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DEVELOPMENT OF FUNGAL CALCIUM OXALATE CRYSTALS ASSOCIATED WITH  
THE BASIDIOCARPS OF *GEASTRUM MINUS* (LYCOPERDALES)

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Abstract

Calcium oxalate crystals were observed on facing peridial surfaces of developing basidiocarps of *Geastrum minus*. Five crystal forms were characterized by their outward shapes and X-ray diffraction patterns: Form One, small bipyramids (COD); Form Two, crystals with split tips (COD); Form Three, crystals with oriented overgrowths (COD); Form Four, large bipyramids (COD); and Form Five, rosettes of plate-like crystals (COM). Forms One and Two were first to appear after peridia separated in unopened basidiocarps, whereas Forms Three, Four and Five were present on exposed peridia of opened basidiocarps. Based on time of appearance during basidiocarp development and crystallography of each form, we believe Form One gives rise to Forms Two, Three and Four. Form Five may arise from dissolution and re-crystallization of pre-existing forms. Farinose or asperate basidiocarps of eