$^b$Berlyn and Miksche (1976).

Table II. Chromocenter number in *Vanilla planifolia* cortical cell nuclei

<table>
<thead>
<tr>
<th>Nuclear diameter class</th>
<th>&gt; 10 $\mu$m$^a$</th>
<th>10-20 $\mu$m</th>
<th>20-30 $\mu$m</th>
<th>&lt; 30 $\mu$m</th>
</tr>
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<tr>
<td>N</td>
<td>50</td>
<td>49</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>Chromocenters $\bar{x}$</td>
<td>7.0</td>
<td>11.7</td>
<td>20.2</td>
<td>23.5</td>
</tr>
<tr>
<td>$s$</td>
<td>1.1</td>
<td>1.7</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>$\frac{s}{\bar{x}}$</td>
<td>0.02</td>
<td>0.06</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>Cov</td>
<td>%16.0</td>
<td>%14.5</td>
<td>%11.9</td>
<td>%8.5</td>
</tr>
</tbody>
</table>

$^a$All are diploid cortical parenchyma interphase nuclei.
DISCUSSION

Increased DNA content in Vanilla planifolia crystal idioblast nuclei is observed, in our study, and this must be a consequence of development, i.e., endopolyploidy. Frequency distribution of individual nuclear DNA content depicts multiple genomes (increasing with geometric periodicity) to the 8C level, followed by less strict DNA replication within the crystal idioblast genome.

Nuclear structures indicate endomitosis (Nagl, 1978) in the low-endopolyploid idioblast nuclei. Preprophasic heterochromatin dispersion, "Zerstäubungsstadium" (Heitz, 1929) or Z-phase (Nagl, 1968; 1978) is the cytological feature by which an endomitotic event, as opposed to endoreduplication (polyteny), is recognized in endo-cycling plant cells (Geitler, 1941; Nagl, 1968, 1978; Tschermak-Woess, 1971). Nagl (1970, 1972) interpreted the endomitosis in angiosperms as a cycle in which mitosis is curtailed by suppression of events later than Z phase. However, we found examples of enlarged idioblast nuclei containing partially condensed chromosomes.

Passage through successive rounds of endomitosis would result in increased numbers of chromocenters as endochromosomes become separated following an endomitotic coiling process. Increased numbers of heterochromatic regions is indicative of polyploid nuclei (Banks and Evans, 1976). All measured idioblast nuclei exhibiting heterochromatin (those not in Z-phase) had higher numbers of chromocenters than diploid interphase nuclei. Based upon the numbers of chromocenters, it is likely that the nuclear
diameter classes in Table II approximate DNA content levels (2C, 4C, 8C, above 8C, respectively) even though a direct correlation has not been made. Separated endochromocenters may fuse secondarily (Geitler, 1939a,b, 1941; Nagl, 1978) accounting for the larger heterochromatic regions observed in idioblast nuclei compared to those found in diploid nuclei.

Endopolyploidy via an endoreplication cycle would usually result in nuclei containing enlarged endochromocenters, but similar in number, to diploid nuclei. Furthermore, we do not see any evidence with TEM of polytene chromosomes at any stage of idioblast development. Therefore, we believe that the change in size and number of chromocenters substantiates the occurrence of an endomitotic cycle to the 8C level.

DNA values above the 8C level do not fit the geometrical order which is found if the total genome is replicated during each endo-cycle; they fall between endopolyploid classes and fail to form peaks in the histogram. The largest nuclei contain the highest DNA content. Neither endomitotic stages or changes in chromocenters were seen in the larger idioblast nuclei. This information offers strong evidence for differential DNA replication (Nagl, 1976, 1978, 1979) during endo-cycles above the 8C level. Apparently, DNA replication above the 8C level does not involve all heterochromatic regions, giving rise to a gradual increase in DNA content commensurate with increasing nuclear diameter but without substantial increase in chromocenters.

Enlarged crystal idioblast nuclei in leaves of Typha angustifolia were found to contain elevated amounts of DNA in comparison to surrounding diploid cells (Kausch, 1980). Developmental studies of these idioblasts
strongly suggested that this increase was also a consequence of differentiation. Frequency distribution of nuclear DNA contents did not indicate entire genomic replication at any stage of development. The occurrence of differential DNA replication was implicated (Nagl, personal communication, University of Kaiserslautern) probably through underreplication of heterochromatic DNA. Increased DNA contents were strongly correlated to increased nuclear diameter and cell length measurements.

The occurrence of nuclear endo-cycles in plants is well-known and their functional significance in cell differentiation has been discussed ever since their discovery (Nagl and Rucker, 1972; Evans and van't Hof, 1975; Magakyan, 1976; Nagl, 1978, 1979) but definitive evidence is lacking. The role of endo-cycles during differentiation of somatic plant cells is usually considered apart from the functioning of the actual extra-replicated DNA sequences and only in terms of coincident developmental events.

Once initiated, *Vanilla* crystal idioblasts never divide (Kausch and Horner, 1983b) and this is a common feature of raphide crystal idioblasts in other plants as well (Franceschi and Horner, 1980a). Intense cytoplasmic fluorescence occurs during all developmental stages of *Vanilla* crystal idioblasts with the acridine orange method indicating a high RNA content and a metabolically active system (Price, 1969). A consequence of endocycling cells is the fact that cell division is inhibited allowing continuous RNA synthesis and cellular differentiation (Pearson, 1974; Nagl, 1978). For example, Walbot et al. (1972) showed that the rate of RNA synthesis was higher in endomitotic suspensor cells than in meristematic
cells in the embryo proper of *Phaseolus*. Also, RNA synthesis has been shown to continue unabated during endomitosis in *Allium* root tips but ceases during mitosis (Nagl, 1973). Nagl (1978) suggested that endocycles may liberate a cell from autosynthetic activities of proliferating cells and allow specific heterosynthetic pathways for specific differentiation processes without abolishing growth. This would be important for cells such as crystal idioblasts which usually differentiate within rapidly proliferating meristematic tissues.

The number of plastids per cell has been correlated to the level of endopolyploidy in the nucleus (Butterfass, 1963, 1967, 1973). Specialized plastids in some crystal idioblasts, called crystalloplastids, have been reported to replicate and increase in number prior to and during crystal formation in a number of plants (Arnott and Pautard, 1970; Kausch and Horner, 1983a,b). Crystalloplastids may be involved in oxalate synthesis in some plants (Kausch, unpublished); thus, their increased numbers might impart a metabolic advantage during differentiation of these cells.

Raphide crystal idioblasts are often elongated and larger than surrounding nonidioblastic cells (Franceschi and Horner, 1980a) and *Vanilla* idioblasts become much larger than surrounding cortical cells (Kausch and Horner, 1983b). A number of investigators have noted a correlation between endopolyploidy and cell elongation; endopolyploid cells are generally larger than diploid cells (Barlow, 1972; Schlichtinger, 1956; Nagl, 1978). Perhaps cell enlargement is another expression of continued, uninterrupted differentiation allowed in endo-cycling cells.
The nuclear condition of differentiating crystal idioblasts in *Vanilla* may be related to other developmental events in these cells, and also may represent a feature common to this cell type in other plants as well. Attempts to reproduce these cells in culture are in progress, and may provide a system that will allow in-depth investigation of various aspects of cytodifferentiation as well as experimental use of crystal idioblasts and nuclear endo-cycles.
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APPENDIX
Figures 14-22. Light microscopy of crystal idioblast nuclei, Feulgen stained.

Figure 23. Telophase nuclei, showing how they were measured individually.

Figure 24. Anaphase nucleus, showing how they were measured together.

Figure 25. Diploid cortical parenchyma nuclei.

Figure 26. Feulgen stained chicken red blood cell nuclei.

"I' the color the tale takes, there's change perhaps;
'Tis natural, since the sky is different,
Eclipse in the air now, still the outline stays."

Robert Browning
Figure 27. Bar graph comparing average DNA content (pg) in telophase (T) and anaphase (A) cortical parenchyma cells, crystal idioblasts (Cc) and chicken red blood cells (Rbc).

If God is God

from J.B.

I heard upon his dry dung heap
That man cry out who cannot sleep:
"If God is God he is not good,
If God is good he is not God;
Take the even take the odd,
I would not sleep here if I could
Except for the little green leaves
in the wood
And the wind on the water."

Archibald Macleish

"Nature is a mutable cloud, which is always and never the same."

Ralph Waldo Emerson
Mean DNA Content (pg)

Type of Nucleus

T, A, Cc, Rbc
PART III: DIFFERENTIATION OF RAPHIDE CRYSTAL IDIOBLASTS IN ISOLATED ROOT CULTURES OF YUCCA TORREYI L. (AGAVACEAE)
Differentiation of raphide crystal idioblasts in isolated root cultures of *Yucca torreyi* L. (Agavaceae)

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Key words: root culture, crystal idioblasts, calcium oxalate, HVEM

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ABSTRACT

Excised primary roots of *Yucca torreyi* were cultured in various media, and development of cortical raphide crystal idioblasts was observed. Idioblasts were consistently produced, required 24 hours to fully differentiate from initials in isolated root cultures, and developed normally relative to those cells in intact roots. In vacuoles of idioblast initials, we observed an electron-dense amorphous material associated with paracrystalline bodies and membranous crystal chambers. Crystallization occurs within, and apparently controlled by crystal chamber membranes. Following crystal production, crystalloplastids differentiate lobed regions, and cables of tubules and mucilage form within the central vacuole. Many aspects of *Yucca* idioblast development are common to differentiation of similar cells in intact organs of other plants. Based on our results, we believe that isolated root cultures of *Yucca* will be useful for future physiological investigations of oxalate metabolism and control of idioblast cytodifferentiation.
INTRODUCTION

Calcium oxalate deposition, common to a large number of higher plant species, is not indiscriminate in plant cells, but is the controlled result of cell differentiation (Kausch and Homer, 1982, 1983a,b). Bundles of needle-shaped calcium oxalate crystals, called raphides (Esau, 1953), are produced within cells, termed raphide crystal idioblasts (Foster, 1956), that are cytologically modified to involve the crystallization process (Franceschi and Homer, 1980a). Crystal idioblast initials are usually formed in meristematic tissues and differentiate precociously in comparison to surrounding cells (Arnott and Pautard, 1970). Despite their widespread occurrence, the role of plant calcium oxalate crystal idioblasts in normal tissue development and functioning is not known.

Cytoplasmic origin of calcium oxalate crystals has been suggested to occur in a number of plants (Netolitzky, 1929; Scott, 1941; Küster, 1956; Wattendorf, 1969, 1979). Crystal formation is thought to occur either inside of cytoplasmic strands, or extracellularly within plasma membrane invaginations, in such a way that the crystals appear to be within central vacuoles. However, a number of investigators (Arnott and Pautard, 1970; Horner and Whitmoyer, 1972; Eilert, 1974; Horner and Wagner, 1980; Horner et al., 1981; Kausch and Horner, 1983a,b) interpret crystal formation as an intravacuolar process facilitated by specialized membranes, called crystal chambers (Arnott and Pautard, 1970), that form prior to crystallization (Franceschi and Homer, 1980a; Horner and Wagner, 1980). Indeed, in many cases this situation is difficult to assess from only convention-
ally processed thin sectioned material for transmission electron microscopy.

Longitudinal files of raphide crystal idioblasts form within the cortex of *Yucca torreyi* roots (Arnott, 1962). Eilert (1974) observed that each file represents an ontogenetic series of idioblast differentiation. Such a system is particularly well-suited for developmental studies of this specialized cell type, as well as experimental physiological investigations of oxalate production and stimulation of cytodifferentiation in plant organs.

In the present study, primary roots of *Yucca torreyi* were cultured under various conditions, and differentiation of crystal idioblasts was observed. The purposes of this paper are to examine the initiation of crystal formation and establish a developmental sequence of events for the raphide crystal idioblasts in isolated root cultures of *Yucca torreyi* using various microscopic techniques. Previously undescribed aspects of crystal idioblast development are emphasized. However, a comparison will be made to results of previous investigations of crystal idioblast development in intact tissues of *Yucca* (Arnott, 1966; Eilert, 1974) as well as other plants. This investigation will be useful for future studies using this system to study oxalate metabolism and idioblast differentiation.
MATERIALS AND METHODS

Dried seeds of *Yucca torreyi* were imbibed overnight in running tap water, surface sterilized 17 min in 2.0% sodium hypochlorite, and grown aseptically for 9-11 days at 27°C on sterile, wet filter paper in glass Petri dishes in darkness. Apical 2.5 cm explants were taken from 4.0 cm primary roots and transferred to 50 ml of liquid medium in 125 ml Erlenmyer flasks. Excised roots were grown in: White's medium (White, 1943, 1963) containing 20 g/l sucrose (pH = 5.7); quarter strength Murashige-Skoog (MS) salts (Murashige and Skoog, 1962) deleting ammonium nitrate, with 3.0 mg/l glycine and 20 g/l sucrose (pH = 5.7); or 50 mM K-phosphate buffer (pH = 7.1). Roots still attached to germinated seeds also were grown on White's medium (pH = 5.7) and 50 mM K-phosphate buffer (pH = 7.1). Culture flasks were kept on orbital shakers in darkness at 25°C. Average growth rate was determined from length measurements taken from ten roots every six hours for 72 h. Isolated roots were kept in culture for up to three months.

Samples for microscopic observations were taken from roots that had been grown in White's medium for 7 days. Some roots from 9 day germinated seeds were also used. Root apices, 5-7 mm long, were excised and fixed in either 3% glutaraldehyde or formaldehyde-glutaraldehyde (4%-1% v/v) in K-phosphate buffer (50 mM, pH = 7.1) at 4°C for 2 h. Formaldehyde was prepared from paraformaldehyde immediately before use. Glutaraldehyde fixed material was transferred into fresh fixative and kept at 4°C overnight. Aldehyde fixed material was followed by three buffer rinses, 15 min each, and post-fixed 2 h at room temperature with 1% OsO₄ (same buffer). Root
tips were then washed in double-distilled water followed by dehydration in an ascending ethanol series. After three rinses in 100% ethanol, samples were subdivided for scanning electron microscopy (SEM) and conventional transmission electron microscopy (CTEM) processing.

Root apices for SEM observation were cryofractured longitudinally in liquid nitrogen (Humphreys et al., 1974), thawed in 100% ethanol, infiltrated with Freon 113 and critical point dried with carbon dioxide. Specimens were mounted on brass discs with silver paste and coated with gold-palladium in a polaron E5100 sputter coating unit. All SEM observations were made on a JEOL JSM-35 microscope at accelerating voltages of 15 or 25 kV.

Root tips for CTEM observation were infiltrated with propylene oxide and then with Spurr's medium (Spurr, 1969). Survey sections for light microscopy (LM) were cut 1-2 μm thick and stained with methylene blue-azure II-basic fuchsin (Humphrey and Pittman, 1974). Histochemical determination of crystals as calcium oxalate was done on similar sections, using the procedure of Yasue (1969) and viewed with brightfield LM. Thin sections were cut with a diamond knife, mounted on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-11C TEM.

Cytochemical selective staining of plasma membranes was done according to Roland et al. (1972) on thin sections (PACP procedure). Thin sections were collected in plastic loops, destained with 1% aqueous periodic acid for 45 min, and rinsed five times, 10 min each, in double distilled water. Plasma membrane specific staining was achieved by treating de-
stained sections for 15 min with 1% phosphotungstic acid in 10% chromic acid, followed by five 10 min rinses with double distilled water. Sections were finally transferred to 200 mesh copper grids and examined with TEM.

Resin embedded material used in LM and CTEM was also used for high voltage electron microscopy (HVEM). Thick sections, 0.25–0.50 μm, were made from ultra-smooth block faces with glass and diamond knives, collected in slotted copper grids, and transferred to a drop of double distilled water on a glass slide. The slide was slightly warmed on a hot plate to expand the sections which were then transferred to Formvar-coated slotted grids and stained with uranyl acetate and lead citrate. Observation and micrography were done on an AEI HVEM operated at 1000 kV.
RESULTS

The growth of primary roots of *Yucca torreyi* is affected by different culture conditions (Figure 1). Growth curves are identical for isolated roots cultured in White's and MS media. A lag period where growth is minimal occurs for 12 h after excision, followed by linear growth that approximates 0.10 mm/h. Linear growth also occurs for roots still attached to germinated seeds, but there is no lag period and the growth rate is slightly greater in comparison to isolated roots (Figure 1). Growth curves for attached roots grown in K-phosphate buffer and White's medium are identical. Excised roots cultured in K-phosphate buffer undergo a lag period similar to the other isolated roots, but grow much more slowly.

Raphide crystal idioblasts form in longitudinal files within the cortex (Figure 2) of isolated and intact primary roots of *Yucca torreyi*. Files consisting of as many as 36 successive idioblasts, and as few as 3, are observed. Single idioblasts, surrounded entirely by cortical parenchyma, usually do not occur. An ontogenetic series, though not complete, is represented in each file where acropetal cells are developmentally youngest. Crystal idioblasts continue to be produced in isolated root cultures, grown in White's and MS media, for up to three months. Roots kept in extended culture in MS medium become swollen at their excised ends and often produce etiolated shoots. Shoot formation did not occur on roots grown in White's medium. Cortical crystal idioblast formation is not affected by extended root culture, or shoot formation. Idioblast dif-
Differentiation is observed as it occurs in isolated roots that have been in culture (White's medium) for 7 days.

Idioblast initials are distinguished from meristematic cortical parenchyma very early (i.e., close to cortical origin) by several ultrastructural characteristics. Idioblast initials (Figure 3) have denser cytoplasm, contain noticeably fewer profiles of spherosomes, and have a more extensive ER system than surrounding parenchyma. Plastids are simple, lack starch inclusions (Figure 4) and resemble eoplasts (Thomson and Whatley, 1980). In comparison, cortical parenchyma plastids occasionally contain starch, but otherwise are similar in size, morphology and apparent number to those in idioblast initials. Young idioblasts also have many small vacuoles (Figure 3), which contain an electron-dense amorphous material and, in some, dense paracrystalline bodies (Figures 3, 5).

As idioblast initials begin development, small vacuoles coalesce and central vacuole formation occurs much earlier than in surrounding cortical cells (Figures 1, 6). One to several paracrystalline bodies are found usually only in the largest vacuole (Figure 6). Electron-dense amorphous material is closely associated with the paracrystalline bodies (Figures 5–7). Periodicity of the crystal lattice is clearly discernible in three dimensional stereoscopic views taken from thick sections with HVEM (Figure 7). Structural units of the crystals are the same width as the interstices. It is also apparent from HVEM observations that the vacuolar sap is continuous with spaces between structural units of the paracrystalline bodies (Figure 7).
Crystal chamber membranes form in the central vacuole in association with the amorphous material, but not necessarily in proximity to para-crystalline bodies (Figure 8). During their formation, the crystal chambers are not always oriented in the same direction relative to each other. Crystal chambers are four-sided in transverse section and tapered at their ends (Figure 8) approximating the shape of raphides. No portion of these chambers has been observed to be contiguous with each other or the tonoplast; i.e., they appear as isolated structures surrounded by the amorphous material within the vacuole. The crystal chambers do not appear to have a unit membrane structure (Figure 8), characteristic of many biological membranes viewed with CTEM, and they measure 50 Å thick. The plasma membrane gives a distinct positive reaction to PACP, whereas the crystal chamber membranes are PACP negative (Figure 9).

Crystallization commences after numerous crystal chambers have been formed (Figure 10). At this stage, the chambers are mostly oriented in the same direction and clustered together to form a bundle. Apparently, raphide crystal initiation is near the centers of the elongated chambers (Figure 10) and crystal growth occurs in length and width. Crystal initiation must be an ephemeral event because well-formed crystals occur in succeeding cells of the same file of idioblasts. Crystal chamber formation, however, continues during the crystallization process, adding new chambers and crystals to the raphide bundle. Eventually, crystals fill and become appressed to the chamber membranes (Figure 11). Paracrystalline bodies are observed in the vacuole, in association with the amorphous material, throughout crystal formation (Figures 11-13).
Idioblasts continue to differentiate during crystal initiation and formation. The central vacuole increases in size (Figure 13) and the idioblast becomes slightly elongate. Plastids and mitochondria are often seen in dumbbell configurations (Figure 13) suggestive of their replication. Plastids develop a number of simple dilated thylakoids (Figure 14), assuming a morphology corresponding to descriptions of undifferentiated crystalloplastids (Arnott and Pautard, 1970; Kausch and Horner, 1983a) and will henceforth be referred to as such. Profiles of microbodies are very uncommon at this stage of idioblast development. However, when they are found, they appear identical in morphology to microbodies in cortical parenchyma cells. Microbodies in Yucca root cortex all contain crystalline inclusion bodies. Spherosomes are not observed in crystal containing idioblasts; i.e., all lipid reserves have been depleted by this stage.

Crystal idioblasts become progressively elongate, and the prominent central vacuole is filled with numerous raphide crystals (Figure 15). Positive reaction with the Yasue technique (Yasue, 1969) demonstrates that the crystals are calcium oxalate. Immature crystals are four-sided with tapered ends and in cross-section have a shape indicative of the monoclinic system of crystallization (calcium oxalate monohydrate, CaC$_2$O$_4$·H$_2$O). Crystal initiation ceases and no new crystal chambers are produced. Size of paracrystalline bodies and amount of amorphous material diminish and neither are found in older idioblasts. The average number of crystals per cell after cessation of crystal initiation is 204±37 (N=53).

At this stage of idioblast development, a number of cytological modifications begin to occur. The crystalloplastids become enlarged and a
layer, dense with plastid ribosomes, is formed at their periphery (Figure 16). One to several lobes are developed which are compartmentalized from the larger remaining portion of the plastid by invaginations of the inner plastid envelope. As lobe formation occurs, the dense peripheral layer diminishes (Figure 16, inset) and is seen as an intermediate to lobed crystalloplastids. The simple thylakoids of the larger plastid portion are tubular invaginations of the inner plastid envelope.

During differentiation of lobed crystalloplastids, the central vacuole invaginates at the opposite ends (Figure 17). A sparse flocculent substance starts to accumulate in the central vacuole. This substance has a different appearance, and is less electron-dense than the amorphous material seen in the vacuoles during crystal initiation. Small tubules, measuring 170 Å in diameter, form in association with this substance. The tubules occur in groups that produce strands or cables (Figures 18-21). The cables become appressed to crystal chamber membranes (Figure 18) and extend into the invaginated portions of the central vacuole (Figure 19). The invaginated tonoplast protrudes through the cytoplasm close to the plasma membrane (Figures 19, 20) but does not fuse with it. Similarly, the cables end abruptly, but never fused to the tonoplast (Figure 21). No attachment site for these tubules is observed. The cables wind through the invaginated tonoplast (Figure 22) which forms a network of vacuolar channels (Figure 23).

A mucilage accumulates in the central vacuole and with SEM appears as a reticular matrix (Figure 24). The crystals become encased in vacuolar mucilage (Figure 25). The cables of tubules are also surrounded with
mucilage. The crystals undergo a change in shape becoming six-sided at their ends (Figures 26, 27) and eight-sided toward their middle regions (Figure 28). The crystals at this stage are ensheathed with secondary lamellated crystal chambers (Figures 30, 31). At the onset of mucilage accumulation, crystalloplastids begin to senesce and degenerate (Figure 29) and resemble plastolysomes (Nagl, 1977). At maturity, *Yucca torreyi* raphide crystal idioblasts are living cells maintaining a peripheral cytoplasm.

Survey observations on crystal idioblast development in intact roots, still attached to germinated seeds, did not reveal any differences compared to idioblasts in isolated root cultures. In fact, we do not observe any ultrastructural differences between intact and cultured roots for any cell type.

Measurements were made on median 1-2 μm thick sections (N=42), using an ocular micrometer, to determine where various stages of crystal idioblast development occur relative to the root apex. Positions of these stages were verified also with electron microscopy. The root cap usually constitutes the apical 0.30 mm±0.07 mm of the root, and the youngest crystal idioblast initials occur 0.65±0.10 mm from the tip of the root cap. Crystal initiation is found at 0.90±0.10 mm; cessation of crystal chamber production, beginning of crystalloplastid lobe differentiation, and appearance of vacuolar tubules occur 1.4±0.12 mm from the apex. Mucilage production and crystalloplastid senescence begin at 1.8±0.20 mm from the root cap tip. Fully developed crystal idioblasts containing bundles of six- and eight-sided crystals surrounded by mucilage are ob-
served at 3.0±0.23 mm. The entire developmental sequence of crystal idioblast differentiation occurs within a 2.3-2.5 mm region of cortex near the root apex. Crystal idioblasts at 50 mm from the apex appear identical to those seen at 3.0 mm.
Figure 1. Growth curves for primary roots of *Yucca torreyi* under various culture conditions; solid squares - roots still attached to germinated seeds grown in K-phosphate buffer or White's medium, open circles - excised roots grown in White's medium, solid triangles - excised roots grown in MS medium, open squares - excised roots grown in K-phosphate buffer. Standard deviations for each point did not exceed 0.20 mm and averaged 0.18 mm for all measurements.
Figures 2-7. Crystal idioblast initials.

Figure 2. Nomarski LM view of a longitudinal file of young crystal idioblasts. Note that central vacuole development is more advanced in crystal idioblasts than surrounding parenchyma. 470X

Figure 3. Ultrastructure of idioblast initial. 9,250X

Figure 4. Eoplast in an idioblast initial. 53,430X

Figure 5. Paracrystalline body in vacuole of idioblast initial. Note the amorphous material closely associated with surface of this structure. 51,400X

Figure 6. Central vacuole formation in an idioblast initial, containing amorphous material, paracrystalline bodies, and crystal chambers. 6,200X

Figure 7. Stereo pair HVEM of paracrystalline body in central vacuole of idioblast initial.
Figure 8. Crystal chambers in vacuole of idioblast initial are associated with amorphous material, but not necessarily with paracrystalline body. Inset is high magnification micrograph of longitudinal section through crystal chamber, note tapered end and shape that approximates raphide shape. 28,200X, 63,500X

Figure 9. Cytochemically treated section for plasma membrane staining with PACP, plasma membrane stains positively, but crystal chambers (arrows) do not. 23,000X

Figure 10. Crystal chambers in vacuole are oblique rectangular in cross section, some contain crystals near their centers indicating initiation of crystal formation. 29,300X

Figure 11. Stereo pair HVEM showing a crystal that has eventually filled crystal chamber; also two crystal chambers with crystals that are closely associated with a paracrystalline body. 15,300X

Figure 12. Paracrystalline body and small raphides seen with SEM, amorphous material appears as a reticular network. 12,000X

Figure 13. Young crystal idioblast with a bundle of raphides that have formed in crystal chambers in central vacuole. Amorphous material and paracrystalline body are still apparent. 7,400X

Figure 14. Undifferentiated crystalloplastid with simple diluted thylakoids. 26,000X

Figure 15. Bundle of raphides in central vacuole of idioblast seen with SEM. 1,800X

Figure 16. Portion of idioblast after cessation of crystal initiation, showing enlarged crystalloplastids with dense peripheral regions. Inset shows beginning of lobe formation. 15,340X, 28,100X

Figure 17. Nomarski LM showing invagination of central vacuole (arrow). 670X

Figure 18. Grazing longitudinal section along a crystal showing cables of tubules adjoining the crystal chamber. 32,930X

Figure 19. Cables of tubules extending into invaginated vacuolar channel. 23,240X

Figure 20. Stereo pair HVEM micrographs showing tubules extending into invaginated vacuole, lack of any attachment site, and mature lobed crystalloplastids. 11,230X

Figure 21. Cable ending abruptly (arrow) without attachment or fusion to any structures. Tonoplast curves out of the plane of section in this micrograph. 37,340X

Figures 22, 23. HVEM micrographs of cables of tubules extending into and ending in vacuolar channel network. 13,340X, 12,600X
Figures 24-30. Maturation of crystal idioblasts, change in crystal shape.

Figure 24. Crystal idioblast at beginning of mucilage accumulation. 1,720X
Figure 25. Raphide bundle encased in mucilage. 7,300X.
Figure 26. Transition region of a crystal which becomes six-sided at top and eight-sided toward bottom. 8,130X
Figure 27. Crystal fractured in its six-sided region. 10,400X
Figure 28. Crystal fractured across its eight-sided region. 9,600X
Figure 29. Senescent crystalloplastids in maturing idioblasts. 17,000X
Figure 30. Cross section trough crystal ensheathed in lamellated material and surrounded by mucilage. Lamellations often extend from one or two sides of a crystal in their six-sided regions. 53,420X
Key to labelling:  c - crystal (or hole left in section where crystal was lost during processing), cc - crystal chamber, CI - crystal idio-blast file, Cp-crystalloplastid, CW - cell wall, Cy - cytoplasm, M - mitochondria, Mb - microbody, Mu - mucilage, N - nucleus, P - plastid, Pb - paracrystalline body, Pm - plasma membrane, T - vacuolar cable of tubules, V - vacuole.
DISCUSSION

Crystal idioblasts are consistently produced in isolated root cultures of *Yucca torreyi*. Calcium oxalate production continues unabated in isolated roots cultured for a three-month duration. This suggests that oxalate synthesis for crystal formation is not necessarily related to metabolism of seed storage reserves (starch and lipids) during germination. Furthermore, since all cultures were dark-grown, photosynthetically derived oxalate (via the glycolate pathway) obviously does not contribute to crystal formation in these cells.

Growth curve data and measurements of anatomical positions of various stages of crystal idioblast differentiation can be used to determine timing of development and the physiochemical events of crystallization. During the linear growth phase, crystal idioblasts in isolated root cultures require approximately 24-25 h to fully differentiate from idioblast initials. Cultured primary roots still attached to germinated seeds, require slightly less time for full idioblast development, approximately 21-23 h, and do not experience a lag period in their growth curve. Crystal chamber production and crystal initiation occur within 2-4 h, but crystal growth continues until the idioblast is fully differentiated.

Crystal idioblast development in *Yucca* root cultures is normal relative to intact roots. Our results generally concur with observations on *Yucca* crystal idioblast development in intact roots (Arnott, 1966; Eilert, 1974), except for certain matters concerning interpretation. In fact, many aspects of *Yucca* crystal idioblast development are also common to the
differentiation of similar cells observed in intact organs of other plants.

Crystal idioblast initials in a number of plants, including *Yucca*, are reported to differentiate earlier than surrounding meristematic cells (Arnott and Pautard, 1970; Eilert, 1974; Kausch and Horner, 1981, 1983a,b). We observed that early idioblast differentiation in *Yucca* root cultures is characterized by depletion of storage reserves (lipid and starch), increase in cytoplasmic density and ER profiles, and formation of a central vacuole which contains paracrystalline bodies and amorphous material.

*Yucca* crystal idioblast nuclei do not become enlarged or structurally modified in comparison to adjacent parenchyma cells. Nuclear enlargement has been often cited as the first cytological modification associated with raphide idioblast initiation (Franceschi and Horner, 1980a). The occurrence of a nuclear endo-cycle is highly suspect in raphide idioblast differentiation (Schlichtinger, 1956; Horner and Whitmoyer, 1972; Kausch, 1980; Kausch and Horner, 1983b) and may represent an important developmental feature of this cell type. The absence of nuclear enlargement in *Yucca* idioblasts does not, however, preclude that these cells are involved in an endo-cycle. The nuclear condition of *Yucca* raphide idioblasts, and crystal idioblasts generally, deserves further investigation.

Initiation of crystal production in *Yucca* is preceded by development of a central vacuole and vacuolar structures involved in crystal formation. Small vacuoles, which contain paracrystalline bodies and amorphous material, coalesce and contribute their contents to a larger vacuole. Vacuolar paracrystalline bodies have been observed in *Psychotria punctata*
raphide idioblasts (Horner, unpublished, Iowa State University) and in Capsicum annuum druse idioblasts (Horner and Wagner, 1980; Horner et al., 1981) but their involvement in crystal production is not clear. In these two plants, membrane complexes form in association with the paracrystalline bodies, which are thought to be precursors of crystal chambers. The paracrystalline bodies in Capsicum are termed nucleation sites (Horner and Wagner, 1980) and are considered to act as central nuclei or "seeds" for calcium oxalate crystallization. Vanilla planifolia crystal chambers are formed in association with electron-dense amorphous material (Kausch and Horner, 1983b) but the vacuoles do not contain paracrystalline bodies at any time in idioblast development.

In vacuoles of Yucca raphide idioblast initials we find amorphous material associated with crystal chambers and paracrystalline bodies. Crystal chamber formation does not necessarily occur in proximity to paracrystalline bodies. Both paracrystalline bodies and amorphous material diminish as formation of new crystal chambers and crystal initiation cease, and are not present in later stages of development. The paracrystalline bodies may represent a crystallized storage form (probably proteinaceous) of the amorphous material, and perhaps it is this material which contributes to or influences crystal chamber formation. This material may also be involved in transport of crystal precursors to the crystal chambers.

Nucleation or initiation of calcium oxalate crystal formation has been examined in experimental animals (Kim and Johnson, 1981) but is not understood in plant cells. Our investigation shows that crystallization
occurs in membranous crystal chambers. These chambers are isolated structures in the vacuole proper and not transvacuolar cytoplasmic strands or invaginations of the plasma membrane. Their formation appears to occur de novo. Crystal chambers are not PACP positive, substantiating that they are not of plasma membrane origin.

Calcium oxalate crystal shape is considered to be genetically determined (Kausch and Horner, 1982); however, the physiochemical control of crystallization is not understood. Arnott and Pautard (1970) suggested that a crystal chamber dictates crystal shape, as a mould, independent of crystal hydration form. It is interesting that prior to crystallization, the crystal chambers in Yucca assume a shape that closely approximates the four-sided raphides which will form within them. It is difficult to understand how a biological membrane could function as a mould and influence physical parameters of crystallization. Bruni et al. (1982) have shown the presence of proteins in crystal chambers of Musa with fluorescence microscopy, thus affirming their membranous characteristics. It seems likely that if crystal chambers function as biological membranes, transport rates of calcium and oxalate could be regulated. Calcium oxalate crystal shape is kinetically controlled and depends upon the rate of mixing of calcium and oxalate (Tomazic and Nancollas, 1980; Cody et al., 1982). Therefore, regulated import of crystal constituents into crystal chambers would influence crystal shape and compartmentalize crystallization. This would also account for genetic determination of crystal shape in plants. However, the specific assumed shape of crystal chambers prior to crystallization remains enigmatic.
Crystalloplastids have been found in developing and mature crystal idioblasts in a number of plants (Mollenhauer and Larson, 1966; Arnott, 1966, 1973; Eilert, 1974; Kausch and Horner, 1983a,b) and are distinguished from plastids in surrounding cells by their morphology and absence of starch inclusions. Kausch and Horner (1983a) examined the origin and development of crystalloplastids in *Typha* and suggested that they may represent multifunctional organelles significant to crystal idioblast functioning. We have observed that *Yucca* crystalloplastids develop from eoplasts, assuming a distinctive appearance compared to plastids in surrounding parenchyma cells, prior to and during crystal production. After cessation of crystal initiation, but before mucilage accumulation, crystalloplastids enlarge and differentiate lobed regions. This pattern of crystalloplastid development has also been observed in *Typha* and *Vanilla* (Kausch and Horner, 1983a,b). Only mucilaginous crystal idioblasts have been reported to contain lobed crystalloplastids (Kausch and Horner, 1983a,b). *Psychotria punctata* raphide idioblasts do not produce mucilage and do not differentiate lobed crystalloplastids (Horner and Whitmoyer, 1972). Crystalloplastids in mucilaginous crystal idioblasts, including *Yucca*, may be involved in synthesis of mucilage precursors. The lobe may be a specialized region for this function.

Vacuolar cables of tubules, which become associated with raphide bundles, were examined with light microscopy in mature crystal idioblasts of a number of monocotyledonous species by Kohl in 1899. Vacuolar tubules were observed with electron microscopy in *Psychotria* raphide idioblasts (Horner and Whitmoyer, 1972) but these did not form cables and measured
100-130 Å in diameter. Cables of tubules were recently observed with TEM in Vanilla raphide idioblasts (Kausch and Horner, 1983b). The tubules in Yucca are larger (170 Å) than those found in Psychotria, but smaller than cytoplasmic microtubules (250 Å). The vacuolar tubules of Yucca and Vanilla are different from any previously reported, but resemble P-protein polymer tubules of sieve elements (Arsanto, 1982). We report that in Yucca, the cables form de novo in the vacuole only after raphide bundle production, and hence do not believe that they function in crystal initiation or growth. Perhaps, these cables anchor and maintain raphide bundle orientation by extending into a ramified vacuolar channel complex. Their formation and cellular role is still unclear, and could provide insight into crystal idioblast functioning.

The production and accumulation of vacuolar mucilage has been observed to be coincident with formation of lamellated crystal sheaths (Eilert, 1974; Wattendorff, 1976a,b, 1979; Tilton and Horner, 1980; Kausch and Horner, 1983a,b) and a change in raphide shape (Horner et al., 1981). The lamellated sheaths in Yucca are identical to those previously reported in other plants. Yucca crystals change shape coincident with mucilage accumulation and lamellated sheath addition, becoming six- and eight-sided. Change in crystal shape can occur by poisoning of existing crystal faces with contaminating atoms (Cody et al., 1982). Components of the mucilage or lamellated sheaths may be responsible for such a poisoning event. The lamellated sheaths may also be involved in stabilization of the calcium oxalate crystals. Neither change in crystal shape nor lamellated sheaths occurs in Psychotria punctata idioblasts that do not produce
mucilage (Horner and Whitmoyer, 1972) implying an interrelationship between these events.

The present study shows that isolated root cultures produce calcium oxalate raphide crystal idioblasts which develop normally in comparison to those in intact roots. We believe that such cultures will be useful as an experimental system for physiological investigations of crystal idioblast metabolism and differentiation.
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Figures 31, 32. Crystal chamber cytochemistry.

Figure 31. Crystal chambers stained with lead and uranyl salts. 26,100X

Figure 32. Crystal chambers (arrows) are PACP negative, whereas the plasma membranes stain positive for PACP marker. 46,000X

"Out of all of this awareness, we must select. What we select and call consciencceness, is never the same as the awareness because the process of selection mutates it. From the endless landscape of awareness we take a handful of sand, and call that handful the world."

Robert Pirsig
Figure 33. Vacuolar cables of tubules extending into the ramified vacuolar channel network. Note where a cable pokes into and apparently ends (arrows) a vacuolar channel that traverses perpendicular to the plane of section. 43,200X

"When brought to meaning, all importance becomes small. As in death, all life seems nothing. Knowing is destroyed by thinking, not destroyed but sterilized. Thinking is the processing of knowing into knowledge."

William Wharton

Wingtip

The birds - are they worth remembering? Is flight a wonder and one wingtip a space marvel? When will man know what birds know?

Carl Sandburg

A cage went in search of a bird.

Franz Kafka
Figure 34. Vacuolar calbes of tubules. 39,340X

Figure 35. High magnification of tubular substructure. 104,320X

[As I] sit quietly, doing nothing
Spring comes and grass grows of itself.

Ch'an Ling Chii Chi

The sound and movement of water is what I know.

Chuang-tzu
Figure 36. Plastids filled with starch grains in a root cup cell. These plastid forms are contrasted to crystalloplastids and eoplasts in the cortex. 31,300X

Let the waters of your soul flow - from joy or from pain - clear, crystalline human liquid, like a spring mornings rain. Cleanse yourself by taking the risk - open your heart and move one step closer to a true emotional bliss.

John Klemmer
PART IV: USE OF THE CERIUM CHLORIDE TECHNIQUE AND ENERGY DISPERSIVE X-RAY MICROANALYSIS IN PLANT PEROXISOME IDENTIFICATION WITH TEM AND SEM
Use of the cerium chloride technique and energy dispersive x-ray micro-analysis in plant peroxisome identification with TEM and SEM

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Key words: cytochemistry; glycolate oxidase; microbody inclusions; peroxisomes; X-ray microanalysis
SUMMARY

Glycolate oxidase activity is demonstrated cytochemically with the CeCl₃ technique in leaf peroxisomes of Nicotiana tabacum, Glycine max, Psychotria punctata and in unspecialized peroxisomes of Yucca torreyi roots. Reaction product deposition occurs throughout peroxisomal matrices, whereas nucleoid inclusion bodies, occurring in all four species, are cytochemically unreactive. We observed reactive and nonreactive microbodies within individual cells in these four plants despite prolonged incubation times and increased CeCl₃ concentration. These results may reflect differences in glycolate oxidase content or peroxisomal differentiation within individual cells of a given tissue. We demonstrate substrate-independent cerium deposits in cell walls, cytoplasmic hoop-shaped structures and chloroplast thylakoids at extended incubation times or increased CeCl₃ concentration, perhaps indicating the presence of endogenous H₂O₂.

Elemental spectral analysis of electron-dense deposits with energy dispersive X-ray microanalysis using a STEM detects five peaks generated from the L-series of cerium. SEM/TEM X-ray mapping for the L₃ peak of cerium verifies peroxisomal localization of reaction product in thin sections. X-ray mapping for cerium on cytochemically reacted, bulk cryo-fractured specimens identifies some spherical bodies as peroxisomes. X-ray localization of cerium is achieved above background when beam penetration is minimized using accelerating voltages of 15-19 kV and the spherical bodies are oriented towards the detector.
INTRODUCTION

Various flavin oxidases, compartmentalized in peroxisomes, catalyze the transfer of substrate hydrogen to molecular oxygen and thereby generate hydrogen peroxide (Tolbert and Essner, 1981). In most cases, hydrogen peroxide subsequently is degraded in a catalase-mediated reaction. Cytochemical techniques for localization of peroxisomal enzymes are useful in developmental and ultrastructural studies of these organelles, providing information often inaccessible by biochemical methods (Beevers, 1979; Tolbert and Essner, 1981).

The majority of electron microscopic cytochemical studies of microbodies have relied upon procedures for detection of catalase activity. Until recently, the ferricyanide method (Shnitka and Talibi, 1971; Burke and Trelease, 1975; Hand, 1975, 1976) has been the only procedure for localization of peroxisomal flavin oxidase activity. However, problems with reaction product diffusion and considerable nonspecific deposits limit the usefulness of this technique.

Consequently, Briggs et al. (1975) introduced a method employing cerium chloride (CeCl₃) to localize an NADH oxidase in mammalian polymorphonuclear leukocytes. Cerium acts as a trapping agent for enzyme-generated peroxide, precipitating cerium perhydroxide as a high-resolution, well-localized, electron-dense reaction product with minimal nonspecific deposition. Veenhuis et al. (1976a) modified the procedure to demonstrate peroxisomal localization of α-hydroxy acid oxidase, D-amino acid oxidase, and methanol oxidase in Hansenula polymorpha yeast cells. The CeCl₃ technique was used subsequently to detect D-amino acid oxidase
activity in peroxisomes of telost kidney (Veenhuis and Wendelaar Bonga, 1977) and, later, for several different oxidases in rat liver, kidney, and glial cells of the central nervous system (Arnold et al., 1977, 1979; Veenhuis and Wendelaar Bonga, 1979; Arnold and Holtzman, 1980). Localization of glycolate oxidase activity with cerium recently was demonstrated in peroxisomes, glyoxysomes and unspecialized peroxisomes in higher plants (Thomas and Trelease, 1981). Our report represents the second investigation to utilize the cerium procedure for localization of plant glycolate oxidase activity.

Investigations concerning microbody distribution would be facilitated greatly by selective localization of these organelles in bulk specimens where several micrometers of cell depth could be analyzed without resorting to serial sections for TEM (transmission electron microscopy). In this report, we verify peroxisomal enzyme-generated reaction product as cerium in thin sections viewed with TEM and analyzed with XRMA (X-ray microanalysis) using both STEM (scanning, transmission electron microscopy) and SEM/TED (scanning electron microscope with transmitted electron detector). The purpose of this study is to evaluate an extension of the cerium technique, using glycolate as a substrate, for visualization of microbodies in cyrofractured bulk specimens with SEM and XRMA. In an attempt to optimize conditions for reaction product deposition and X-ray mapping, various modifications in the procedure utilized by Thomas and Trelease (1981) were made. The cytochemical effects of these modifications are examined also.
MATERIALS AND METHODS

Mature, fully-expanded leaves of *Nicotiana tabacum* L. and *Glycine max* L., and young, developing leaves of *Psychotria punctata* Vatke were obtained from plants grown in greenhouses at Iowa State University, Ames, Iowa. Roots from seeds of *Yucca torreyi* L. were used also. Dried seeds were imbibed overnight in running tap water, surface sterilized 17 min in 10% sodium hypochlorite, and grown aseptically for 7-9 days at 27 C on wet filter paper in glass Petri dishes in darkness, until each primary root was about 3 cm long.

Leaves were placed in formaldehyde-glutaraldehyde (4%-1%, v/v) buffered with 20 mM K-phosphate (pH 6.9) at 4 C and leaf discs were made with a 0.5 mm (I.D.) needle punch. Formaldehyde was prepared fresh from paraformaldehyde immediately before use. Primary roots of *Y. torreyi* were immersed in the same fixative and the terminal 2 mm of each root were excised and then cut longitudinally with a razor blade. Aldehyde fixation proceeded for 5-35 min, starting when plant materials first were exposed to fixative, and followed by three 5 min rinses in 50 mM K-phosphate buffer (pH 6.9) at 21 C, and one 20-min wash in 100 mM Tris-maleate (pH 7.5).

The plant materials were then preincubated 1, 2, 3, or 4 h in 100 mM Tris-maleate (pH 7.5) containing 50 mM of 3-amino-1, 2, 4 triazole (a potent catalase inhibitor) and 2.5, 5.0, 7.5 or 10.0 mM CeCl₃. Some samples were placed in this mixture which also contained 10% dimethoxy-sulfoxide (DMSO). All solutions were made with double distilled water,
boiled to remove CO\textsubscript{2}. The pH of Tris-maleate buffer was adjusted with fresh 5N NaOH. Furthermore, all solutions containing CeCl\textsubscript{3} were aerated thoroughly with CO\textsubscript{2}-free air, and filtered.

Tissues were then transferred into fresh reaction medium for durations of 1, 2, 3, 4, 9, 10, 11, 17, 18, 19 or 36 h at 21 C. Hourly changes into fresh medium were made for incubation times less than 4 h. The 9-19 h incubations were changed once; 36 h incubations twice. The reaction solution was prepared from preincubation medium and contained 50 mM glycolic acid.

This procedure essentially is that described by Thomas and Trelease (1981) except for these variations: extension of preincubation and incubation times; variation of CeCl\textsubscript{3} concentrations, and addition of DMSO to the preincubation medium. Control preparations were made by preincubation at 70 C for 4 h; omission of CeCl\textsubscript{3}; omission of glycolate in reaction mixture; or aldehyde fixation in excess of 18 h.

Following incubation, specimens were rinsed three times, for 15 min each, in 100 mM sodium cacodylate (pH 6.0) and then for 15 min in the same buffer at pH 7.2. Tissues were then post-fixed in 1% OsO\textsubscript{4} (same buffer, pH 7.2) for 1 h, rinsed with distilled water, then subdivided for separate TEM and SEM processing.

The TEM specimens were dehydrated in a graded ethanol-propylene oxide series, embedded in Medcast resin and thin sectioned with a diamond knife. Sections were collected on uncoated or Formvar-coated copper grids. The majority of sections were viewed without post-staining, but some were also stained with uranyl acetate and lead citrate.
The SEM specimens were dehydrated in a graded ethanol series to 100% ethanol and then quick frozen in liquid nitrogen. Leaf discs and roots were then fractured (Humphreys et al., 1974), thawed in 100% ethanol, placed in Freon 113 (TF), and critical point dried with liquid CO$_2$. All specimens were mounted on brass discs with silver cement and sputter coated with 150 Å Ag-Pd in a Polaron E5100 unit.

Conventional TEM was conducted on a Hitachi HU-11C TEM operated at accelerating voltages of 50 or 75 kV. Energy dispersive X-ray microanalysis of thin sections was carried out on both a JEOL 100 CX STEM with Kevex X-ray detector and Ortec multichannel analyzer, and on a JEOL JSM-35 SEM operated at 10-39 kV, and equipped with TED and Kevex X-ray detector and multichannel analyzer.
RESULTS

The metabolic functions of glyoxysomes in seed lipid-storage cells and leaf-type peroxisomes in photosynthetic cells have been relatively well established (Beevers, 1979); whereas, the function of microbodies in other plant tissues has not been specifically determined. These microbodies are named "unspecialized peroxisomes" to specify their peroxisomal enzyme content and undetermined metabolic function (Huang and Beevers, 1971; Beevers, 1979). All microbodies were observed in Nicotiana, Glycine, and Psychotria specimens correspond in size (Table 1) and description to leaf-type peroxisomes (Beevers, 1979) whereas those in Yucca roots could be classified as unspecialized peroxisomes (Beevers, 1979) and henceforth are referred to this way. Reaction product deposition clearly distinguishes leaf peroxisomes, exemplified in young leaves of Psychotria (Figure 1), as well as unspecialized peroxisomes, exemplified in roots of Yucca (Figure 2), from other organelles.

Reaction penetrability is not complete through entire tissue samples of Psychotria and Yucca, but limited to a layer, usually 10-15 cells thick, inward from the cut edge. Penetration in these tissues was not affected by duration of aldehyde fixation (up to 35 min), preincubation, or reaction medium incubation, CeCl₃ concentration, or DMSO treatment. Therefore, all observations on these two species are limited to the peripheral cell layers near cut edges of tissues. Reaction penetration is observed repeatedly to be complete throughout Nicotiana and Glycine leaf disc samples.
Nucleoid inclusion bodies occur in peroxisomes of all four species examined, and reaction product deposition occurs throughout the peroxisomal matrices (Table 1, Figures 3-7). Leaf peroxisomes of *Nicotiana* contain crystalline nucleoids which appear as less intensely stained areas within the matrix (Figure 3). The periodicity of the crystal lattice is difficult to discern because of the relatively small units of crystal structure. Staining within the crystal in Figure 3 is more intense at its periphery than towards its center. Unspecialized peroxisomes in developing cortical root cells of *Yucca* also contain crystalline nucleoid inclusions (Figures 4 and 5). However, interstices of these crystals are substantially wider than the structural units of the crystal rendering the inclusion as a uniform, negatively stained structure within the peroxisomal matrix. The matrix is contiguous with the interstitial crystal spaces. Reaction product is present in interstices between structural units of the crystals and hence the crystal lattice is not obliterated by reaction product. Leaf peroxisomes of *Psychotria* and *Glycine* both contain amorphous nucleoids (Table 1, Figures 6-8) readily observed as less intense, slightly stained regions in contrast to the more reactive peroxisomal matrix. The amorphous nucleoids of *Glycine* appear as dense regions in minus CeCl₃ control sections that have been post-stained with uranyl acetate and lead citrate (Figure 8).

Variations in staining characteristics among peroxisomes within individual cells are apparent in all four species examined (Figures 9-12). Figure 9 shows a portion of a *Glycine* cell with a peroxisome that does not contain any reaction product adjacent to a stained peroxisome and, in
proximity, is a very densely stained peroxisome. This variation is observed in cells well within the penetration range of the reaction, and it is apparent in samples subjected to all extended preincubation and/or incubation times, increased CeCl₃ concentrations, and DMSO treatment.

Leaf-type peroxisomes stain at all examined incubation times and CeCl₃ concentrations. However, 1-4 h incubation in reaction medium containing 5 mM CeCl₃ achieves optimum results for leaf peroxisomes. Extended incubation times and/or increased CeCl₃ concentrations sometimes result in large amounts of deposition which make the material difficult to section (Figure 13). These latter conditions also result in slight deposition of reaction product in peroxisomes in minus glycolate control samples (Figure 14).

Optimum conditions for leaf peroxisomes result in relatively few stained unspecialized peroxisomes in Yucca roots, and these occur in disrupted cells at the cut surface. Unspecialized peroxisomes stain optimally at 9-19 h incubation in 5 mM CeCl₃ reaction medium (Figures 2, 4, 5, 12). Incubation longer than 9 h sometimes results in appearance of globular material (Figures 2, 3, 12, 19) which often is membrane associated. Diffusion of reaction product away from any peroxisomes does not occur regardless of conditions.

Electron-dense deposits are not limited to peroxisomes when incubation times and/or CeCl₃ reaction medium concentrations are increased from these optima. As judged by control samples, there are three specific sites of substrate independent electron-dense accumulations. At incubation times exceeding 4 h, deposition is apparent within thylakoid spaces
of all chloroplasts (Figures 1, 9-11, 15, 16). Deposits are strictly confined to thylakoid spaces and do not accumulate in the plastid stroma (Figures 15 and 16). Increased concentrations of CeCl₃ (7.5 and 10 mM) have no effect on thylakoid staining apart from extended incubation times. Deposition is not visible in any portion of amyloplasts in Yucca root cortical cells regardless of incubation time or CeCl₃ concentration (Figure 2). Electron-dense deposits also accumulate under these same conditions along the plasma membrane and within the cell wall (Figures 17 and 19). Lastly, structures which are hoop-shaped in section (Figure 17) and clearly are membrane-bound (Figure 18) also accumulate electron-dense deposits. These hoop-structures occur only in Psychotria mesophyll cells but there is no apparent association between them and any other organelles.

All electron-dense deposits in thin sections were analyzed in a STEM with energy dispersive X-ray microanalysis at an accelerating voltage of 40 kV. Elemental spectral analysis of a reacted peroxisome of Psychotria clearly shows detection of five peaks generated from the L-series of cerium (Figure 20). The two strongest signals are generated from Lα₁ and Lβ₁. Similar analyses verify that the reaction product is in peroxisomes of the other three species, as well as in chloroplast thylakoids, cell walls, and hoop-structures.

Spectral analysis was also conducted on the same and similar thin sections with SEM/TED and XRMA (Figure 21). The spectrum is less complex, showing only three of the five L-series peaks of cerium and fewer detected elements (Figure 21). Signal/noise ratio is maximized at accelerating voltages between 20 and 25 kV. The Lα₁ and Lβ₁ cerium peaks are resolved
clearly whereas the third L cerium "peak" probably represents a combination of the remaining four L-series peaks shouldered together (Figure 22). Figures 23 and 24 compare conventional TEM and SEM/TED images (respectively) of the same cell. Relatively low resolution of the X-ray mapping system and low cerium signal counts prevent adequate cerium mapping at these magnifications even though deposits are clearly visible. Two peroxisomes from this same cell were photographed at higher magnification (Figures 25, 26) and X-ray mapping for the \( L_{\alpha 1} \) peak verifies the peroxisomal localization of the cerium reaction product (Figure 27). Still, low cerium signal counts account for relatively low resolution X-ray mapping despite tilt angles between 30° and 45°. When \( L_{\alpha 1} \) and \( L_{\beta 1} \) peaks are used together for X-ray mapping purposes, background becomes excessive, precluding localization. Concentration of cerium deposits outside of microbodies are too small to be X-ray mapped.

Elemental spectral analysis with SEM on cryofractured, critical point dried, bulk specimens shows an elemental array different than that from resin-embedded, thin-sectioned material (Figure 28). Optimum signal/noise ratio is achieved at accelerating voltages between 15 and 19 kV.

In comparison to the X-ray spectrum from sectioned material, this spectrum notably shows an elevated background (Figure 28) and resolution of the third cerium "peak" consequently is lost (Figure 29). However, the \( L_{\alpha 1} \) and \( L_{\beta 1} \) peaks are stronger due to the presence of more cerium and beam penetration. The \( L_{\alpha 1} \) peak is limited for use in X-ray mapping (Figure 30). Figure 31 shows a spherical structure protruding into the central vacuole of a Glycine leaf mesophyll cell. Higher magnification and X-ray mapping,
using the L_{\text{u1}} cerium peak, clearly show cerium localization (Figures 32, 33). However, such cerium X-ray localizations are accomplished only infrequently and with significant difficulty. Localization can be achieved above background only when beam penetration is minimized using accelerating voltages of 15-19 kV and when the spherical body in question is oriented properly toward the X-ray detector. Various tilt angles also are used to minimize background noise from beam penetration. Thin-sectioned material viewed with TEM shows that peroxisomes occasionally protrude into the central vacuole (Figure 34). This may represent an artifact of inadequate fixation because the tonoplast of this cell is disrupted.

All successful X-ray maps of cerium-stained microbodies are achieved on leaf-type peroxisomes incubated for 9-18 h in 5 mM CeCl₃ reaction medium. This period of incubation achieves an apparent overloading of peroxisomes with cerium reaction product as indicated in sectioned material (Figures 13, 23-28, 34). SEM images of spherical bodies and X-ray mapping localization for cerium in *Psychotria* and *Nicotiana* verifies them as peroxisomes (Figures 35-41). Use of high magnification in the SEM is necessary for adequate mapping (Figures 27, 33, 38, 41). The unspecialized peroxisomes in *Yucca* root cells proved too small (Table 1) to distinguish above background signals in XRMA.
# Table 1. Summary of data about peroxisomes from four taxa

<table>
<thead>
<tr>
<th>Genus/species</th>
<th>Plant source</th>
<th>Peroxisomal type</th>
<th>Peroxisomal size(^a)</th>
<th>Nucleoid inclusion body crystalline/amorphous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana tabacum</em> L.</td>
<td>mature leaves</td>
<td>leaf</td>
<td>0.90 μm±0.06</td>
<td>present; C</td>
</tr>
<tr>
<td><em>Glycine max</em> L.</td>
<td>mature leaves</td>
<td>leaf</td>
<td>1.15 μm±0.11</td>
<td>present; A</td>
</tr>
<tr>
<td><em>Psychotria punctata</em> Vatke</td>
<td>young leaves</td>
<td>leaf</td>
<td>0.55 μm±0.06</td>
<td>present; A</td>
</tr>
<tr>
<td><em>Yucca torreyi</em> L.</td>
<td>primary roots</td>
<td>unspecialized</td>
<td>0.35 μm±0.90</td>
<td>present; C</td>
</tr>
</tbody>
</table>

\(^a\)Represents mean diameter of largest peroxisomes (n=25) and standard error.
Figure 1. Portion of young Psychotria punctata leaf mesophyll cell. Reaction product clearly distinguishes peroxisomes from other organelles; incubation was 9 h in 5 mM CeCl₃. Bar = 1.0 μm.

Figure 2. Portion of Yucca torreyi root cortical cell; incubation was 18 h in 5 mM CeCl₃. Bar = 0.5 μm.
Figure 3. *Nicotiana* leaf peroxisome with crystalline nucleoid; incubation was 2 h in 5 mM CeCl$_3$. Crystal interstices stain more intensely at periphery. Bar = 0.5 μm.

Figure 4. *Yucca* peroxisome with crystalline nucleoid; incubation was 17 h in 5 mM CeCl$_3$. Bar = 0.1 μm.

Figure 5. *Yucca* peroxisome in different plane of section than Figure 4; incubation was 19 h in 5 mM CeCl$_3$. Reaction product deposition is relatively even within crystal lattice; resultant negatively stained crystal has wider interstices than structural units. Bar = 0.1 μm.

Figure 6. *Psychotria* leaf peroxisome with amorphous nucleoid; incubation was 4 h in 5 mM CeCl$_3$. Bar = 0.25 μm.

Figure 7. *Glycine* leaf peroxisome with amorphous nucleoid, incubation was 4 h in 5 mM CeCl$_3$. Slight reaction product deposition occurs within nucleoid. Bar = 0.25 μm.

Figure 8. *Glycine* control specimen, minus glycolate substrate, post-staining with uranyl acetate and lead citrate shows nucleoid appearing as dense inclusion with conventional TEM. Bar = 0.5 μm.

Figure 9. Portion of *Glycine* leaf mesophyll cell with a nonreactive peroxisome (arrow) adjacent to a peroxisome containing reaction product and near a very densely stained peroxisome; incubation was 36 h in 5 mM CeCl$_3$. Bar = 1.0 μm.

Figure 10. Portion of *Nicotiana* leaf mesophyll cell with variably stained peroxisomes; incubation was 19 h in 7.5 mM CeCl$_3$. Bar = 1.0 μm.

Figure 11. Portion of *Psychotria* leaf mesophyll cell with variably stained peroxisomes. Dark spot in less intensely stained peroxisome is not reaction product. Incubation was 19 h in 5 mM CeCl$_3$. Bar = 1.0 μm.

Figure 12. Portion of *Yucca* root cortical cell with variably stained peroxisome; incubation was 36 h in 7.5 mM CeCl$_3$. Note accumulation of globular material along plasmalemma. Bar = 0.5 μm.
Figure 13. Excess deposition of reaction product in *Glycine* resulting in over-stained peroxisomes. Clear areas are where reaction product chipped during sectioning; incubation was 19 h in 10 mM CeCl₃. Bar = 1.0 μm.

Figure 14. Slight deposition of reaction product in *Psychotria* peroxisome in minus-glycolate substrate control sample, indicating detection of H₂O₂ only in presence of endogenous glycolate; incubation was 19 h in 7.5 mM CeCl₃. Bar = 0.5 μm.

Figure 15. Cerium deposition in *Psychotria* chloroplast thylakoids, in minus-glycolate substrate control sample, is confined to thylakoid spaces; incubation was 19 h in 7.5 mM CeCl₃. Bar = 0.5 μm.

Figure 16. High magnification of *Nicotiana* thylakoid sacs containing cerium deposition; incubation was 11 h in 5 mM CeCl₃. Bar = 0.1 μm.

Figure 17. *Psychotria* leaf mesophyll cell hoop structures containing cerium deposits; the central stained structure may be a hoop perpendicular to plane of section. Note also fine cerium precipitation along cell wall in lower left; incubation was 19 h in 7.5 mM CeCl₃. Bar = 0.5 μm.

Figure 18. High magnification of hoop structure shows delimiting membrane (arrow); incubation was 17 h in 5 mM CeCl₃. Bar = 0.1 μm.

Figure 19. Precipitation of cerium along plasma membrane and cell wall in *Yucca* root cortex; incubation was 36 h in 7.5 mM CeCl₃. Bar = 0.5 μm.
Figure 20. Elemental X-ray spectral analysis of reacted Psychotria peroxisome in a resin embedded thin section, mounted on a copper grid. Peaks 1-6: $L\alpha$ and $L\beta$-Cu, $K\alpha$ and $K\beta$-Al, $K\beta$ and $K\gamma$-Si, M-Os, $L\alpha$ and $L\beta$-Mo; and $K\alpha$ and $K\beta$-Cr; peaks 7-11: $L\alpha_1$-Ce, $K\beta_1$-Ce, $L\beta_2$-Ce, $L\gamma_1$-Ce, $L\gamma_2,3,4$-Ce; peaks 12 and 13: $K\alpha$-Cu, $K\beta$-Cu (respectively). Analysis was at 40 kV with an STEM.
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[Graph with labels indicating settings and data points]

PS: OFF  CPS: 1902  QT: 16  FS: 4k  LINEAR  ORTEC

V: 9.075
Figure 21. Elemental X-ray spectral analysis of resin embedded thin section of reacted Glycine peroxisome; analyzed at 20 kV with SEM/TED. Elements with low emission energies, seen in Figure 20, are not detected; copper, from the grid, and three L-cerium peaks still are clearly evident.

Figure 22. Enlarged X-ray spectrum shows third cerium peak (arrow) is only slightly above background and is probably a combination of higher emission energy L-cerium signals. The Kα–Cu peak is at extreme right.

Figure 23. Conventional TEM of a thin section through Glycine leaf mesophyll cells containing over-stained peroxisomes. Bar = 2.5 μm.

Figure 24. SEM/TED image of same cells as in Figure 23. Resolution is slightly diminished but cerium deposits still are discerned easily at this magnification. Bar = 2.5 μm.

Figure 25. High magnification conventional TEM of the same two peroxisomes seen in center cell in Figures 23 and 24. Bar = 0.5 μm.

Figure 26. SEM/TED image of identical peroxisomes seen in Figure 25. Bar = 0.5 μm.

Figure 27. SEM/TED X-ray map for the Lα1 cerium peak showing peroxisomal localization of reaction product. Bar = 0.5 μm.
Figure 28. Elemental X-ray spectral analysis of cryofractioned bulk 
Glycine leaf specimen, analyzed at 20 kV with SEM.

Figure 29. Enlarged X-ray spectrum shows \( \text{L}_{\alpha 1} \) and \( \text{L}_{\beta 1} \) cerium peaks are 
easily discerned (arrows), whereas remaining L-series peaks are lost in background noise.

Figure 30. Limitation of \( \text{L}_{\alpha 1} \) cerium peak for X-ray mapping.

Figure 31. Secondary electron image of spherical structure protruding 
into the vacuole of a Glycine leaf mesophyll cell. Bar = 5.0 \( \mu \text{m} \).

Figure 32. Higher magnification of structure in Figure 31. Bar = 1.0 \( \mu \text{m} \).

Figure 33. X-ray map for \( \text{L}_{\alpha 1} \)-cerium signal shows that structure shown in 
Figures 31 and 32 contains significant amounts of cerium. Bar = 1.0 \( \mu \text{m} \).

Figure 34. Section through reacted Glycine leaf mesophyll cell, showing 
reacted peroxisomes protruding into central vacuole. Note 
discontinuities in tonoplast (arrows). Bar = 1.0 \( \mu \text{m} \).
Figure 35. Secondary electron image of *Psychotria* leaf mesophyll cell; arrow indicates suspected peroxisome. A number of other similar structures occur in the cell. Bar = 5.0 μm.

Figure 36. Higher magnification of Figure 35 showing spherical structure in association with chloroplast; X-ray localization for cerium could still not be achieved above background at this magnification. Bar = 1.0 μm.

Figure 37. High magnification SEM of same structure shown in Figures 35 and 36. Specimen was rotated and tilted 17° towards detector. Bar = 0.5 μm.

Figure 38. X-ray map for $L_{3\alpha}$-cerium signal shows cerium localization. Bar = 0.5 μm.

Figure 39. Secondary electron image of two spherical bodies protruding into central vacuole of *Nicotiana* leaf mesophyll cell. Bar = 2.5 μm.

Figure 40. Higher magnification of structures seen in Figure 39. Bar = 1.0 μm.

Figure 41. X-ray map for $L_{3\alpha}$-cerium signal showing cerium localization within the two structures seen in Figures 39 and 40. Bar = 1.0 μm.

Key to labelling: C - chloroplast; Cm - chloroplast envelope; CW - cell wall; ER - endoplasmic reticulum; M - mitochondria; N - nucleus; P - amyloplast; S - starch grain; V - vacuole
Cytochemical localization of glycolate oxidase activity in leaf-type peroxisomes and unspecialized peroxisomes is demonstrated with the \( \text{CeCl}_3 \) technique in TEM and SEM using XRMA. Reaction product in these peroxisomes is similar in appearance to \( \text{CeCl}_3 \) reacted peroxisomes of methanol-grown \textit{Hansenula polymorpha} (Veenhuis et al., 1978, 1979) and peroxisomes, glyoxysomes and unspecialized peroxisomes in various higher plant species (Thomas and Trelease, 1981).

Thomas and Trelease (1981) do not mention reaction penetration difficulties associated with this procedure, but we find that penetrability of the reaction is not complete in samples of young developing tissues, such as those from \textit{Psychotria} and \textit{Yucca}. Furthermore, reaction penetration in these tissues is not affected by length of aldehyde fixation, preincubation or incubation, \( \text{CeCl}_3 \) concentration, or DMSO treatment. Reaction product, however, is observed in peroxisomes throughout mature leaf samples of \textit{Nicotiana} and \textit{Glycine}. Penetrability of the reaction solutions may be limited in immature plant tissue by minimal intercellular spaces.

Reaction product deposition due to glycolate oxidase activity occurs throughout the matrices of peroxisomes in the four species examined. Nucleoid inclusions occurring within the matrices are similar to those observed in previous investigations (Veenhuis et al., 1978, 1979; Thomas and Trelease, 1981) even though the negatively stained images of these structures are interpreted differently. Veenhuis et al. (1978, 1979) suggested that the yeast crystalline nucleoids represent a highly ordered organizati-
tion of several peroxisomal oxidases and catalase. Thomas and Trelease
(1981), however, argue that if this is true and the crystal retains enzymatic activity, then cytochemical localization would obscure the crystal lattice structure. This seems to be the case when catalase activity is localized in crystal nucleoids with the DAB reaction (Frederick and Newcomb, 1969; Vigil, 1973). Thomas and Trelease (1981) observed that nucleoids are cytochemically unreactive to glycolate oxidase localization, and we concur with their results.

Briggs et al. (1975) observed that reaction product does not diffuse from the surface of polymorphonuclear leukocytes even after thorough rinsing and processing for electron microscopy. Accordingly, we did not see any evidence for reaction product diffusion outside of peroxisomal membranes under various treatment conditions. This agrees with previous investigations using the CeCl₃ procedure (Arnold et al., 1977, 1979; Veenhuis et al., 1978, 1979; Arnold and Holtzman, 1980; Thomas and Trelease, 1981). Therefore, Thomas and Trelease (1981) consider reaction product diffusion unlikely and postulate that crystal interstices could accommodate active glycolate oxidase molecules, resulting in observable reaction product deposition between structural units.

We observe uneven staining of Nicotiana crystalline nucleoids and believe this could be caused by inhibition of reactant diffusion to the inner portion of the crystal as reaction product accumulates at the periphery. A crystal lattice with wider interstitial spaces, such as those seen in Yucca unspecialized peroxisomes, might allow diffusion throughout its structure during reaction product deposition. Slight
staining of amorphous nucleoids in Psychotria and Glycine also may indicate the presence of glycolate oxidase within these inclusions. We share the contention of Thomas and Trelease (1981) that glycolate oxidase is associated intimately with peroxisomal nucleoids. However, unequivocal demonstration of a peroxisomal enzyme within plant nucleoid inclusions has not been accomplished in any species (Gerhardt, 1978).

Glycolate oxidase is considered a marker enzyme for plant peroxisomes (Beevers, 1979; Tolbert and Essner, 1981), and Thomas and Trelease (1981) cytochemically have shown CeCl₃ localized glycolate oxidase activity in peroxisomes, glyoxysomes and unspecialized peroxisomes from a variety of higher plant tissues. In our study, we find cells which contain cytochemically reactive and unreactive microbodies, for glycolate oxidase activity, as well as peroxisomes that stain with various intensities. Thomas and Trelease (1981) also note unstained and stained unspecialized peroxisomes within a single barley coleoptile cell after incubation in a reaction mixture containing 5 mM CeCl₃ for 18 h. They suggest that whereas this may indicate not all microbodies contain glycolate oxidase in a given plant cell, they suspect a limitation in the technique which might be overcome by longer incubation times. We observe variously reactive and nonreactive peroxisomes in tissues of all four species at incubation times up to 36 h. This situation remains unchanged when CeCl₃ concentrations are varied. We believe these results indeed reflect differences in glycolate oxidase content within peroxisomes of individual cells and that variously stained peroxisomes indicate variation in physiological condition of these organelles. Heterogeneity among a cellular peroxisome population would
not be discerned in biochemical fractionation studies and deserves further investigation.

Extended incubation times and/or increased CeCl₃ concentrations may increase the sensitivity of the CeCl₃ technique. Leaf-type peroxisomes contain higher levels of glycolate oxidase activity than other types of plant microbodies (Beevers, 1979). Glycolate oxidase activity is detected reliably after 1 h in 2.5 mM CeCl₃ reaction medium, the shortest incubation time in the lowest CeCl₃ concentration examined. Leaf peroxisomes have been shown to contain approximately 30-300 times more glycolate oxidase activity than unspecialized peroxisomes (Huang and Beevers, 1971). This relatively low glycolate oxidase activity in unspecialized peroxisomes may account for inadequate staining observed at shorter incubation times. Thomas and Trealease (1981) recognize similar reaction difficulties for glycolate oxidase localization in glyoxysomes and unspecialized peroxisomes. Longer incubation times (9-19 h) and higher CeCl₃ concentrations (7.5 and 10 mM) result in reliable glycolate oxidase localization. These conditions also result in a slight deposition of reaction product in leaf peroxisomes of control specimens minus glycolate. This suggests increased sensitivity of the technique to enable detection of glycolate oxidase activity in the presence of only endogenous glycolate.

We think the presence of substrate-independent cerium deposits in thylakoid spaces of chloroplasts and hoop-structures, observed at extended (9-36 h) incubation times, indicates endogenous H₂O₂ and increased sensitivity of the CeCl₃ technique at extended incubation periods. Reduction of oxygen by the electron transport chain of chloroplasts during CO₂
assimilation results in formation of superoxide radicals and/or $\text{H}_2\text{O}_2$ (Egneus et al., 1975). Production of $\text{H}_2\text{O}_2$ at the inner plastid membrane surface would result in a cerium perhydroxide "pseudo-reaction product." The amount of $\text{H}_2\text{O}_2$ may be insufficient to detect by cerium precipitation at shorter incubation times. The presence of cerium deposition within hoop-structures of *Psychotria* leaf mesophyll cells remains enigmatic. These structures resemble ER, which may also contain endogenous $\text{H}_2\text{O}_2$. Their identification in post-stained sections is difficult.

Energy dispersive XRMA on thin sections clearly identifies the cerium reaction product. Furthermore, spectral analysis identifies the $\text{L}_{\text{u}}\text{I}$ peak as the strongest generated signal.

Localization of cerium stained peroxisomes occluded from view by cytoplasm or other organelles in SEM-prepared bulk specimens would be ideal for many investigative purposes involving peroxisomal distribution. We demonstrate that present technology is available for visualization of peroxisomes with SEM and XRMA; however, ideally reliable localization is limited by two factors. First, peroxisomes must be recognizable protruding spherical structures and, in some cases, this may represent a fixation artifact. Unfortunately, any given structure having a spherical appearance, when viewed with SEM, may or may not be a peroxisome. Secondly, not all peroxisomes are found to be reactive with the $\text{CeCl}_3$ technique using glycolate as a substrate. While some plant material seems more conducive to location of these structures, these are recognized as major limitations of SEM peroxisome identification and localization.
The XRMA localization of peroxisomes is achieved best on cerium overloaded specimens. This also results in cerium deposition in other structures, especially chloroplasts and cell walls, which contribute to background signals and mapping difficulties. However, this background is minimal when compared to nonspecific staining properties observed with other cytochemical methods for enzyme activities. Detection of catalase activity (Frederick and Newcomb, 1969; Vigil, 1973) is accomplished by osmium precipitation of polymerized DAB (osmium black); osmium also stains most other cellular structures during post-fixation. The ferricyanide technique (Shnitka and Talibi, 1971; Hand, 1975, 1976) for peroxisomal oxidase activity results in considerable nonspecific staining. Beam penetration on bulk prepared specimens also greatly contributes to background interference, allowing only some structures to be mapped. These factors seriously limit the SEM localization procedure for widespread investigative use. However, modifications and refinement of the CeCl₃ technique and XRMA procedures remain as possibilities which require further investigation.
ACKNOWLEDGMENTS

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APPENDIX
Figures 42-45. Specificity of CeCl$_3$ technique for glycolate oxidase activity in *Psychotria* leaves.

Figure 42. 21,200X  
Figure 43. 12,400X  
Figure 44. 22,300X  
Figure 45. 26,220X

Nature, in her blind thirst for life, has filled every possible cranny of the rotting earth with some sort of fantastic and complex creature.

Joseph Wood Krutch
Figures 46-52. CeCl₃ reacted *Psychotria* leaf-type peroxisomes.

Figures 46, 47. Peroxisomes stained for glycolate oxidase activity with cerium. 42,100X, 44,980X

Figures 48-50. Peroxisomes with amorphous nucleoid inclusion bodies that appear as negatively-stained structures contrasted from surrounding peroxisomal matrix. 31,900X, 32,000X, 55,000X

Figure 51. Stained peroxisome which appears to contain substructure. 69,440X

Figure 52. Glycolate-minus control stained peroxisome indicating endogenous glycolate oxidase activity. 32,000X

Into every empty corner, into all forgotten things and nooks, Nature struggles to pour life, pouring life into the death, life into life itself.

Henry Beston
Figures 53-55. Variously reacted and unstained peroxisomes within individual cells for glycolate oxidase activity.

Figure 53. *Glycine max.* 36,240X
Figure 54. *Psychotria punctata.* 39,950X
Figure 55. *Nicotiana tabacum.* 37,250X

The smallest sprout shows there is really no death. And if ever there was it led forward life, and does not wait at the end to arrest it.

Walt Whitman
PART V. ABSENCE OF CeCl₃-DETECTABLE GLYCOLATE OXIDASE ACTIVITY IN DEVELOPING RAPHIDE CRYSTAL IDIOBLASTS OF PSYCHOTRIA PUNCTATA AND YUCCA TORREYI
Absence of CeCl₃-detectable glycolate oxidase activity in developing raphide crystal idioblasts of Psychotria punctata and Yucca torreyi

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Key words: glycolate oxidase, peroxisomes, cytochemistry, calcium oxalate, crystal idioblasts

Abbreviations: AMPD = 2-amino-2-methyl-1,3-propanediol; CTEM = conventional transmission microscopy; DAB = 3,3′-diaminobenzidine tetrahydrochloride; HVEM = high voltage electron microscopy
ABSTRACT

Cytochemical localization of three peroxisomal enzymes, glycolate oxidase, urate oxidase, and catalase, is performed on tissues from *Psychotria punctata* leaves and *Yucca torreyi* roots, which contain developing and mature calcium oxalate raphide crystal idioblasts. Enzyme content and distribution of peroxisomes in crystal idioblasts and surrounding cells are described. Glycolate oxidase and catalase activities are cytochemically localized in leaf-type peroxisomes in nonidioblastic mesophyll cells in *Psychotria* leaves. Urate oxidase activity could not be cytochemically demonstrated in these organelles. Unspecialized peroxisomes in *Yucca* root cortical parenchyma cells are shown to exhibit activities of all three enzymes. Reaction product deposition attributable to glycolate oxidase activity is never observed in peroxisomes of any developing or mature crystal idioblasts of *Psychotria* and *Yucca* with CTEM and HVEM. Catalase localization indicates that idioblast microbodies are functional peroxisomes. The apparent absence of glycolate oxidase in raphide idioblasts of *Psychotria* and *Yucca* casts serious doubt that pathways involving glycolate or glyoxalate intermediates are operational in oxalic acid synthesis for calcium oxalate crystal production.
INTRODUCTION

Oxalic acid [(COOH)₂] accumulates in plants as the free acid (Soderstrom, 1962), ionic soluble salts (i.e., Na⁺, K⁺), or precipitated as an insoluble salt (Bornkamm, 1965). The insoluble salts have been more thoroughly investigated than other oxalate forms (Arnott and Pautard, 1970) and calcium oxalate is the most commonly occurring insoluble calcium salt in plants (Arnott, 1973). Deposits of calcium oxalate occur as microscopic crystals in many different organs and diverse tissues in a wide variety of plant taxa (Arnott, 1973; Franceschi and Horner, 1980a,b). The amount of accumulated calcium oxalate varies among species and may comprise up to 85 percent of a plant's dry weight (Czapek, 1921). Calcium oxalate deposition is not indiscriminate in plants, but occurs in vacuoles of specialized cells, called crystal idioblasts (Foster, 1956), differentiated to involve the crystallization process (Kausch and Horner, 1982, 1983a,b). Despite their widespread occurrence, the role of plant calcium oxalate crystals and crystal idioblasts in normal tissue development and functioning is not known. Furthermore, the specific origin and pathway of oxalate used in crystal synthesis has not been determined.

Oxalate is produced in plants through at least five biochemical pathways (Hodgkinson, 1977; Franceschi and Horner, 1980a; Huang, 1982). Oxalic acid is formed in some plants via enzymatic cleavage of oxaloacetate (Chang and Beevers, 1968) and ascorbate metabolism (Wagner and Loewus, 1973). However, it is generally accepted that the major intermediate precursors of oxalate in higher plants are glycolate and glyoxylate (Kpodar et al., 1978). These precursors may arise from the glycolate pathway
(i.e., photorespiration; Tolbert, 1973), the glyoxylate bypass (Kornberg and Krebs, 1957) or ureide metabolism from purine degradation (Huang, 1982). Certain pathological fungi oxidize glyoxalate to oxalic acid in the presence of NAD-glyoxalate dehydrogenase (Armentrout et al., 1978) but this system has not been demonstrated in any plant species (Huang, 1982). Therefore, in higher plants the conversion of glycolate to glyoxylate, and subsequently to oxalate, is mediated exclusively by peroxisomal glycolate oxidase (Tolbert, 1980; Huang, 1982).

Ultrastructural and developmental studies of calcium oxalate crystal idioblasts have shown that peroxisomal profile frequency is similar (Mollenhauer and Larson, 1966; Horner and Whitmoyer, 1972; Horner and Wagner, 1980) or lower than surrounding nonidioblastic cells (Frank and Jensen, 1970; Eilert, 1974; Kausch and Horner, 1983a,b). In fact, Kausch and Horner (1983a) did not observe any peroxisomes in Typha raphide crystal idioblasts at any time in their development. These observations are of special interest because peroxisomal development, number, and enzyme content are generally believed to be substrate-dependent and metabolically induced (Tolbert and Essner, 1981). Therefore, if oxalate for crystal synthesis is derived via glycolate oxidase mediated reactions within developing crystal idioblasts, a higher number of peroxisomal profiles might be expected in comparison to cells in surrounding tissues.

In the present study, three peroxisomal enzymes, glycolate oxidase, catalase, and urate oxidase, were cytochemically localized in tissues containing developing raphide crystal idioblasts. Photosynthetic leaves of Psychotria punctata and dark-grown primary roots of Yucca torreyi were
examined. The purposes of this investigation were to determine cytochemically detectable peroxisomal enzyme content in developing idioblasts, and examine approximate peroxisome number and distribution in these cells and the surrounding nonidioblastic tissue. At present, this information is inaccessible by standard biochemical procedures.
MATERIALS AND METHODS

Tissue Preparation

Dried seeds of *Yucca torreyi* L. were imbided overnight in running tap water, surface sterilized 17 min in 2.0% sodium hypochlorite, and grown aseptically for 9-11 days at 27°C on sterile, wet filter paper in glass Petri dishes in darkness. Young, developing, but photosynthetic leaves of *Psychotria punctata* Vatke were obtained from glass house grown plants at Iowa State University, Ames, Iowa. Dissection and fixation for electron microscopy was done by placing leaves in buffered fixative at 4°C and making leaf discs with a 0.5 mm (I.D.) needle punch. Primary roots were immersed in the same fixative and the terminal 3 mm of each root was excised and cut longitudinally with a razor blade.

Catalase Cytochemistry

Tissue samples were fixed in 3% glutaraldehyde in 100 mM K-phosphate buffer (pH = 7.2) for 30 min and tested for catalase activity in accordance with the procedure of Vigil (1970). Following aldehyde fixation, tissues were washed with the same buffer three times, 5 min each rinse, and equilibrated in 100 mM AMPD buffer at pH = 9.0 for 15 min. Tissues were incubated for 60 min at 37°C in the following freshly prepared medium as described by Frederick and Newcomb (1969): 1) standard test for catalase: 10.0 mg DAB plus 0.1 ml 3% H₂O₂ diluted fresh from 30% superoxal were added to 5 ml of 50 mM AMPD buffer at pH = 10.0. The pH was readjusted to 9.0 before tissue incubation; 2) control tests: standard
reaction medium minus H$_2$O$_2$ or minus DAB. Post-incubation rinses were in 100 mM AMPD (pH = 9.0), followed by 100 mM K-phosphate buffer (pH = 7.4); three rinses each buffer for 5 min each. Post-fixation, and osmium black reaction product formation, were in 2% OsO$_4$ (w/v) in 100 mM K-phosphate buffer (pH = 7.4) for 1 h, followed by processing for TEM.

Urate Oxidase Cytochemistry

Unfixed leaf discs and root segments were incubated immediately after dissection for 150 min at 27 C in reaction medium. Urate oxidase is extremely labile to aldehyde fixation (Yokota and Nagata, 1977); control samples were made by fixing tissues for 2 h prior to incubation. Two separate reaction mixture solutions were prepared individually and consisted of 6.0 ml of 25.0 mM CeCl$_3$·7H$_2$O and 10 mM 3-amino-1,2,4 triazole (a potent catalase inhibitor) in 100 mM tris buffer (pH = 9.6) and 40 ml of 5 mg/ml sodium urate in warmed double distilled water. Double distilled water used for all solutions was boiled to prevent carbonate precipitation. To avoid precipitation of reaction components, these two solutions were mixed dropwise under constant gentle stirring. Tissues were thoroughly aerated with CO$_2$-free air for the duration of incubation. Following the incubation period, tissues were fixed in formaldehyde-glutaraldehyde (2%-1%, v/v) buffered with 50 mM K-phosphate buffer (pH = 7.2) for 4 h at 4 C. Formaldehyde was prepared fresh from paraformaldehyde immediately before use. Tissues were then rinsed three times with the same buffer, 5 min each rinse, and then three times with 50 mM Na-cacodylate buffer (pH = 6.0) for 10 min each rinse, to remove nonspecific
cerium precipitates. Tissues were then equilibrated 10 min in 50 mM Na-cacodylate buffer (pH = 7.2), post-fixed for 2 h in 1% OsO₄ (w/v), and processed for TEM observation. The above procedure for urate oxidase localization has been modified from Vaughn et al. (1982).

**Glycolate Oxidase Cytochemistry**

Leaf discs and root segments were made in formaldehyde-glutaraldehyde (4%–1%, v/v) in 50 mM K-phosphate buffer (pH = 6.9) at 4°C. Aldehyde fixation was for 7 min, starting when plant materials were first exposed to fixative, and followed by three 5-min buffer rinses (same buffer) at 21°C, and one 20-min wash in 100 mM Tris-maleate (pH = 7.5). Samples were then preincubated 1 h in 100 mM Tris-maleate (pH = 7.5) containing 50 mM of 3-amino-1,2,4 triazole and 5.0 mM CeCl₃·7H₂O. Tissues were then transferred into fresh, reaction medium for 11 h. The reaction medium was made from preincubation medium and contained 50 mM glycolic acid. Control samples were made by omitting the glycolate substrate from the reaction medium. All solutions containing CeCl₃ were continuously aerated with CO₂-free air and filtered before use. Specimens were rinsed three times after incubation, for 15 min each rinse, in 100 mM Na-cacodylate (pH = 6.0), and then 15 min in the same buffer at pH = 7.2. Tissues were then post-fixed in 1% OsO₄ (w/v; same buffer and pH) for 1 h and processed for electron microscopic observation. This procedure for glycolate oxidase localization utilizes optimal reaction mixture concentrations and incubation times as previously determined for these tissues (Kausch et al., 1983).
Electron Microscopy

Following osmication, specimens were dehydrated in a graded ethanol-propylene oxide series, embedded in Medcast resin and sectioned with a diamond knife. Thin sections (silver reflectance) were collected on Formvar-coated grids and post-stained with uranyl acetate and lead citrate. All cytochemically prepared specimens were viewed without post-staining. Conventional TEM was done with a Hitachi HU-11C, at accelerating voltage of 50 or 75 kV. The same material used for CTEM was also used for HVEM. Thick sections, 0.25-5.00 μm, were made from ultra-smooth block faces, collected in slotted copper grids, and transferred to a drop of double distilled water on a glass slide. The slide was slightly warmed on a hot plate to expand the sections, which were then transferred to Formvar-coated slotted grids and carbon coated. Observation and micrography were done on an AEI HVEM operated at 800 kV.
RESULTS

Crystal idioblasts occur scattered in both the palisade and spongy mesophyll of *Psychotria* leaves (Horner and Whitmoyer, 1972) and as longitudinal files in root cortex of *Yucca* (Eilert, 1974). Cytochemical reaction product deposition in nonidioblastic cells indicates peroxisomal enzyme activity and verifies the various localization procedures for these tissues by reaction specificity. In photosynthetic *Psychotria* leaf mesophyll cells, reaction product deposition from glycolate oxidase activity with CeCl₃ (Figure 1) and catalase activity with DAB (Figure 2) clearly distinguishes leaf-type peroxisomes from other organelles. A positive reaction for urate oxidase activity is not observed in any mesophyll cells under the conditions used for that localization procedure. However, a positive reaction indicating urate oxidase activity in unspecialized peroxisomes in *Yucca* root cortical parenchyma cells is obtained (Figure 3) using the identical cytochemical localization conditions used for *Psychotria* leaves. These peroxisomes also were stained for glycolate oxidase (Figure 4) and catalase (Figure 5) activities.

Single membrane-bound organelles morphologically discernible as microbodies have been previously reported in developing raphide crystal idioblasts of both *Psychotria* (Horner and Whitmoyer, 1972) and *Yucca* (Eilert, 1974). Microbodies in developing idioblasts are ultrastructurally identical to those in nonidioblastic cells, exemplified in *Yucca* (Figures 6-8). All microbodies in *Yucca* root cortical cells have crystalline nucleoid inclusions (Figures 7, 8), whereas those in *Psychotria* leaf mesophyll contain amorphous inclusions (Kausch et al., 1983).
Reaction penetrability is not complete through entire tissue samples of either *Psychotria* or *Yucca* for any of the three cytochemical procedures applied in this investigation. Localization of urate oxidase activity, in *Yucca*, is particularly limited in this regard and only a very small number of cells near the cut tissue surface occasionally contain stained peroxisomes. This erratic staining property renders this reaction unacceptable for reliable localization of peroxisomes, and their distribution, in the tissues used in this study. Reactions for glycolate oxidase and catalase activities occur in a layer of 10-15 cells inward from cut tissue surfaces. This characteristic is consistent, and reliable peroxisomal enzyme localization always occurs in cells at or very close to cut surfaces. Therefore, all observations on these latter two enzymes in *Psychotria* and *Yucca* are restricted to peripheral cell layers of tissue samples.

Reaction product deposition from glycolate oxidase activity is never observed in any developing or mature raphide crystal idioblasts of *Psychotria* or *Yucca*. Observations on all crystal idioblasts are well within the penetration range of the reaction as verified by stained peroxisomes in surrounding cells (Figures 9, 10). A large number of thin sections and samples, viewed with CTEM, does not show any peroxisomal cerium reaction product indicative of glycolate oxidase activity in any idioblasts. Peroxisomes that do not contain reaction product are very difficult to identify in sections viewed without post-staining (Figures 9, 10, 12), and we find that post-staining with lead and uranyl salts leaves cytochemically stained peroxisomes difficult to distinguish. Even ruptured idioblasts at cut surfaces, which still contain intact cytoplasmic organ-
elles and are directly exposed to reaction mixture components, do not contain any reacted peroxisomes. Furthermore, reacted peroxisomes in adjacent cells do not show any definitive spatial orientation or concentration relative to developing idioblasts in either Psychotria or Yucca (Figures 11, 12). Leaf peroxisomes in Psychotria are generally associated with chloroplasts and mitochondria (Figure 11) indicative of their photorespiratory role, whereas unspecialized peroxisomes of Yucca do not appear to be specifically associated with any other organelles (Figures 10, 12). Nonspecific cerium deposits accumulate within the cell walls of Yucca under the applied cytochemical conditions; control samples show that these deposits are substrate independent and not related to enzyme activity.

Catalase activity is localized in peroxisomes with the DAB reaction in Psychotria and Yucca crystal idioblasts (Figures 13, 14). Peroxisomes in idioblasts generally stain less intensely with DAB than peroxisomes in surrounding cells. In Yucca idioblasts, the catalase reaction was particularly weak (Figure 14).

The possibility persists that glycolate oxidase reactive peroxisomes occur in idioblasts of both species and are merely not observed despite a thorough examination of many cells and sections. The potential occurrence of such reactive peroxisomes must be addressed, particularly since the apparent number of peroxisomes may be low in developing crystal idioblasts and unstained peroxisomes are difficult to see in thin sections viewed with CTEM. Also, crystal idioblasts in both species are large cells and hence, serial sectioning does not offer an acceptable practical solution.
However, up to 5.0 μm of cell depth can be viewed at once with HVEM and the presence of any reaction product deposition is easily observed.

Thick sections observed with HVEM clearly substantiate the absence of any cerium reaction product deposition attributable to enzyme activity in Yucca crystal idioblasts (Figures 15, 16). Increased cytoplasmic density generally characterizes crystal idioblast development (Franceschi and Horner, 1980a) and is obvious in sections viewed with HVEM (Figures 15, 16). This cytoplasmic density does not, however, interfere with localization of reaction product. We do not find glycolate oxidase activity in peroxisomes of idioblasts during any stage of their development. However, in this study, the presence of crystals within the central vacuole is the criterion used to distinguish idioblasts from undifferentiated cells and by this stage in their development significant differentiation has already occurred (Franceschi and Horner, 1980a).
Figures 1-5. Cytochemical characterization of peroxisomal enzymes in nonidioblastic cells of *Psychotria punctata* leaves and *Yucca torreyi* roots.

Figure 1. Glycolate oxidase localization using CeCl₃ in *Psychotria* leaf mesophyll cell clearly distinguishes peroxisome from other organelles. Thylakoid staining has been previously noted (Kausch et al., 1983). 12,430X

Figure 2. Catalase localization with DAB in leaf peroxisome of *Psychotria* mesophyll cell. 13,500X

Figure 3. Urate oxidase localization with CeCl₃ in root parenchyma cell of *Yucca*. Small granular cerium deposits occur nonspecifically throughout cytoplasm and do not indicate enzyme activity as deduced from control sections. This effect is probably a result of incubating unfixed cells. 15,400X

Figure 4. Glycolate oxidase localization in *Yucca* root parenchyma cell with CeCl₃. Note how clean this preparation is in comparison to Figure 3. 18,960X

Figure 5. Catalase localization with DAB in *Yucca*. Note apparent staining of peroxisomal membrane (arrow) which is characteristic of this reaction. 38,840X
Figures 6-8. Comparison of *Yucca* root cortical parenchyma and crystal idioblast microbody ultrastructure.

Figure 6. Cytoplasm of developing crystal idioblast showing microbody near a crystalloplastid. 12,400X
Figure 7. Microbody containing a crystalline nucleoid inclusion in a developing crystal idioblast. 52,100X
Figure 8. Microbody in a cortical parenchyma cell. 51,870X
Figures 9-14. Distribution of glycolate oxidase and catalase activities in and around developing crystal idioblasts, note that electron-dense material in vacuoles of idioblasts is calcium oxalate and not reaction product.

Figure 9. Reaction product indicating glycolate oxidase activity clearly distinguishes peroxisomes in Psychotria mesophyll cell adjacent to a crystal idioblast. Arrow points to an unstained single membrane-bound organelle in idioblast cytoplasm. 8,200X

Figure 10. Glycolate oxidase stained peroxisomes in parenchyma cell adjacent to a crystal idioblast. Arrow points to an unstained single membrane-bound organelle in idioblast cytoplasm. 9,820X

Figure 11. Example of peroxisome orientation in mesophyll cell of Psychotria, peroxisome associated with chloroplast. 6,900X

Figure 12. Peroxisomes in Yucca root parenchyma cells are not associated with any organelle in particular and do not show any orientation to crystal idioblasts. 7,210X

Figures 13, 14. Positive reaction with DAB indicating catalase activity in idioblast peroxisomes of Psychotria and Yucca, respectively. 10,200X, 16,800X
Figures 15, 16. HVEM of thick sections showing absence of cerium reaction product attributable to glycolate oxidase activity in Yucca crystal idioblasts. Key to labeling: c - crystal (or hole left in section where crystal fell out during processing), CI - crystal idioblast, Cp - chloroplast, ER - endoplasmic reticulum, L - lipid body, M - mitochondria, Mb - microbody, N - nucleus, P - plastid, V - vacuole.

Figure 15. Thick section, 2.5 μm, showing stained peroxisomes (arrows) parenchyma cell adjacent to crystal idioblast. Cerium deposits in cell wall are not a consequence of enzyme activity.

Figure 16. Thick section, 5.0 μm, showing absence of cerium stained peroxisomes in crystal idioblast cytoplasm; cerium stained peroxisomes in adjacent cells (arrows) are clearly visible.
Peroxisomal deposition of reaction products attributable to the various enzyme activities (i.e., glycolate oxidase, urate oxidase, catalase) tested cytochemically in this study, verifies the localization procedures in nonidioblastic Psychotria leaf cells and Yucca root tissues. Furthermore, positive reactions for these enzymes provide an internal standard of peroxisomal enzyme content to which developing crystal idioblasts can be compared. Psychotria leaf-type peroxisomes show cytochemical localization of glycolate oxidase and catalase, but are not reactive to urate oxidase localization.

The cytochemical procedure for urate oxidase used in this study is a modification of the CeCl₃ technique used to localize glycolate oxidase. Both utilize cerium as a trapping agent, precipitating enzyme-generated peroxide as electron-dense cerium perhydroxide. Activity of urate oxidase has been shown to be about 100 times less than glycolate oxidase in leaf-type peroxisomes isolated from spinach (Huang and Beevers, 1971). It is possible that the CeCl₃ technique is incapable of resolving inherently low enzyme activities (Thomas and Trelease, 1981); however, reliable detection of glycolate oxidase in unspecialized peroxisomes with the CeCl₃ procedure has been demonstrated (Thomas and Trelease, 1981; Kausch et al., 1983). Glycolate oxidase and urate oxidase activities are similar in unspecialized peroxisomes of some plants (Huang and Beevers, 1981; Huang, 1982). Kausch et al. (1983) reported detection of endogenous H₂O₂ with CeCl₃ in four higher plant species, suggesting significant sensitivity of the reaction. Therefore, this may indicate that Psychotria leaf peroxisomes lack urate
oxidase activity; however, further investiga
coupled with biochemical enzyme assays are n
localization of peroxisomal enzymes with low

Unspecialized peroxisomes in nonidiobla
partmentalize catalase, glycolate oxidase, a
ble by cytochemistry. This further supports
since glycolate oxidase and urate oxidase ac
are likely to be comparable, and both inhere

Developing crystal idioblasts in both P
microbodies that are identical in size and u
rounding nonidioblastic cells. Despite a th
some containing reaction product attributabl
ity was found in any developing or mature cry
acteristic is not explained by inability of
penetrate the idioblasts, since ruptured cel
organelles were observed. Also, the possib
blast peroxisomes in out-of-plane thin sect:
umber of examined sections and samples as w
conclude, therefore, that crystal idioblast
lack any significant glycolate oxidase acti
technique. These idioblast microbodies are
ever, as indicated by the fact that they st
with DAB.

Glycolate oxidase is considered a mark
peroxisomes (Beevers, 1979; Tolbert and Ess
(1983) found cells which contained cytochemically reactive and unreactive peroxisomes, for glycolate oxidase activity, as well as peroxisomes that stained with various intensities in four higher plant species. Reaction product in peroxisomes was verified as cerium with X-ray microanalysis. Thomas and Trelease (1981) have also noted unstained and stained peroxisomes within a single barley coleoptile cell. They suggested that while this may indicate that not all peroxisomes of a given plant cell contain glycolate oxidase, they suspected a limitation in the CeCl₃ technique which might be overcome by adjusting reaction mixture concentrations or incubation times. In the study by Kausch et al. (1983), a wide range of modifications in the CeCl₃ procedure were made and variously stained and unreactive peroxisomes were still consistently observed. This implies differences in glycolate oxidase content, and/or variations in physiological condition of peroxisomes within individual plant cells. Such heterogeneity among a cellular peroxisomal population would not be discerned in biochemical fractionation studies. Glycolate oxidase may not be a ubiquitous higher plant peroxisomal enzyme.

Peroxisomes in a tissue with a specific function (e.g., leaves and photorespiration; Tolbert, 1973 and root nodules and ureide metabolism; Vaughn et al., 1982) usually have enzymes associated only with that metabolic pathway, and other enzymes may be either completely missing or greatly repressed (Tolbert, 1980). It is not unreasonable, therefore, that crystal idioblast peroxisomes may be differentiated to contain an enzyme complement different from those cells in the surrounding tissue. It is generally accepted that the major oxalic acid precursors in higher
Plants are glycolate and glyoxylate (Kpodar et al., 1978) and that oxidation of these precursors is mediated by peroxisomal glycolate oxidase (Tolbert, 1981). Glycolate oxidase, then, is the key enzyme for oxalate synthesis via three pathways: the glycolate pathway, the glyoxylate bypass, and ureide metabolism. The apparent absence of glycolate oxidase in raphide idioblasts of Psychotria and Yucca casts serious doubt that any of these pathways involving glycolate or glyoxylate intermediates are operational in oxalic acid synthesis for calcium oxalate crystal production. Inability to effectively localize urate oxidase in this study, therefore, is not significant to this conclusion since glycolate oxidase would still be required for oxalate synthesis via ureide metabolism since glyoxylate is one of the products of that pathway (Huang, 1982).

It is of significant interest that cells which accumulate large amounts of insoluble oxalate differentiate to exclude a key enzyme involved in three major pathways which produce oxalate. Possibly, oxalate generated by glycolate oxidase mediated reactions may inhibit by feedback or interfere with a preferred pathway of oxalate synthesis for crystal production. Most crystal idioblasts differentiate specialized plastids (Kausch and Horner, 1983a,b), called crystalloplastids (Arnott, 1966), which are probably nonphotosynthetic in that they do not develop thylakoid granal systems. Therefore, glycolate biosynthesis from photorespiration and subsequent metabolism may not occur in these cells, even within photosynthetic tissues, such as Psychotria leaves. Also, one of the first recognizable events of crystal idioblast initiation is loss of storage reserves (i.e., starch and lipids). Glyoxylate could be generated in de-
veloping crystal idioblasts during β-oxidation of fatty acids in the
glyoxylate bypass as well as during purine degradation in ureide metabo-
ism. Assuming a sufficient nitrogen source and the absence of glycolate
oxidase, any metabolically produced glyoxylate could be efficiently
utilized for serine and glycine synthesis in transamination reactions.
The functional role of peroxisomes in crystal idioblasts remains enig-
matic.

It is possible that oxalate for crystal synthesis is generated in
surrounding tissue and transported into developing idioblasts. However,
addition of oxalic acid to *Psychotria punctata* callus culture growth media
did not stimulate crystal idioblast differentiation (Franceschi and
Horner, 1979) whereas a number of other media addenda and modifications
are known to do so (Franceschi and Horner, 1979; Kausch, unpublished, The
Rockefeller University). Also, developing crystal idioblasts are general-
ly not reported to be highly endocytotic. Furthermore, in the present
study, we show that there is no specific orientation of peroxisomes in
surrounding cells that might warrant their involvement in oxalate produc-
tion for developing crystal idioblasts in either plant examined. There-
fore, tentatively, we assume that oxalic acid synthesis for calcium
oxalate crystals occurs within developing idioblasts by a pathway which
does not involve glycolate oxidase mediated reactions. Certainly, the
synthetic pathway of oxalic acid synthesis for calcium oxalate production
in plants and the functional role of peroxisomes in crystal idioblasts
deserves further attention.
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APPENDIX
Figure 17. Supplementary micrographs substantiating the absence of glycolate oxidase activity in crystal idioblasts in *Psychotria* leaves.

A. 13,300X  
B. 15,600X  
C. 12,260X  
D. 13,100X

It's not true that life is one damn thing after another - its one damn thing over and over.

   Edna St. Vincent Millay

Beware of the undertoad.

   John Irving
Figure 18. Supplementary micrographs substantiating the lack of any specific orientation of *Psychotria* leaf peroxisomes to crystal idioblasts.

A. 8,200X  
B. 4,100X  
C. 9,400X  
D. 4,300X

The crows maintain that a single crow could destroy the heavens. Doubtless that is so, but it proves nothing against the heavens, for the heavens signify simply: the impossibility of crows.

Franz Kafka
Figure 19. Supplementary micrographs substantiating the absence of glycolate oxidase activity in crystal idioblasts in *Yucca* root cortex.

A. 15,500X  
B. 16,200X  
C. 14,900X  
D. 15,500X

The True way goes over a rope which is not stretched at any great height but just above the ground. It seems more designed to make people stumble than to be walked upon.

*Franz Kafka*
Figure 20. Supplementary micrographs substantiating the absence of glycolate oxidase activity in crystal idioblasts in *Yucca* root cortex.

A. 10,900X  
B. 14,300X  
C. 13,900X  
D. 16,200X

The decisive moment in human development is a continuous one.

Franz Kafka
Figure 21. Supplementary HVEM showing stained peroxisomes for glycolate oxidase adjacent to crystal idioblast (CI). 15,200X

If it had been possible to build the Tower of Babel without ascending it, the work would have been permitted.

Franz Kafka

What are you building? - I want to dig a subterranean passage. Some progress must be made. My station up there is much too high.
   We are digging the pit of Babel.

Franz Kafka
PART VI. BIOGENESIS AND CYTOCHEMISTRY OF UNSPECIALIZED PEROXISOMES IN ROOT CORTICAL CELLS OF YUCCA TORREYI L.
Biogenesis and cytochemistry of unspecialized peroxisomes
in root cortical cells of *Yucca torreyi* L.

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ABSTRACT

Microbodies containing bipyramidal crystalline nucleoid inclusions occur within every cortical cell in roots of *Yucca torreyi*. Deposition of reaction product attributable to catalase, glycolate oxidase, and urate oxidase activities are cytochemically localized to *Yucca* root microbodies and classifies them as unspecialized peroxisomes on the basis of their enzyme complement and tissue origin. Crystalline nucleoids do not stain for glycolate or urate oxidase activities and, as such, appear as negatively-stained inclusions, but the nucleoids are reactive for catalase activity. This indicates that the nucleoids may be composed at least partially of catalase that retains enzymatic activity in crystalline form. Development of unspecialized peroxisomes in *Yucca* roots is consistent with all evidence for glyoxysome and leaf-type peroxisome biogenesis from ER. Dilated ends of ER cisternae accumulate cytochemically detectable glycolate oxidase activity. After considerable dilation, paracrystalline precursors to nucleoids form within the bulge, and the inclusion enlarges to comprise the majority of peroxisomal volume. Peroxisomes that are not attached to ER are observed with high voltage electron microscopy and in serial thin sections, implying that eventually the budding peroxisomes are vesiculated. The functions of these unspecialized peroxisomes are suggested based upon cytochemical detection of their partial enzyme complement and their spatial and developmental timing relationships within developing *Yucca* root cortical parenchyma cells.
INTRODUCTION

Microbodies are now generally recognized as ubiquitous subcellular organelles in plant cells (Tolbert and Essner, 1981). The metabolic functions of glyoxysomes in seed lipid-storage cells and leaf-type peroxisomes in photosynthetic cells have been relatively well-established (Beevers, 1979; Tolbert, 1980; Tolbert and Essner, 1981). However, the functions of microbodies in most other plant cells has not been elucidated, and these organelles are named unspecialized peroxisomes (Tolbert, 1980; Huang, 1982) to specify their peroxisomal morphology, enzyme content, and as yet undetermined metabolic function (Thomas and Trelease, 1981). Similarly, the biogenesis of glyoxysomes during seed germination and of leaf-type peroxisomes during cotyledon greening have been investigated in some detail (Trelease et al., 1971; Vigil, 1973; Beevers, 1979). However, development of unspecialized peroxisomes is virtually unexamined despite their widespread occurrence in diverse plant cell types and their unknown physiological functions.

For over a decade, evidence for plant peroxisomal origin from ER has been accumulating from physiological, cytochemical, and ultrastructural investigations (Vigil, 1970; Lord et al., 1973; Beevers, 1975, 1979). Although most developmental studies of plant microbodies have examined glyoxysomes, and to a lesser extent leaf-type peroxisomes, peroxisomes are generally envisaged to form from dilated ends of ER cisternae (Vigil, 1970; Beevers, 1979; Tolbert, 1980). Cisternal ends enlarge while continually accumulating peroxisomal enzymes (Lord et al., 1973; Beevers, 1978, 1979). In mammalian cells, nucleoid inclusions are observed to form
in the bulged ER cisternae after significant dilation has occurred (Essner, 1967; Tsukada et al., 1968). Amorphous or crystalline nucleoid inclusions are common in plant peroxisomes in many cell types (Thomas and Trelease, 1981; Kausch et al., 1983) but the development or composition of these structures has not been determined.

At least some peroxisomal enzymes appear to be cotranslationally segregated into the budding peroxisome through ER channels (Beevers, 1975, 1979; Bowden and Lord, 1976, 1977, 1978). Goldman and Blobel (1978) have suggested that catalase and uricase may be posttranslationally inserted from the cytoplasm, where they are synthesized on free ribosomes, through animal peroxisomal membranes. Post-translational insertion of plant peroxisomal enzymes has not been demonstrated. Current models of peroxisomal ontogeny and maintenance within eukaryotic cells (Beevers, 1979; Tolbert and Essner, 1981) necessitate knowledge of continued or severed ER attachment to these organelles. Vesiculation of peroxisomes from ER immediately precludes supplementation or alteration of the cotranslationally inserted peroxisomal enzyme complement in response to developmental or environmental changes.

Generally, peroxisome biogenesis occurs during tissue development and differentiation in both plants and animals (Tolbert and Essner, 1981). In the present study, the ontogeny and cytochemistry of unspecialized peroxisomes is examined in developing parenchyma cells near the cortical origin of primary roots of Yucca torreyi. The purposes of this investigation are to establish a developmental sequence of events for these organelles in comparison to current evidence for glyoxysomal biogenesis in
other plants and gain insight into their function by cytochemical detection of their partial enzyme complement. Further indication of their function will be supplemented by observations of their spatial and developmental timing relationships to other organelles during differentiation of *Yucca* cortical parenchyma cells.
MATERIALS AND METHODS

Dry seeds of *Yucca torreyi* L. were imbibed overnight in running tap water, surface sterilized 17 min in 2.0% commercial bleach, and grown aseptically for 9-11 days at 27 C on sterile wet filter paper in glass Petri dishes in darkness. Primary roots were immersed in 3% (V/V) glutaraldehyde in 100 mM K-phosphate buffer (pH = 7.2) and the terminal 3 mm of each root were excised and cut longitudinally with a razor blade. Aldehyde fixation was for 4 h at 4 C. Tissues were then washed three times in the same buffer, 10 min each rinse, and post-fixed in 1% (W/V) OsO₄ (same buffer and pH) for 2 h at room temperature. Specimens were then rinsed three times with distilled water, dehydrated in a graded ethanol-propylene oxide series and embedded in Medcast resin.

Procedures for cytochemical localization of catalase, urate oxidase, and glycolate oxidase have been described elsewhere (Kausch et al., 1983; Kausch and Horner, 1983c).

Thin sections (silver reflectance) were made with a diamond knife, collected on copper grids, and stained with uranyl acetate and lead citrate. However, cytochemically prepared sections were viewed without post-staining. Thin sections were viewed in a conventional transmission electron microscope (CTEM), Hitachi HU-11C, operated at accelerating voltage of 50 or 75 kV. The same material used for CTEM was also used for high voltage electron microscopy (HVEM). Sections of cytochemically prepared specimens were cut 0.5-5.0 μm thick from ultra-smooth block faces, collected in slotted copper grids, and transferred to a drop of double
distilled water on a glass slide. The slide was warmed slightly on a hot plate to expand the section which was then transferred to Formvar-coated slotted grids and coated with a thin layer of carbon to stabilize the sections. Observation and micrography were done on an AEI HVEM operated at 800-1000 kV. Bas-relief photographic processing (Kausch and Horner, 1983b) is used for image enhancement of cytochemically stained features of some HVEM micrographs. Positive and negative stereo pair images, taken 7° from each other, are interfaced slightly out of register to produce side-lit, relief micrographs. The degree of relief is directly related to electron opacity; very electron-dense structures are seen in greatest relief and almost white.
RESULTS

Longitudinal files of parenchyma cells and raphide crystal idioblasts comprise the cortex and are produced at the cortical origin near the apex of Yucca torreyi roots. An ontogenetic series is represented in this tissue where acropetal cells are developmentally youngest. Cells nearest the apex are ultrastructurally similar and have an organellar complement and appearance that is characteristic, generally, of meristematic plant cells (Figure 1). The region of the root near the apex is meristematic and mitotic figures occur frequently (Figure 1, inset). The nuclei of interphase cells in this region are centrally situated and spherically-shaped. Cytoplasmically, these cells contain numerous small membrane-bound spherosomes and small vacuoles (Figures 1, 2). Plastids are simple, relatively small, and correspond to descriptions of eoplasts (Thomson and Whatley, 1980) although some contain small starch deposits. Mitochondria have a sparse and dispersed system of cristae. Both plastids and mitochondria are often seen in dumbbell configurations in these cells, suggestive of their replication. Numerous active dictyosome profiles are also observed. Single membrane-bound organelles which are morphologically identified as microbodies are found in every cell. Microbodies are often associated with ER, which is throughout the cytoplasm, but are generally not associated with any other organelles. Observations on microbody development are made in these cells within the meristematic region of root growth, particularly near the apex.

All microbodies in the cortex of Yucca roots contain crystalline nucleoid inclusion bodies (Figures 2, 3). In most sections, the crystal-
line inclusion appears to fill the microbody and only a small amount of matrix remains between the crystal and the microbody membrane. The nucleoid crystal is bipyramidal; sections through four-sided (Figure 2) and six-sided planes (Figure 3) are observed. In current usage, the term microbody is assigned to single membrane-bound organelles on a strictly morphological basis and have not been biochemically characterized. Peroxisomes are defined as microbodies which are known to compartmentalize catalase and at least one flavin oxidase (Tolbert and Essner, 1981). Microbodies in Yucca cortical parenchyma cells accumulate reaction product attributable to enzyme activities of all three enzymes cytochemically localized in this investigation (i.e., glycolate oxidase, urate oxidase, and catalase). Reaction product deposition clearly distinguishes Yucca microbodies from other subcellular structures, and classifies them as un-specialized peroxisomes on the basis of their enzyme complement and tissue origin.

Peroxisomal glycolate and urate oxidase activities are localized with the CeCl₃ technique (Figures 4–6). Cerium acts as a trapping agent for enzyme-generated peroxide with the procedure, precipitating cerium perhydroxide as a high resolution, well-localized, electron-dense reaction product with minimal nonspecific deposition. Cerium deposition from glycolate oxidase activity occurs throughout the peroxisomal matrices (Figure 4) rendering the crystalline inclusion as a uniform, negatively-stained structure. The matrix is contiguous with the interstitial crystal spaces and reaction product occurs between structural units of the crystal proper, in the interstices. The crystal lattice is not occluded by
reaction product accumulation. The cerium reaction product from urate oxidase activity is less granular in appearance (Figures 5, 6) than that resulting from glycolate oxidase activity. The crystal lattice of the inclusion is not clearly distinguished in peroxisomes stained for urate oxidase, but is discerned in some sections (Figure 6). The unfixed tissue incubated for urate oxidase localization results in an accumulation of globular material along the plasma membrane (Figure 5) and associated with some organellar membranes. This material does not interfere with identification of reaction product in peroxisomes having a lower electron opacity.

Catalase activity is localized in *Yucca* peroxisomes (Figure 7) with 3,3-diaminobenzidine tetrahydrochloride (DAB). The DAB reaction product, osmium black, does not share the high resolution qualities of the CeCl$_3$ technique. However, in lightly-reacted peroxisomes, the crystalline nucleoid inclusion appears as a positively-stained structure within the matrix (Figure 7); the crystal lattice structure appears less defined and stained by reaction product accumulation.

Attachments of rough ER to dilated cisternae (Figures 8, 9) and peroxisomes (Figure 10) suggest a direct mode of peroxisomal origin from ER. Some ends of dilated ER cisternae contain a slightly electron-dense material (Figure 9) and may represent an accumulation of peroxisomal matrix proteins and initiation of peroxisomal biogenesis. Attachments of ER to peroxisomes are difficult to discern in post-stained thin sections viewed with CTEM because ER, peroxisomes and cytoplasmic ground substance are all of relatively equal electron density. Associations of ER cisternae with peroxisomes are common and ER is often observed appressed to peroxi-
somal membranes. Carefully viewed serial sections can elucidate a difference between connected and closely associated ER cisternae to peroxisomes. Figure 10 is a representative micrograph from a series of successive sections showing a peroxisome (to the left) which is actually connected, whereas the one to the right is appressed to ER membranes.

The high-resolution and specificity of the CeCl₃ technique for glycolate oxidase localization provides samples that are best sorted for developmental study of peroxisomes in Yucca roots. Optimal conditions allowing maximum peroxisomal staining have been previously determined for this tissue and are reported elsewhere (Kausch et al., 1983).

Bulged ER cisternae accumulate cerium reaction product, indicating glycolate oxidase activity (Figure 11). Flanking serial sections (Figures 12, 13) show that this bulge is not a portion of a larger peroxisome, out of the plane of section, and that it is not spherical, but almost flat. Similar stages can be seen in thick sections viewed with HVEM, and processed for image enhancement. Slight amounts of reaction product are seen within ER cisternae (Figures 11, 14-18) and contrast the ER to surrounding unstained cytoplasm (Figures 11, 14, 17). Small paracrystalline inclusions are seen in slightly larger ER-attached bulges (Figure 15) and occur as negatively-stained structures for glycolate oxidase activity (Figures 16, 17). These must represent precursors to the nucleoid inclusions that comprise the majority of matrix volume in mature peroxisomes. Developing peroxisomes, attached to ER, are also seen with HVEM (Figure 18).

Peroxisomes with nucleoid inclusions in meristematic parenchyma cells are often observed in association with a profusion of ER (Figure 19).
Thick sections viewed with HVEM (Figure 20, 21) often show peroxisomes that apparently are attached to ER, as well as some that appear isolated (i.e., not attached to ER). Both attached and isolated peroxisomes are found in meristematic cells near the cortical origin, and mature, vacuolated parenchyma cells 3.0 to 6.0 mm from the root apex. Associations between peroxisomes and organelles other than ER are not seen at any stage of cortical parenchyma cell development. Observations of isolated peroxisomes are substantiated also in serial sections viewed with CTEM. Less intensely stained blebs are seen on some peroxisomes (Figures 20, 21) and sometimes these may be separated from one another by tortuous connections.

Figure 1. Portion of meristematic cell near the cortical origin in a primary root of Yucca. Inset shows mitotic cell (telophase) found in the same region. 10,200X, 4,200X

Figure 2. Portion of meristematic cell cytoplasm showing organellar complement. Note microbody is not associated with any other organelles and contains a four-sided crystalline nucleoid inclusion body. Inset shows microbody with four-sided nucleoid at a higher magnification. 22,342X, 51,867X

Figure 3. Another example of meristematic cell cytoplasm with some addition features, including lipid bodies which do not exhibit any specific orientation to microbodies in these cells. Inset shows higher magnification of a section through six-sided plane of a crystalline nucleoid inclusion body of a microbody. 22,500X, 50,120X

Figure 4. Cerium reaction product attributable to glycolate oxidase activity in peroxisomal matrix. Note crystalline nucleoid appears as a negatively stained structure. 79,300X

Figure 5. Positively stained peroxisome indicating urate oxidase activity. Slight substrate independent cerium precipitate occurs in the cytoplasm, and globular material accumulates along the plasma membrane in all cells; this may be a consequence of incubation of unfixed tissue. 52,520X

Figure 6. Cerium stained peroxisome for urate oxidase. Note smooth appearance of precipitate and negatively stained nucleoid inclusion (arrow). 67,410X

Figure 7. Lightly stained peroxisome with DAB, indicating catalase activity. Crystalline nucleoid inclusion is positively stained. 84,330X

Figures 8, 9. Dilated end of ER cisternae, which are presumed preoxisomal precursors. 71,230X

Figure 10. Peroxisome at left is connected to ER (small arrows), whereas ER is only closely associated with peroxisome at right (large arrow). 67,920X

Figure 11. Bulged ER cisternae showing cerium reaction product attributable to glycolate oxidase activity. Note ER connection to the bulge (arrow). 76,200X

Figures 12, 13. Flanking serial sections to Figure 11. 76,200X

Figure 14. Bas-relief HVEM micrograph showing bulged ER cisternae obviously connected to ER. The bulge contains a slight amount of reaction product from glycolate oxidase active, as does the ER itself, giving these structures electron density and relief from this image processing procedure. Peroxisome at top of this micrograph is heavily reactive for glycolate oxidase activity and very electron dense. 75,120X

Figure 15. Paracrystalline structure in peroxisomal matrix in a section stained with uranyl and lead salts and viewed with CTEM. 44,200X

Figure 16. Peroxisome stained for glycolate oxidase activity and of similar size as the one in Figure 15. Paracrystalline inclusion (arrow) is negatively stained and contrasted against peroxisomal matrix. 59,937X

Figure 17. Glycolate oxidase reacted peroxisome containing a small nucleoid inclusion body, and connected to ER. 42,000X

Figure 18. Developing peroxisome connected to ER as viewed with HVEM in a thick section. 61,243X

Figure 19. Peroxisome surrounded by ER. 55,340X

Figures 20, 21. Thick sections viewed with HVEM show peroxisomes which are apparently connected to ER (large arrows) as well as some which show no apparent ER association (i.e., vesiculated; small arrows). 39,734X
DISCUSSION

The earliest cytological reports of plant cell microbodies described their occurrence in meristematic cells of root tips (Mollenhauer et al., 1966). However, the development, enzyme content, and physiological functions of root microbodies, and unspecialized peroxisomes generally, have scarcely been investigated.

Development of unspecialized peroxisomes in Yucca roots is consistent with ultrastructural and physiological evidence for glyoxysome and leaf-type peroxisome biogenesis from ER (Beevers, 1979). Dilations of ER cisternal ends form bulges and apparently accumulate peroxisomal enzymes. During early stages of glyoxysome development, phospholipid components of the glyoxysomal membranes are made on the ER, and ER cisternae themselves contain glyoxysomal enzymes (Beevers, 1975; Gonzalez and Beevers, 1976; Donaldson and Beevers, 1977; Bowden and Lord, 1977, 1978). Cerium reaction product deposition occurs in small bulges at the ends of ER cisternae in Yucca root cells, and denotes glycolate oxidase activity in developing peroxisomes. After considerable dilation, a crystalline nucleoid inclusion forms within the bulge, and eventually comprises the majority of peroxisomal volume. A similar developmental sequence is depicted for ontogeny of glyoxysomes in castor bean endosperm (Vigil, 1970) and animal liver peroxisomes (Essner, 1967; Tsukada et al., 1968).

A slight cerium reaction product deposition from glycolate oxidase localization is visible within ER cisternae attached to budding peroxisomes in Yucca root cortical cells. This deposition may represent in situ
glycolate oxidase activity within ER cisternae, or diffusion of reaction product from developing peroxisomes.

A number of previous investigations using the CeCl₃ technique have not detected reaction product diffusion through peroxisomal membranes (Arnold et al., 1977, 1979; Veenhuis et al., 1978, 1979; Arnold and Holtzman, 1980; Thomas and Trelease, 1981) under various procedural conditions including DMSO treatment (Kausch et al., 1983). Briggs et al. (1975) observed that reaction product does not diffuse from the surface of polymorphonuclear leukocytes even after thorough rinsing and processing for electron microscopy. Thomas and Trelease (1981) even considered reaction product diffusion into nucleoid inclusions unlikely, and Kausch et al. (1983) demonstrated support for this contention. If cerium precipitate in ER of Yucca cells is not an artifact of diffusion, then it indicates enzymatic activity of glycolate oxidase before it is compartmentalized within the peroxisome proper. This is a reasonable possibility since glycolate oxidase activity is localized within even very small ER dilations, and ER cisternae have been shown to contain peroxisomal enzymes (Bowden and Lord, 1977, 1978). This suggests that Yucca root glycolate oxidase may be cotranslationally inserted and delivered to budding peroxisomes through ER channels.

Only preliminary investigations have been conducted on the enzyme complements of unspecialized peroxisomes, particularly those in roots, because of the difficulty in homogenizing the tissue without disrupting peroxisomal integrity. Partial characterizations have shown that unspecialized peroxisomes from root tissues contain malate dehydrogenase (Rocha
and Ting, 1970), urate oxidase (Parish, 1972a, b; Vaughn et al., 1982), catalase and glycolate oxidase (Huang and Beevers, 1971). The present study shows that unspecialized peroxisomes in *Yucca* roots contain, at least, catalase, urate oxidase and glycolate oxidase.

Unequivocal determination of plant peroxisomal nucleoid inclusion composition has not been accomplished (Gerhardt, 1978). The crystalline nucleoids of *Yucca* peroxisomes do not stain for urate or glycolate oxidase activities, appearing as negatively-stained inclusions, but are reactive for catalase activity. This implies that the nucleoid is at least partially composed of catalase. Several investigators with similar results have proposed that some peroxisomal nucleoid inclusions are composed of catalase protein presumably crystallized from matrix enzyme (Vigil, 1969, 1970; Frederick and Newcomb, 1969; Matsushima, 1971; Fukui et al., 1975). Veenhuis et al. (1978, 1979) have suggested that the crystalline nucleoids in yeast cells represent a highly ordered organization of several peroxisomal oxidases and catalase. This also may be true in *Yucca* root cell peroxisomes; however, apparently only catalase returns enzymatic activity in the crystalline form. The diminished resolution and definition of the nucleoid in peroxisomes stained for urate oxidase does not appear to be from enzymatic activity within the nucleoid, but a consequence of the localization procedure. The cerium reaction product from urate oxidase activity is less granular and lacks resolution qualities of the same reaction product formed in the glycolate oxidase localization procedure. This difference in cerium precipitation may be a result of alkaline conditions (pH = 9.6) during urate oxidase localization (K. C. Vaughn, personal
communication, Southern Weed Science Laboratory). Glycolate oxidase localization with cerium utilizes solutions that are close to neutral (pH = 7.5).

Unspecialized peroxisomes occur that are not attached and apparently vesiculated from the ER system in meristematic and mature cortical parenchyma cells. Observations of vesiculated peroxisomes with HVEM are substantiated in serial sections viewed with CTEM. Lord et al. (1973) and Beevers (1975) proposed that glyoxysomal membranes are derived rather directly, by a process of vesiculation and excision, from specific regions of the ER in vivo. If vesiculation of unspecialized peroxisomes in Yucca is a real cellular event (i.e., not an artifact of chemical fixation), then the possibility of new cotranslationally inserted enzymes is eliminated. Perhaps the mode of peroxisomal enzyme insertion changes after excision. Most peroxisomes in Yucca root cells are found in association with ER. It is conceivable that peroxisomal enzymes may be transferred through ER membranes appressed to vesiculated peroxisomes. The composition of ER and peroxisomal membranes is sufficiently similar (Lord et al., 1973; Beevers, 1975, 1979) to allow such a process. Alternatively, peroxisomal vesiculation may represent a senescence or turnover event, and now peroxisomes must be continually formed to replace them. Vesiculated Yucca peroxisomes retain glycolate oxidase activity as evidenced by accumulation of cerium reaction product. Certainly, in vivo vesiculation of peroxisomes from ER deserves further investigation.

It is presumed that unspecialized peroxisomes must have important physiological functions because of their ubiquitous distribution among plant cells. Their metabolic roles in Yucca root parenchyma cells can be
deduced by the cytochemical detection of catalase, glycolate oxidase and urate oxidase, as well as their apparent lack of spatial relationship to organelles other than ER.

Various flavin oxidases, compartmentalized in peroxisomes, catalyze the transfer of substrate hydrogen to molecular oxygen, and thereby generate hydrogen peroxide (Tolbert and Essner, 1981). In most cases, hydrogen peroxide subsequently is degraded in a catalase-mediated peroxidative reaction. Indeed, de Duve and Baudhuin (1966) proposed that a peroxisome is defined by the presence of at least one \( H_2O_2 \) producing flavin oxidase and catalase; catalase is thought to be, generally, a characteristic peroxisomal enzyme. Although catalase deficient microbodies have been reported in some fungi (Maxwell et al., 1977; Theimer and Beevers, 1971), the presence of catalase most probably indicates a functional peroxisome involved in metabolic processes that utilize at least one flavin oxidase.

In photosynthetic plant cells, peroxisomal glycolate oxidase is the initiating flavin oxidase which forms \( H_2O_2 \) and directs carbon flow to glycine during photorespiration (Tolbert, 1980). Glycolate oxidase also oxidizes glyoxylate to oxalate, since glyoxylate is an \( \alpha \)-hydroxy acid analogue. In the event of an insufficient glutamate pool for rapid conversion of glyoxylate to glycine in an aminotransferase reaction, oxalate accumulates in spinach leaves and this situation is alleviated by nitrogen fertilization (Tolbert, 1980). Therefore, in nonphotosynthetic cells, such as Yucca cortical parenchyma, without glycolate production and photorespiration, peroxisomal glycolate oxidase activity must be involved in oxalic acid synthesis from glyoxylate. Glyoxylate could be derived via
glyoxylate bypass reactions during β-oxidation of fatty cells within the cortial parenchyma cells. This implies at least partial "glyoxysomal" function to these organelles, although no associations between them and spherosomes was observed in these cells at any stage in their development. It is possible also that these peroxisomes metabolize transported intermediates of glyoxylate bypass reactions in lipid-storing Yucca seeds during germination. It would be of interest to know if the unspecialized peroxisomes of Yucca roots also contain other enzymes of the glyoxylate bypass. The function of derived oxalate, in any form, in plants is controversial and not well-understood (Franceschi and Horner, 1980a).

The metabolic pathway of ureides, allantoin and allantoic acid, involve enzymes including xanthine oxidase, urate oxidase, allantoinase, and allantoicase which are all compartmentalized in animal peroxisomes (Tolbert and Essner, 1981). In plants, urate oxidase activity has been demonstrated in glyoxysomes (Theimer and Beevers, 1971), leaf peroxisomes (Huang and Beevers, 1971) and unspecialized peroxisomes (Huang and Beevers, 1971; Parish, 1972a,b). Peroxisomal urate oxidase activity in cells of some nitrogen-fixing root nodules is especially high (Hanks et al., 1981) readily allowing cytochemical localization of that enzyme with the CeCl₃ technique (Vaughn et al., 1982). Although allantoinase activity was demonstrated in glyoxysomes (Theimer and Beevers, 1971) and soybean root nodule peroxisomes (Hanks et al., 1981), peroxisomal localization of a complete ureide pathway enzyme complement has not been demonstrated in plants. The role of ureide metabolism in plants is understood mostly in relation to nitrogen transport (Huang, 1982) as allantoin and allantoic
acid are the major transport forms of nitrogen in some plants (Rawsthorne et al., 1980; Thomas and Schrader, 1981). It is possible that urate oxidase in Yucca peroxisomes is involved in such a process as well. The end products of the complete ureide pathway are CO₂, NH₃, and glyoxylate. If glyoxylate is generated in these cells through that pathway, it could be subsequently utilized for oxalate production, or serine and glycine synthesis.

It is conceivable that unspecialized peroxisomes in Yucca are multifunctional organelles compartmentalizing enzymes involved in several pathways. Perhaps, no specific massive metabolite flux occurs in these organelles such as those that characterize glyoxysomes and leaf peroxisomes, but metabolism of products from several different pathways may be in operation. This notion is supported by the fact that Yucca unspecialized peroxisomes are not specifically associated with other organelles.

It is possible also that these organelles could be the major intracellular site of an enzyme activity that awaits to be disclosed. I believe that further study of the function and physiology of unspecialized peroxisomes would facilitate a better understanding of plant cell functioning generally.
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Figure 22. Supplementary HVEM micrographs of peroxisomal development from ER (arrows).

A. 67,930X  
B. 61,243X  
C. 35,230X

No thought, no reflection, no analysis
No cultivation, no intention;
the Tao settles.

Alan Watts

"Wu-nien"
Figure 23. Peroxisomal formation appears to occur in crystal idioblasts in a fashion similar to that in cortical parenchyma. These micrographs show idioblast microbodies connected (in A) and associated with (in B) ER cisternae.

A. 41,300X
B. 58,920X

When everyone recognizes beauty as beautiful, there is already ugliness;
when everyone recognizes goodness as good, there is already evil.
To be and not to be arise mutually;
Difficult and easy are mutually realized;
Long and short are mutually contrasted;
High and low are mutually posited,
Before and after are in mutual sequence.

Tao Te Ching

"Yin-Yang"
Figure 24. Microbody in developing crystal idioblast of Yucca. Note phragmoplast formation in adjacent telophase cell in upper left.

At one stroke I forgot all my knowledge! There is no use for artificial discipline, For, more as I will, I manifest the ancient way [Tao].

Hsiang-yen
Figure 25. Cytoplasm of crystal idioblast initial with well formed microbody.

The Tao, without doing anything [wu-wei] leaves nothing undone.

Lao-tzu
Figure 26. Peroxidase localization, according to Roland et al. (1972), shows activity in vacuoles and cell walls of all cells including crystal idioblast initials, in Yucca root cortex.

It ain't why, why, why, why, why, why; it just is.

Van Morrison
PART VII. A COMPARISON OF CALCIUM OXALATE CRYSTALS ISOLATED FROM CALLUS CULTURES AND THEIR EXPLANT SOURCES
A comparison of calcium oxalate crystals isolated from callus cultures and their explant sources

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Short title: Plant and callus crystals
ABSTRACT

Callus cultures were derived from explant tissues of nineteen angiosperm and gymnosperm species known to produce calcium oxalate crystals of different habits. Calluses were dark-grown in controlled-environment growth chambers on a variety of defined solid media. Eight species produced crystals in callus culture: Ginkgo biloba, Canavalia ensiformis, Glycine max, Phaseolus vulgaris, Cissus quadrangularis, Malus domestica, Capsicum annum, and Psychotria punctata. Comparisons of crystals isolated from explant sources and calluses derived from them generally show maintenance of crystal habit in culture. Several species which produced callus crystals were cultured on media which were suboptimal for callus growth. Three species produced callus crystals which were abnormal compared to their intact plant counterparts. We believe that crystal production and habit generally are species specific, under genetic control, and influenced by the metabolic status of the cells.
INTRODUCTION

Calcium oxalate crystals occur as intracellular inclusions in diverse tissues and organs of a large number of plant species. The occurrence and distribution of plant calcium oxalate crystals have attracted much investigative interest regarding their formation and possible functions in normal plant growth (Arnott and Pautard, 1970; Franceschi and Horner, 1980b; Pentecost, 1980). Numerous ultrastructural studies concerning cell development show calcium oxalate does not precipitate indiscriminately in plant tissues; rather, crystallization usually occurs within special membranes inside vacuoles of highly specialized, differentiated cells (Franceschi and Horner, 1980b), sometimes called crystal idioblasts.

Calcium oxalate deposits occur as various crystal habits; their generalized shapes and aggregates have been described (Franceschi and Horner, 1980b). The habit of calcium oxalate usually is consistent within a given plant species and often within a given taxonomic group (Arnott and Pautard, 1970). However, crystals of different habits occurring within adjacent cells were observed in a number of plants (McNair, 1932; Scurfield et al., 1973; Horner and Wagner, 1980) and the question of general genetic control of crystal habit was raised by Franceschi and Horner (1980b).

The distribution of crystal cells throughout any plant is characteristic of its species, and therefore predictable (Arnott and Pautard, 1970; Franceschi and Horner, 1980b), the number of crystals or crystal idioblasts in any one plant, however, is not fixed. The number of cells which differentiate into crystal cells was found to be affected, for instance, by availability of nutritional calcium in intact plants (Frank, 1972) as
well as ascorbate in callus cultures (Franceschi and Horner, 1979, 1980a). These studies suggest many parenchymatous cells are capable of differentiating into crystal cells if the proper inductive stimulus is provided. The reverse process may be true also. It is not known what extracellular or environmental parameters are involved with induction of crystal cell differentiation or crystal habit even though some abnormal raphide crystals were produced in callus culture (Franceschi and Horner, 1979).

This study is a survey of callus cultures from various crystal-containing explant sources established on standard and modified solid defined media. The purposes of this study are to evaluate the occurrence of differentiated crystal cells in various tissue cultures and to examine the relationship between intact plant and callus-derived crystal habits. Tissue culture results of this study are used to serve also as an evaluation of suitable systems for future experiments on crystal cell induction and differentiation.
MATERIALS AND METHODS

Tissues of nineteen higher plant species were used to initiate callus cultures on various media. Specific explant sources, media, growth regulator concentrations, media modifications and original tissue cultures are referenced (where applicable) in Table 1. Plants and explant tissues or organs were selected on the basis that they are known to contain calcium oxalate crystals (Table 2). Various generalized plant calcium oxalate crystal habits (i.e., raphides, prismatic, druses and crystal sand) are represented (Table 2).

Plant material was from either seedlings or mature plants grown in a greenhouse. Vegetative and floral organs were surface sterilized 5-17 min with 2.0% sodium hypochlorite (prepared from commercial bleach and double-distilled water; 0.1% Tween 20 was added as a wetting agent) followed by 3 rinses with sterile-distilled water for a total elapsed time of 25 min. Explants were prepared aseptically according to Yeoman and Macleod (1977) and placed on solid media in sterile disposable Petri dishes in a laminar-flow transfer hood. Inorganic macro- and micro-nutrients and organic constituents were supplied according to four separate basal culture media and modifications thereof: Murashige and Skoog (1962) medium (MS); Linsmaier and Skoog (1965) medium (LS); Schenk and Hildebrandt (1972) medium (SH); and Gamborg (1970) medium (B5). Media modifications are not necessarily those suggested by the authors for optimal growth. Media pH was adjusted to 5.7 with 1.0 N KOH and 1.0 N HCl after addition of growth regulators (prior to autoclaving) and Difco bacto-agar (8 g/l). All media were autoclaved 15 min at 120 C, dispensed, and allowed to age 4-7 days.
All cultures were initiated and maintained routinely at 25.2±0.7°C in controlled-environment growth chambers in the dark. Inoculum for subcultures was taken 20-47 days after callus induction; original explant tissue was carefully excluded. Subculturing for callus maintenance took place after 21-62 days, depending on callus growth of individual species. The calluses were then removed and the crystals were isolated and observed by the following procedures.

Crystal occurrence was determined by squashing small amounts (less than 0.01 g fresh weight) of callus on glass slides in 0.1 M phosphate buffer (pH 7.2) and viewing squashed cells with polarized light. Numerous squash preparations were made for each species since, in many cases, crystal number per callus was very low.

Crystal isolates from tissues of intact plants (specific explant sources, see Table 2) were obtained using a modification of the simple technique devised by Franceschi (1978): tissue segments were placed in a drop of water on a small piece of coverglass and teased apart to liberate the crystals. Pieces of tissue then were removed with tweezers, a few minutes were allowed for crystals to settle, and the water drawn off with filter paper. To wash the preparation, drops of water or of 75% ethanol were then applied and drawn off repeatedly. Crystals in calluses generally were present in much lower number and as a result had to be isolated by a modification of this procedure. Several pieces of callus were minced in distilled water with a razor blade in a glass Petri dish and the resultant homogenate was passed through a loosely meshed gauze to remove larger tissue fragments. Filtrate was then centrifuged at low speed for
5-10 min and washed repeatedly to eliminate some cellular debris. Final pellet was pipetted dropwise onto pieces of coverglass and dried down. All coverglass pieces with fresh (unfixed) crystals were affixed to brass discs and gold-palladium sputter coated for observation.

Chemical tests for calcium oxalate were preformed on all isolated crystals on glass coverslips according to Pohl (1965). The calcium constituent of isolated crystals was identified on the SEM with a 5000 Å Kevex X-ray energy dispersive analysis unit.

Callus pieces were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12-14 hrs at 4°C. The pieces were washed with the same buffer, three times each for 20 min. Post-fixation was in 1% OsO₄ (same buffer, pH and temperature) 1-2 hrs, followed by ethanol dehydration. After 3 changes of 100% ethanol, callus pieces were cryofractured in liquid nitrogen according to Humphreys et al. (1974). Fractured specimens were infiltrated with freon TF (113) and critical point dried with liquid CO₂. Specimens were mounted on brass discs and sputter coated with gold-palladium. Scanning electron microscopy (SEM) of callus and crystal isolates was done using a JEOL JSM-35 SEM operated at an accelerating voltage of 20 kV and 80 μA beam current. Results were recorded on Polaroid type 665 film.
Table 1. Summary of plant and explant sources for callus production, media sources and their modifications

<table>
<thead>
<tr>
<th>Plant genus/species</th>
<th>Explant source(s)</th>
<th>Media</th>
<th>Growth regulators (mg·l⁻¹)</th>
<th>Media modifications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Medicago</em> sativa</td>
<td>hypocotyl; first trifoliolate leaves</td>
<td>MS 0.2 2,4-D/0.2 K/2.0 IAA</td>
<td>B₁, (1 mg·l⁻¹) niacin (5 mg·l⁻¹)</td>
<td>glycine (2 mg·l⁻¹)</td>
<td>Churova and Holickova (1980)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>LS &quot;</td>
<td></td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>2. <em>Phaseolus vulgaris</em> (Great Northern)</td>
<td>&quot;</td>
<td>MS 0.5 2,4-D/1.0 K</td>
<td>B₁, (mg·l⁻¹) niacin (5 mg·l⁻¹)</td>
<td></td>
<td>Mok and Mok (1977)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>LS &quot;</td>
<td></td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>3. <em>Canavalia ensiformis</em></td>
<td>&quot;</td>
<td>SH 0.5 2,4-D/0.1 K/2.0 pCPA</td>
<td>none</td>
<td>glycine (2 mg·l⁻¹)</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>LS 1.0 2,4-D/0.5 BA</td>
<td></td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>4. <em>Glycine max</em> (Amsoy)</td>
<td>&quot;</td>
<td>SH 0.5 2,4-D/0.1 K/2.0 pCPA</td>
<td>none</td>
<td></td>
<td>Schenk and Hildebrandt (1972)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>LS 1.0 2,4-D/0.5 BA</td>
<td>B₁ (1 mg·l⁻¹); niacin (5 mg·l⁻¹)</td>
<td>glycine (2 mg·l⁻¹)</td>
<td>---</td>
</tr>
<tr>
<td>5. <em>Lycopersicon esculentum</em></td>
<td>young leaves, young stem internodes</td>
<td>MS 0.5 NAA/1.0 BA</td>
<td>none</td>
<td></td>
<td>Behki and Lesley (1976)</td>
</tr>
<tr>
<td>6. <em>Nicotiana glauca</em></td>
<td>young leaves</td>
<td>MS 2.0 IAA/0.2 K</td>
<td>none</td>
<td></td>
<td>Murashige and Skoog (1962)</td>
</tr>
<tr>
<td>7. <em>Capsicum annum</em></td>
<td>young leaves, young stems</td>
<td>LS 1.0 2,4-D/0.5 BA</td>
<td>glycine (2 mg·l⁻¹)</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>MS 2.0 IAA/0.2 K</td>
<td>none</td>
<td></td>
<td>---</td>
</tr>
</tbody>
</table>
Table 1. (continued)

<table>
<thead>
<tr>
<th>Plant genus/species</th>
<th>Explant source(s)</th>
<th>Media</th>
<th>Growth regulators (mg·l⁻¹)</th>
<th>Media modifications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. <em>Vitis vinifera</em></td>
<td>young leaves, petioles</td>
<td>MS</td>
<td>0.1-1.0 2,4-D/0.1 BA</td>
<td>none</td>
<td>Krul and Worley (1977)</td>
</tr>
<tr>
<td>9. <em>Cissus quadrangularia</em></td>
<td>young leaves, petioles, stem internodes</td>
<td>MS</td>
<td>&quot;</td>
<td>none</td>
<td>---</td>
</tr>
<tr>
<td>10. <em>Psychotria punctata</em></td>
<td>young leaves, young stem internodes</td>
<td>LS¹</td>
<td>2.0-5.0 NAA/0.3 K</td>
<td>none</td>
<td>---</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>LS²</td>
<td>1.0 2,4-D/0.5 BA</td>
<td>none</td>
<td>LaMotte and Lersten (1972) modifications</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>LS³</td>
<td>5.0 IAA/0.5 BA</td>
<td>none</td>
<td>---</td>
</tr>
<tr>
<td>11. <em>Malus domestica</em> (mac 9)</td>
<td>young leaves</td>
<td>LS</td>
<td>4.0 NAA/0.3 K</td>
<td>LaMotte and Lersten (1972) modifications</td>
<td>---</td>
</tr>
<tr>
<td>12. <em>Citrus aurantium</em></td>
<td>young stem internodes</td>
<td>MS</td>
<td>4.0 NAA/1.0 BA</td>
<td>Murashige and Tucker (1969) modifications</td>
<td>Kockba and Spiegel-Roy (1977)</td>
</tr>
<tr>
<td>13. <em>Beta vulgaris</em></td>
<td>petioles, anthers</td>
<td>LS</td>
<td>1.0 NAA/0.5 BA</td>
<td>Rogozinska et al. (1977) modifications</td>
<td>Rogozinska et al. (1977)</td>
</tr>
<tr>
<td>14. <em>Allium cepa</em></td>
<td>inner bulb scales</td>
<td>B5</td>
<td>1.0 2,4-D/0.1 K</td>
<td>4 g/l sucrose; pH 5.5</td>
<td>Fridborg (1971)</td>
</tr>
<tr>
<td>15. <em>Zebrina pendula</em></td>
<td>very young stem</td>
<td>MS</td>
<td>1.0-3.0 2,4-D/0.3 BA</td>
<td>Hunault (1979a) modifications</td>
<td>Hunault (1979a,b,c)</td>
</tr>
<tr>
<td>Plant genus/species</td>
<td>Explant source(s)</td>
<td>Media</td>
<td>Growth regulators (mg·l⁻¹)</td>
<td>Media modifications</td>
<td>References</td>
</tr>
<tr>
<td>--------------------</td>
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<td>------------</td>
</tr>
<tr>
<td>16. <em>Ornithogalum caudatum</em></td>
<td>inflorescence stems</td>
<td>MS</td>
<td>1.5-2.0 2,4-D/0.04 K</td>
<td>none</td>
<td>Hussey (1975)</td>
</tr>
<tr>
<td>17. <em>Sansevardia trifasciata</em></td>
<td>young leaves, stems</td>
<td>MS</td>
<td>1.0-3.0 2,4-D/0.3 BA</td>
<td>Hunault (1979a) modifications</td>
<td>Hunault (1979a,b,c)</td>
</tr>
<tr>
<td>18. <em>Hyacinthus</em> sp.</td>
<td>inflorescence stems</td>
<td>MS</td>
<td>1.5-2.0 2,4-D/0.04 K</td>
<td>none</td>
<td>Hussey (1975)</td>
</tr>
<tr>
<td>19. <em>Ginkgo biloba</em></td>
<td>young leaves, petioles</td>
<td>LS</td>
<td>0.1-1.0 2,4-D/0.5 BA</td>
<td>glycine (2 mg·l⁻¹)</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 2. Summary of plant sources, crystal habits and quality of callus growth and crystal production on different defined media

<table>
<thead>
<tr>
<th>Plant genus/species</th>
<th>Crystal habit in explant tissue</th>
<th>Reference</th>
<th>Media</th>
<th>Callus growth(^a)</th>
<th>Friability(^b)</th>
<th>Relative crystal content per callus(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Medicago sativa</td>
<td>prismatic</td>
<td>Metcalfe and Chalk (1950)</td>
<td>MS</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LS</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2. Phaseolus vulgaris</td>
<td>prismatic</td>
<td>Horner and Zindler-Frank (1982)</td>
<td>MS</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LS</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SH</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4. Glycine max</td>
<td>prismatic</td>
<td>Metcalfe and Chalk (1950)</td>
<td>LS</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SH</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5. Lycopersicon esculentum</td>
<td>crystal sand</td>
<td>Metcalfe and Chalk (1950)</td>
<td>MS</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6. Nicotiana glauca</td>
<td>crystal sand</td>
<td>Franceschi and Horner (1980b)</td>
<td>MS</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>7. Capsicum annuum</td>
<td>crystal sand, druse, prismatic</td>
<td>Horner and Wagner (1980)</td>
<td>LS</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MS</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\)Relative callus growth: 1-5 = slow-fast; based on growth of Nicotiana callus.

\(^b\)Relative callus friability: 1-5 compact-friable; based on quality of Nicotiana callus.

\(^c\)Relative crystal number per callus: 0-5 = none-many; based on crystal content of Psychotria LS3 callus.
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Plant genus/species</th>
<th>Crystal habit in explant tissue</th>
<th>Reference</th>
<th>Media</th>
<th>Callus growth</th>
<th>Friability</th>
<th>Relative crystal content per callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. <em>Vitis vinifera</em></td>
<td>raphide</td>
<td>Arnott (1973)</td>
<td>MS</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9. <em>Cissus quadrangularia</em></td>
<td>raphide and druse</td>
<td>Kausch (unpublished, The Rockefeller University)</td>
<td>MS</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>10. <em>Psychotria punctata</em></td>
<td>raphide</td>
<td>Horner and Whitmoyer (1972)</td>
<td>LS^1</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LS^2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LS^3</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>11. <em>Malus domestica</em></td>
<td>druse</td>
<td>Metcalfe and Chalk (1950)</td>
<td>LS</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>12. <em>Citrus aurantium</em></td>
<td>prismatic</td>
<td>Arnott (1973)</td>
<td>MS</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>13. <em>Beta vulgaris</em></td>
<td>various</td>
<td>Al-Rais et al. (1971)</td>
<td>LS</td>
<td>1</td>
<td>4</td>
<td>0(^d)</td>
</tr>
<tr>
<td>15. <em>Zebrina pendula</em></td>
<td>raphide</td>
<td>Wheeler (1979)</td>
<td>MS</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>16. <em>Ornithogalum caudatum</em></td>
<td>raphide</td>
<td>Tilton and Horner (1980)</td>
<td>MS</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>17. <em>Sansevaria trifasciata</em></td>
<td>raphide</td>
<td>---</td>
<td>MS</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^d\) Some cells in some cultures contained crystals, see results.
<table>
<thead>
<tr>
<th>Plant genus/species</th>
<th>Crystal habit in explant tissue</th>
<th>Reference</th>
<th>Media</th>
<th>Callus growth</th>
<th>Frailty</th>
<th>Relative crystal content per callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>18. <strong>Hyacinthus sp.</strong> raphide</td>
<td>Kohl (1899)</td>
<td>MS</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19. <strong>Ginkgo biloba</strong> druse</td>
<td>Arnott (1973)</td>
<td>LS</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

At the end of the second subculture passage (culture specific) calluses were examined for presence of calcium oxalate crystals. Of the nineteen species which produced callus (Table 1), eight contained crystals (Table 2). These eight species included: Ginkgo biloba, Canavalia ensiformis, Glycine max, Phaseolus vulgaris, Cissus quadrangularia, Malus domestica, Capsicum annuum, and Psychotria punctata.

In general, crystal cells were about the same size as surrounding cells of the same callus which do not contain crystals. However, crystal cells of Ginkgo were always small and spherical, despite a high variability in size and shape of other callus cells. Some cells in Ginkgo callus were long and tubular; but these never were observed to contain crystals. Differentiated, lignified xylem elements also were distinguished easily under polarized light, and were commonly observed in Canavalia, Glycine, Malus, Capsicum, and Psychotria callus cultures. Crystal cells often were located near xylem nodules which did not separate readily during squash preparation in these species. Furthermore, except for Psychotria, these species produced relatively compact callus (Table 2).

Micrographs of cryofractured callus cultures show general cell morphology, cell size variability and relative callus friability (Figures 1–8). The purposes for processing and cryofracturing callus tissues for SEM were to observe crystals in situ and gain information about cells which form them. Impetus for this examination was gained from earlier studies of Psychotria cultures (Franceschi and Horner, 1979, 1980a,b;
Horner and Franceschi, 1981). However, except for Psychotria, cultures either did not contain sufficient numbers of crystal cells (Table 2) or they were too friable (Figures 1, 3, 4, 5) to realize these types of observation. When cryofractured by conventional procedures (Humphreys et al., 1974), friable callus breaks apart without exposing internal features of many of its cells.

Comparisons between crystal isolates from explant source-tissues and calluses generally show maintenance of crystal habit in culture (Figures 9-16; 17-25).

Ginkgo biloba produces two sizes of druse crystals in leaves of intact plants. Large, 80-120 μm druses (Figure 9) are found in vein sheath cells and smaller, 7-10 μm druses are formed in files of phloem cells. Ginkgo callus crystals measure 50-80 μm (Figure 10). Ginkgo callus crystals have numerous small facets associated with the larger crystals of a druse (Figure 10) giving these crystals a rough appearance.

The three legumes species (Canavalia ensiformis, Glycine max, and Phaseolus vulgaris) all produce twin prismatic crystals in leaf tissues of intact plants as well as in callus cultures (Figures 11-16). These crystals in intact plants are known to be surrounded by a cellulosic sheath (Horner and Zindler-Frank, 1982) making them difficult to isolate. Ensheathed crystals isolated from callus cells were obtained only from Phaseolus culture (Figure 17). It is not known if a similar sheath is formed in all cultured cells of the legume species. Canavalia and Phaseolus both produced callus crystals identical to those isolated from tissues of intact plants whereas Glycine callus produced larger prismatic
crystals which lacked the prominent central ridge of crystals from intact plants. We allowed some cultures to grow continuously on the same medium without subculturing for several months. In these older cultures of *Glycine*, small acicular crystals formed in many cells. Similar crystals were not observed in other aged cultures.

*Cissus quadrangularia* produced both raphides and druses in stems and leaves of intact plants (Figure 17). The raphides are notched at one end and pointed at the other (Figure 17); these crystals may be twins along their longitudinal axis. Raphide crystals are especially abundant in stem tissues. Callus cells of *Cissus* did not produce either raphides or druses, but single styloid crystals instead (Figure 18). The predominant crystals observed in *Cissus* callus were styloids of various lengths similar to that represented in Figure 18. However, a few cells observed in squash preparations contained a number of very small needle-shaped crystals. These crystals were not stationary, but drifted about, apparently under Brownian influence, within the central vacuole. It is not likely that these are young raphide idioblasts as no cells with large raphides were observed.

Most calluses produced from *Beta vulgaris* anthers and petioles did not produce crystals. Interestingly, however, some calluses contained a number of crystal cells. These crystal cells were from callus grown on the same medium, from the same explant source, and processed identically to those that did not produce crystals. Occasionally, they were found in the same culture dish. Large cells from *Beta* callus contained numerous
(5-20) bipyramidal-shaped crystals, but this did not occur consistently as already mentioned.

Druse crystals from intact leaves of *Malus* (Figure 19) were indistinguishable from those isolated from the slow-growing callus of that species (Figure 20).

The individual crystals which comprise the crystal sand from leaves, petioles and stems of *Capsicum annuum* (Figure 21) are highly variable in shape and separate from each other when liberated from crystal cells. The crystals isolated from callus cells were similar and also variable in shape, but generally smaller (Figure 22).

Extensive observations have been previously reported on four-sided raphide crystals of *Psychotria* (Horner and Whitmoyer, 1972; Franceschi and Horner, 1979, 1980a,b; Figure 23). Raphide crystals from *Psychotria* callus were similar in morphology, yet again generally smaller (both in length and width) than those formed in an intact plant (Figure 24). Not all callus lines of *Psychotria* produce crystals (see Tables 1 and 2). Crystals were found only in those cultures grown on LS media containing either 1.0 (mg·l⁻¹) 2, 4-D; 0.5 (mg·l⁻¹) BA (LS²) or 5.0 (mg·l⁻¹) IAA: 0.3 (mg·l⁻¹) K (LS³). The latter culture (LS³) was grown on LS medium modified according to LaMotte and Lersten (1972) and contained a noticeably higher number of crystals than other culture lines. This medium was utilized by Franceschi and Horner (1979; 1980a) and Horner and Franceschi (1981). The cultures grown on this medium exhibited better growth than *Psychotria* cultures grown on other media or on different growth regulator
combinations and produced large, friable calluses suitable for suspension culture initiation.

Several species (e.g., *Medicago sativa*, *Phaseolus vulgaris*, *Canavalia ensiformis*, *Glycine max*, and *Capsicum annuum*). Different media produced calluses with different growth characteristics (i.e., rate of growth, friability, and production of callus crystals). *Medicago* callus did not produce crystals on either LS or MS medium, but xylem differentiated on both of these media. *Canavalia* and *Glycine* produced crystals as shown previously (Figures 12, 14) and xylem only on LS medium. Both species produced callus crystals and only small amounts of xylem on the SH medium and no callus crystals. However, acicular crystals were produced in aged *Glycine* cultures grown on the SH medium. *Phaseolus* and *Capsicum* produced both callus crystals and xylem on both LS and MS media.

None of the dark-grown calluses developed buds or shoots on the media used (Table 1). This is probably due to the widespread use of the auxin 2, 4-D for callus initiation in most cultures. Roots were formed only on some of the calluses including: *Phaseolus vulgaris*, *Glycine max* and *Psychotria punctata*. All of the callus cultures were growing with a healthy appearance at the time evaluated for the presence of crystals (during the second subculture passage). However, several underwent senescence with the growth regulator conditions specified in Table 1 during the fourth to sixth subculture passage. These species include: *Ginkgo biloba*, *Cissus quadrangularis*, *Beta vulgaris*, *Zebrina pendula*, *Ornithogalum caudatum*, *Hyacinthus* spp., and *Sansaeveria trifaciata*. We believe this is because of the relatively high concentrations of auxin
used to initiate callus for these species. No attempt was made to optimize either growth conditions, or crystal formation for any cultures grown during this investigation.
Figures 1-8. Scanning electron micrographs of portions of callus cultures from eight species. See Table 2. Cultures were cryofractured during processing to show degree of cell compactness within mass. Bars equal 100 µm for all figures.

Figure 1. Ginkgo biloba
Figure 2. Canavalia ensiformis
Figure 3. Glycine max
Figure 4. Phaseolus vulgaris
Figure 5. Cissus quadrangularis
Figure 6. Malus domestica
Figure 7. Capsicum annuum
Figure 8. Psychotria punctata
Figures 9-16. Scanning electron micrographs of isolated crystals from intact plants (IP) and tissue cultures (TC) from four species. Bars equal 10 μm for Figure 9, 10; 5 μm for Figure 11-16. Cracks in crystals shown in Figures 12 and 16 are result of electron heat damage.

Figure 9. *Ginkgo biloba* druse; IP
Figure 10. *Ginkgo biloba* vein sheath druse; TC
Figure 11. *Canavalia ensiformis* prismatic crystal; IP
Figure 12. *Canavalia ensiformis* prismatic crystal; TC
Figure 13. *Glycine max* prismatic crystal; IP
Figure 14. *Glycine max* prismatic crystal; TC
Figure 15. *Phaseolus vulgaris* ensheathed prismatic crystal; IP
Figure 16. *Phaseolus vulgaris* ensheathed prismatic crystal; TC
Figures 17-25. Scanning electron micrographs of isolated crystals from intact plants (IP) and tissue cultures (TC) from four species. Bars equal 10 μm for Figures 18, 19; 5 μm for Figure 17, 20-22, 24, 25; 1 μm for Figure 23.

Figure 17. *Cissus quadrangularia* notched raphides; IP
Figure 18. *Cissus quadrangularia* druse; IP
Figure 19. *Cissus quadrangularia* styloid crystal; TC
Figure 20. *Malus domestica* druse; IP
Figure 21. *Malus domestica* druse; TC
Figure 22. *Capsicum annuum* crystal sand; IP
Figure 23. *Capsicum annuum* crystal sand; TC
Figure 24. *Psychotria punctata* raphide crystal; IP
Figure 25. *Psychotria punctata* raphide crystal; TC
DISCUSSION

The value of tissue cultures as experimental systems for the study of calcium oxalate crystal idioblasts has already been established (Paupardin, 1964; Franceschi and Horner, 1979, 1980a; Horner and Franceschi, 1981), and this investigation reinforces earlier contentions.

Crystals isolated from calluses were similar than those in parts of whole plants from which the callus had originated. Whole plants that produce calcium oxalate crystals of specific habit apparently do so under genetic control. This control is reflected in the fact that crystal habit is usually consistent within a given plant species and/or is tissue specific and predictable (Franceschi and Horner, 1980b). This notion is further supported by the observations presented in this study that crystal habit is maintained in calluses grown on defined media. Heritable expression of crystal shape could result from gradients of components involved in calcium oxalate crystal formation established because of species specific cell and tissue arrangements. Species-specific maintenance of crystal shape in a callus would occur if these gradients were mimicked in culture although this seems unlikely. Alternatively, genetic expression of crystal habit may occur within individual cells, during differentiation of the complex structures and cell components (apparently necessary for the crystallization process) in crystal idioblasts. If this is so, then media conditions which support crystal idioblast differentiation should produce crystals indicative of that species.

Crystal idioblasts are highly specialized, differentiated cells which contain an array of unique structures and modifications involved in the
crystallization process (Franceschi and Horner, 1980b). Evidently, calcium oxalate typically does not precipitate in vacuoles of nonspecialized cells. Crystal idioblasts of *Psychotria punctata* callus exhibit modifications similar to cells in the intact plant (Franceschi and Horner, 1979, 1980). We presume isolated callus crystals also were produced in differentiated crystal idioblasts (Franceschi and Horner, 1979, 1980a).

The presence of calcium oxalate crystals probably is related to specific media or other callus growth conditions. We observed that in the same species a specific medium produces callus with crystals whereas another does not (see Table 2). A medium which evokes crystal formation by callus of one species may not do so for callus of another species. The reasons for this could be manifold but are not known. Previous studies suggest that many parenchymatous cells have the capability of differentiating into crystal idioblasts under proper inductive conditions (Paupardin, 1964; Frank, 1972; Franceschi and Horner, 1979, 1980a). Since it is not known if crystal idioblasts are terminally differentiated, it is also possible that these specialized cells might later undergo dedifferentiation. If this is true, then any callus initiated from crystal-containing explants might be capable of forming crystal idioblasts under culture conditions which favor their differentiation. However, it is also possible that the media which produce calcium oxalate cells in culture select for growth of cells in original explant tissues that possess this capability or genetic competency (Street, 1977). This might explain why calluses derived from explants containing more than one crystal habit (*Capsicum annuum* and *Cissus quadrangularia*) produced only one type of
crystal in culture. The differences in these possibilities may be interrelated, but have not been discerned.

The three lines of *Psychotria* callus described in this study all were grown on different modifications of LS media and possess different capacities to form calcium oxalate crystals. *Psychotria* calluses LS\(^1\) and LS\(^2\) (see Table 1) differed only in type of growth regulator and its concentration supplied. The LS\(^1\) medium contained 4.0 (mg\(\cdot l^{-1}\)) NAA and 0.3 (mg\(\cdot l^{-1}\)) K, while the LS\(^2\) medium contained 1.0 (mg\(\cdot l^{-1}\)) 2, 4-D and 0.5 (mg\(\cdot l^{-1}\)) BA. Only LS\(^2\) produced raphide crystals whereas LS\(^1\) grew more rapidly and presented a more suitable system for suspension cell culture initiation. Apparently, crystal idioblast differentiation is related to the type and/or concentration of growth regulator(s) in the medium, and perhaps to its ratio. Numerous reports point to the putative role of phytohormones as important to differentiation of vascular elements in culture (Reinert et al., 1977). For example, Dalessandro and Roberts (1971) found xylem differentiation was induced at low concentration of 2, 4-D in *Lactuca* pith parenchyma explants, but not with IAA or NAA. However, it generally has been concluded (Reinert et al., 1977) that growth regulator concentrations certainly are not the only factor involved in formation of specialized cells from undifferentiated tissues. The third *Psychotria* culture (LS\(^3\)), grown on LS medium with LaMotte and Lersten (1972) modifications with 5.0 (mg\(\cdot l^{-1}\)) IAA and 0.3 (mg\(\cdot l^{-1}\)) K, produced a noticeably larger quantity of crystals than did callus grown on LS\(^2\). The modifications of the basal LS medium used by LaMotte and Lersten (1972) include deletion of all NH\(_4\)NO\(_3\), and the addition of 1.32 (g\(\cdot l^{-1}\)) lactalbumin hydrolysate. This medium
also was used (with different growth regulators) for *Malus domestica*, which also produced callus crystals. Both of these modifications may affect idioblast differentiation and currently are under further investigation in our laboratory. It is our contention that *Psychotria punctata* represents the best system available to date for investigation of calcium oxalate crystal formation in culture situations.

Three species we investigated produced callus crystals which were abnormal with respect to their intact-plant counterparts. It is not known whether these types of crystals are produced in tissue(s) of the intact plant other than the explant source tissues we examined. *Glycine* callus produced prismatic crystals, which were modifications of those in intact leaf tissue. Also, this species produced acicular crystals in older cultures. *Cissus* callus produced styloid crystals in culture when its explants contained raphides and druses. Perhaps styloid crystals are merely individual raphide crystals produced one to a cell. It is interesting that some cells contained a number of very small needle-shaped (raphide?) crystals. It should be noted that this callus grew very slowly and could not be maintained further than four subculture passages. Similarly, *Beta* callus underwent senescence on the applied medium of Rogozinska et al. (1977; see Table 1) after four passages. This culture inconsistently produced bipyramidal crystals in some calluses. Cells in these cultures were extremely large.

We do not understand why some calluses contained crystal-containing cells and others did not. Abnormal raphide crystals also have been previously reported in *Psychotria* callus (Franceschi and Horner, 1979). The
formation of abnormal callus crystals may reflect mutations induced during culture and/or the metabolic condition of the callus. It is also possible that physical and chemical conditions of the medium on which the callus is grown affects crystal shape (see Cody et al., 1982).

The presence of calcium oxalate crystals in callus may be useful for evaluation of callus differentiation, or differentiation potential, similar in this regard to xylogenesis and production of secondary metabolites (Aitchison et al., 1977). The production of a large number of secondary metabolites by plant cultures has been reported (for reviews see: Constable et al., 1974; Stohs and Rosenberg, 1975; Butcher, 1977). Calcium oxalate crystals could be regarded as secondary metabolites (Esau, 1977). It has been observed repeatedly that maximal production of secondary metabolites in callus cultures does not coincide with optimal cell or tissue growth conditions (Aitchison et al., 1977). For example, production of trigonelline by tissue cultures of *Trigonella foenum-graceum* was inversely proportional to the growth index of those cultures (Khanna and Jain, 1972, 1973). We noted that a number of calluses which produce crystals are those that grow under suboptimal conditions (see Table 2). Conversely, cultures growing under conditions suggested to be optimal by the authors who had previously established these conditions did not produce crystals.

In many cases, the production of secondary metabolites in slow-growing cultures has been correlated with an increased level of histological and morphological differentiation (Aitchison et al., 1977) and/or the appearance of specialized cells (Neuman and Mueller, 1974; Becker, 1970;
Reinhard et al., 1968). Aitchison et al. (1977) suggested that this apparent correlation may be explained by the ability of vacuolated cells to accumulate, rather than simply produce these metabolites. In the case of calcium oxalate idioblasts, the cells would become specialized for the accumulation of these metabolites. Furthermore, the conditions which favor calcium oxalate formation also may favor structural differentiation, as both may be considered expressions of biochemical specialization that are not necessarily linked.
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DISCUSSION WITH REVIEWERS

J. Arditti: Since not all plant hormones survive autoclaving, why were they not cold sterilized?

Authors: The literature presents conflicting information on the heat stability of growth regulators used in this study. We have autoclaved our media containing the growth regulators in accordance with original culture references (Table 1). We continued this practice to maintain consistency for other nonreferenced media which contained the same growth regulators.

J. Arditti: The authors note that more than one crystal form has been observed in different cells of some intact plant tissues. How can this fact be reconciled with the hypothesis that control of crystal shape is strictly genetic?

Authors: We believe that crystal shape is an expression of specific cell differentiation. Intact plant tissues often contain adjacent cells which are considerably differentiated from one another. In callus cultures derived from explants containing more than one crystal habit, differentiation of crystal cells which form one habit versus another may be influenced by a large number of factors including medium constituents.

H. J. Amott: Is it possible that the bipyramidal crystals produced in Beta vulgaris callus were not calcium oxalate?

Authors: Yes. The crystals in Beta calluses were not produced in sufficient number to be isolated by our technique. The chemical determination for calcium oxalate (Pohl, 1965) was not applied to the acicular crystals in aged Glycine calluses, the small "raphides" in Cissus callus or the
bipyramids in Beta callus. The bipyramids of Beta are not as birefringent under polarized light as the calcium oxalate crystals we observed, and hence may be composed of other compounds. However, similar appearing bipyramids have been identified as calcium oxalate dihydrate.

V. R. Tilton: It is well-known in in vitro systems that individual genotypes within a species may respond differently to identical conditions. What safeguards does your experimental design incorporate to preclude this prejudice in your results?

Authors: The objectives of this study were to determine if crystal habit was maintained in callus culture and to find a suitable system for future experiments on crystal cell induction and differentiation. Other genotypes of the same species (Table 1) might well produce crystals under the same conditions (Table 2); also, altered conditions might result in crystal production in those species which did not form crystals in our study. We do not think this a prejudice in our results.

V. R. Tilton: Which endogenous phytohormone(s) do you think has the greatest influence on crystal production in vivo?

Authors: Evidence for growth regulator effects on crystal cell differentiation in this study is inconclusive. The effects of different auxins and cytokinins (supplied at various ratios) on callus crystal and oxalate production are currently under examination in our laboratory. We have noted that a number of cultures in this study produce crystal cells when grown under suboptimal conditions. It is possible that a balance between endogenous growth regulator concentrations and secondary metabolites and
their precursors, is more important to crystal cell differentiation than rate of tissue growth or metabolism.

E. Zindler-Frank: You state that it is possible that the media which produce calcium oxalate cells in culture select for growth of cells in original explant tissues that possess this capability. Isn’t this unlikely in view of the situation in Canavalia? In this species, crystals are predominantly formed in the epidermis and epidermal cells usually do not contribute much to callus growth.

Authors: Cells or tissues capable of forming crystal cells in culture need not necessarily be crystal-containing cells or tissues in the intact plant. It is conceivable that some cells in a leaf could retain the ability for differentiation into a wide range of cell types, while others may not. Selection and proliferation of such cell populations may be favored on one medium and excluded on another. The ability for crystal cell differentiation may be also influenced within the callus during growth as endogenous levels of medium components, including growth regulators, are continually changing.

E. Zindler-Frank: What is the distribution of crystals in the calluses?

Authors: Distribution and orientation of callus crystal cells were not determined in this study. However, further investigations in our laboratory are specifically concerned with these aspects and how distribution and number are affected by media modifications.
E. Zindler-Frank: The individual crystals of the crystal sand of Capsicum are very different in size. Is this a speciality of this species, or is this known of other species with crystal sand, too?

Authors: The crystal sand of Lycopersicon esculentum and Nicotiana glauca is composed of individual crystals which are also highly variable in shape and size; Franceschi and Horner (1980b) show crystal sand of Nicotiana glauca with SEM. The variability in size and shape of individual crystals of crystal sand may be characteristic of this crystal habit and not specifically of Capsicum crystals.
APPENDIX
Figures 25-32. Paired brightfield and polarized light micrographs of differentiated crystal cells from callus cultures of eight species. Bars equal 10 μm for all figures.

Figure 25. Ginkgo biloba
Figure 26. Canavalia ensiformis
Figure 27. Glycine max
Figure 28. Phaseolus vulgaris
Figure 29. Cissus quadrangularia
Figure 30. Malus domestica
Figure 31. Capsicum annuum
Figure 32. Psychotria punctata

The highest good is like water,
for the good of water is that it nourishes everything without striving.
It occupies the places of lowest level.
It is thus that Tao in the world is like a river going down the valley to the ocean.
The most gentle thing in the world overrides the most hard.
Nothing in the world is weaker than water,
but it has no better in overcoming the hard.

Chuang-tzu
This dissertation presents a wide array of developmental and physiological aspects about calcium oxalate crystal idioblasts. Conclusions are presented in some detail within each discussion section of the various parts. It is not reasonable that details be reiterated here, but some generalities concerning development, physiology and function of these cells in higher plants may be reached and clarified from overview. Also, because of time requirements, two incomplete investigations could not be formally presented, and those results will be briefly described here to supplement some general conclusions. The results of those investigations will be prepared for publication in the near future.

In the first three parts, morphological and developmental aspects of calcium oxalate crystal idioblast differentiation are described. Knowledge of these events provides a degree of understanding concerning plant cell specializations, and insight into crystal idioblast functioning during normal plant tissue and organ development. A summary of the developmental aspects presented here may be useful for understanding generalities of crystal idioblast differentiation.

Vanilla planifolia root crystal idioblast initials are first distinguished from surrounding cells by intense fluorescence of the cytoplasm with the acridine orange method, indicating high RNA content and a metabolically active system. Nuclear and nucleolar enlargement gradually ensues and continues throughout idioblast development. Crystal chambers form de novo in association with electron-dense amorphous material within the central vacuole. The chambers approximate crystal shape even before
crystallization commences inside the chambers. After formation of a raphide bundle, vacuolar cables of tubules become associated with the crystal chambers and extend into a ramified vacuolar complex. Crystalloplastids differentiate lobed regions similar to those seen in other mucilaginous raphide idioblasts, and their development precedes mucilage accumulation. During mucilage production, the raphides become enveloped in lamellated sheaths and change from being four-sided in cross-section to six- and eight-sided. Many aspects of crystal idioblast development in aerial roots of Vanilla planifolia, described in Part I, are common to differentiation of similar cells observed in other plants.

In Part II, results of the first cytophotometric study on enlarged crystal idioblast nuclei are presented. Idioblast nuclear DNA contents ranged from 4 C to 32 C (106 pg) and averaged 5.9 times that of parenchyma telophase nuclei. Frequency distribution of individual DNA content measurements depicts multiple genomes (increasing with geometric periodicity) to the 8C level, followed by less strict DNA replication within the idioblast genome. The largest nuclei had the highest DNA content. Endomitotic stages are observed with light and electron microscopy. DNA content values above the 8C level do not fit the geometrical order which is expected if the total genome is replicated during each endo-cycle, indicating differential DNA replication. Chromocenter counts substantiate the occurrence of endomitosis to the 8C level, and suggest heterochromatin underreplication in higher endopolyploid nuclei. It would be of interest to learn how widespread endopolyploidy occurs during crystal idioblast development and to understand its function at a molecular level.
The results of the investigation presented in Part III provide an experimental system for further study of idioblast cytodifferentiation in intact plant tissues and reiterates several aspects of idioblast development seen in other plants. Excised primary roots of *Yucca torreyi* were cultured in various media. Microscopic observations and growth curve information were used to deduce that idioblast initials require about 24 hours to fully differentiate. Crystal chamber production and crystal initiation occur within 2-4 h, but crystal growth continues until the idioblast is fully differentiated. Crystal idioblast development in isolated root cultures occurs normally relative to those cells in intact roots. In vacuoles of idioblast initials, an electron-dense amorphous material was observed in association with paracrystalline bodies and membranous crystal chambers. It is suggested that the paracrystalline bodies represent a crystallized storage form (probably proteinaceous) of the amorphous material, and perhaps it is this material which contributes to or influences crystal chamber formation. Crystallization occurs within, and apparently controlled by crystal chamber membranes, as in *Vanilla* idioblasts. Many aspects of *Yucca* idioblast development are common to differentiation of similar cells in intact organs of other plants. Based upon the results of this study, the isolated root culture system of *Yucca* will be useful for future physiological investigations of oxalate metabolism and control of idioblast cytodifferentiation.

The current knowledge of raphide crystal idioblast differentiation, including results from this dissertation and previous investigations, shows a series of ultrastructural events which appear common to many
plants and may be generalized. These events occur chronologically as
summarized in the following sequence:

1) Meristematic cells differentiate early in comparison to sur-
rounding nonidioblastic cells. Idioblast initials may be characterized by
a dense protoplasm, high cytoplasmic RNA content, eoplasts or undifferen-
tiated crystalloplastids, enlarged nuclei and nucleoli, and low peroxi-
somal profile frequency.

2) Small vacuoles, which apparently are dictyosome products, coalesce
to form a single, large central vacuole. Central vacuole formation is
precocious relative to surrounding cells. Vacuolar material of an unknown
composition, or vacuolar vesicles, contributes or facilitates crystal
chamber formation. Crystal chambers may be peculiar biological membranes
(if indeed they are membranes); they are definitely not derived from in-
vaginations of the plasma membrane and are truly intravacuolar. Para-
crystalline bodies may represent a crystallized form of the amorphous
vacuolar material observed in association with crystal chambers. In the
cytoplasm, eoplasts assume a distinctive appearance of crystalloplastids.

3) Nuclear enlargement occurs in many (but certainly not all) raphide
crystal idioblasts, and endopolyploidy was observed in *Vanilla*. Perhaps
in some plants, endo-cycling cells are necessary for rapid and continuous
differentiation in meristematic tissues, while other plants have evolved
similar mechanisms to achieve the same effect. Alternatively, endo-cycles
may occur in all plant cells as a consequence of differentiation, but at
levels not readily detectable in all tissues.
4) Crystal formation occurs within and apparently controlled by the crystal chambers. The crystal chambers may function as calcium oxalate molds for crystallization or as membranes which control transport rates of crystal precursors. In any case, the crystal chambers seem responsible for control of crystal shape. Crystal shape is apparently under general genetic control by the idioblast during development, and not greatly influenced by environmental factors.

5) In mucilaginous crystal idioblasts, crystalloplastids develop lobed regions and become enlarged, and mucilage accumulates within the central vacuoles. Vacuolar cables of tubules, observed only in monocotyledonous crystal idioblasts, become attached to crystal chambers and extend into a ramified vacuolar channel network. Secondary lamellations form around crystal chambers and crystals may change shape.

6) At maturity, most crystal idioblasts are reportedly living cells, although syncitial tubes formed by dead raphide idioblasts have been reported in some plants.

Obviously, calcium oxalate does not merely precipitate indiscriminantly in plant cells or tissues. The cells involved in calcium oxalate accumulation are quite specialized for that function, and this degree of cell specialization is, no doubt, energy expensive. Therefore, one must assume that formation of these cells is necessary to normal growth and development in plants where they are found. However, the functional role of these cells remains uncertain.

It is of significant interest that cells which accumulate large amounts of insoluble oxalate differentiate to exclude a key enzyme,
glycolate oxidase, involved in three major pathways of oxalic acid biosynthesis. It is possible that oxalate for crystal synthesis is generated in surrounding tissues and transported into developing idioblasts. This is especially reasonable since unspecialized peroxisomes in those cells are shown to contain glycolate oxidase activity; expression of glycolate oxidase activity in nonphotosynthetic cells (i.e., root tissues) strongly suggests that oxalate is generated in those cells via a glyoxylate intermediate.

Recent autoradiographic studies have shown that primary roots of *Yucca torreyi* readily absorb and utilize L-"C\(^{14}\)-L-ascorbic acid for crystal synthesis (Figures 1-8). Generation of oxalic acid via ascorbate metabolism does not depend on peroxisomal enzymes (i.e., glycolate oxidase), and it is possible that this is a preferred pathway for oxalate synthesis for crystals in some plants. Perhaps, oxalate generated by glycolate oxidase mediated reactions may inhibit by feedback or interfere with oxalate synthesis via ascorbate breakdown. Furthermore, the autoradiograms suggest that the crystalloplastids may be the site of ascorbate breakdown. If, indeed, ascorbate metabolism is the preferred pathway for oxalic acid synthesis in crystal idioblasts, then the question is immediately raised about the consequence of tartaric acid production. Furthermore, perhaps by deleting glycolate oxidase, any glyoxylate generated in these cells must be shunted into glycine and serine synthesis given a nonlimiting glutamate supply. One might imagine, therefore, that glutamate might be necessary for cytodifferentiation of crystal idioblasts.
Figures 1-8. Autoradiograms of 1-\textsuperscript{14}C-ascorbate labelled \textit{Yucca torreyi} roots, showing incorporation of label, after 3 h limited exclusively to developing crystal idioblasts. Developing crystal idioblasts at all stages of crystal formation incorporate label.

Figures 1, 2. Incorporation of label into young crystal idioblasts. Focus on section and silver grains (respectively). 470X

Figures 3, 4. Incorporation of label into maturing crystal idioblasts. Focus on section and silver grains (respectively). 470X

Figures 5, 6. Incorporation of label during mucilage production. Focus on section and silver grains (respectively). 470X

There is only a spiritual world; what we call the physical world is the evil in the spiritual one, and what we call evil is only a necessary moment in our endless development. In a light that is fierce and strong one can see the world dissolve. To weak eyes it becomes solid, to weaker eyes it shows fists, before still weaker eyes it feels ashamed and smites down him who dares to look at it.

Franz Kafka
Figures 7, 8. Enlargement of incorporation of label into young crystal cells. Note an accumulation of label over crystalloplastids. Focus on section and silver grains (respectively). 770X

Like the wind, a brook exists only through motion. Down the narrow groove it has worn in the earth, hurrying toward the greater valleys of the rivers that will carry it to the sea, all the dark water foaming and gurgling below me rushes away into the night. The stream flows on and on. So the long life of the ever-renewing brook extends through the years. But it continues without awareness, sensation or emotion. Its existence is one of action, of music, of beauty; but it is life without life. The great gift of our lives is the gift of awareness.

Edwin Way Teale
The survey of callus cultures grown from crystal containing explants presented in this dissertation (Part VII) provides information from which several conclusions about idioblast differentiation in culture can be drawn. Crystal idioblast differentiation is observed to be media dependent; different media did not have the same capacity to produce idioblasts from identical explant sources. Also, crystal idioblast production on a specific medium is species dependent; a medium which supports idioblasts for one species may not do so for another. Cytodifferentiation of crystal idioblasts is generally favored by suboptimal growth conditions. Furthermore, it was observed that the type of growth regulators may have an effect on differentiation potential. In conclusion, the survey and comparison of crystals isolated from callus cultures and their explant sources demonstrated that crystal production and shape are species specific, under genetic control, and influenced by the metabolic status of the cultures.

Subsequent to the callus survey investigation, media modification experiments were run on *Psychotria punctata* callus and *Yucca torreyi* isolated root cultures. Oxalate content of these tissues has been determined by gas chromatography of the butylated ester derivative of oxalic acid in freeze-dried samples. These studies indicate that the type of auxin used in the culture influences oxalate content per cell in callus cultures (Figures 9-14) and potential for structural differentiation. Latin square analysis of various cytokinin to auxin regimes clearly demonstrates that IAA most strongly favors oxalate accumulation and idioblast structural differentiation. The ability of 2,4-D in this capacity was significantly less
Figures 9-14. Effects of various growth regulator concentrations in Latin square arrangements on *Psychotria* callus culture growth, expressed in change in fresh weight (g) over 6 wks, and oxalate content, expressed as μmoles/mg dry callus·10^-2. Callus was grown on media according to specifications in Kausch and Horner (1982).

Figure 9. Graph of growth curve in response to IAA and K concentrations. These cultures differentiated many crystal idioblasts.

*Nature is not fixed, but fluid crystal.*

Ralph Waldo Emerson
Figure 10. Same graph as Figure 9, showing oxalate content to be constant, despite positive growth response.

New ways of seeing can disclose new things: the radio telescope revealed quasars and pulsars, and the scanning electron microscope showed the whiskers of a dust mite. But turn the question around: Do new things make for new ways of seeing?

William Least Heat Moon
Figure 11. Graph of growth curve in response to NAA and K concentrations. These cultures did not support any crystal idioblast differentiation.

See the sun in the midst of the rain;
Scoop clear water from the heart of the fire.

Mu-chou Lu
Figure 12. Same graph as Figure 11, showing oxalate content to be constant, despite positive growth response.

Like an eye that sees but cannot see itself.

Ch'uan Teng Lu
Figure 13. Graph of growth curve in response to 2,4-D and K concentrations. These cultures differentiated only very few crystal idioblasts.

You cannot get it by taking thought;
You cannot seek it by not taking thought.

from ancient Zenrin poem
Figure 14. Same as Figure 13, showing oxalate content to be constant, despite positive growth response.

A trout leaps;
Clouds are moving
In the bed of the stream.

from Zenrin poem
Change in fresh wt/callus (g)

Oxalate Content

BA (mg/l) 5.0 1.0

2,4-D (mg/l) 5.0 1.0

5.0 2.0
Figure 15. Effect of alteration of KNO₃/NH₄NO₃ ratio in medium on Psychotria cultures grown according to Kausch and Horner (1982).

Come forth into the light of things,
let Nature be your teacher.

Wordsworth
and produced very slowly growing callus. The use of NAA produced callus with the lowest oxalate content per cell of any cultures examined, and did differentiate any crystal idioblasts.

Modification of the ammonium to nitrate ratio in the medium provided a strong indication that the presence of ammonium decreases oxalate content (Figure 15) and potential for idioblast differentiation. This effect may be understood as an internal tissue response to pH changes during nitrogen metabolism. Nitrate reduction results in hydroxyl ion formation. Perhaps, oxalate is synthesized in response to increased internal pH as an organic buffer.

A host of other media modifications, including media pH changes, ascorbic acid addition, calcium addition, use of various oxalate synthesis inhibitors (lycorine, allopurinol, and αHPMS), and glutamate and glycine additions, await gas chromatographic analysis. Certainly, the results of these studies will provide some further insight into the control and inductive factors influencing idioblast differentiation in culture as well as in intact plant tissues.

If all of the parameters governing idioblast initiation are determined, and cytodifferentiation could be stimulated at will, an important system for plant cell developmental studies will be in hand and the possibilities become endless. Such a system could be used to investigate in greater detail some of the interesting developmental aspects of plant cell specialization, including endo-cycling nuclei, plastid transformations, plant calcification, mucilage production, peroxisomal differentiation, and many specifics of idioblast development. Indeed, such a system would be a
very powerful tool, unprecedented in plant cell biology, to examine molecular aspects of differentiation. Heterogeneous plant tissues do not provide good systems for molecular studies of development, and this fact, coupled with incredibly poor funding and interest, leaves plant cell biologists lacking knowledge attainable in other organisms.

I feel that calcium oxalate crystal idioblasts represent a specialized cell type that could be manipulated to the goal expressed above. The parameters governing cytodifferentiation are most likely understood through knowledge of development and metabolic functioning of these cells, or maybe any cell type. This investigation provides inroads into that knowledge.

As mentioned previously, the function of calcium oxalate crystal idioblasts during normal plant growth and development remains enigmatic. Clearly, they are not imperative for all plants, since all plants do not possess them, and have evolved different methods to accommodate their functions. Whether they are absolutely necessary in plants that produce them is still an open question; mutant selection of idioblast deficient plants would certainly provide interesting answers concerning their function. It is likely that these cells are multifunctional, and any suggestion of a specific role need not be mutually exclusive of another role. The results of my observations, however, strongly suggest that these cells are not depositories for oxalate as a waste product in any of the plants that have been observed in this investigation. However, unless granting agencies show some affection for problems of cytodifferentiation in plants, and/or
problems concerning development, physiology and function of crystal idioblasts generally, answers to many broad-based biological questions may need to await an approach from a different angle.
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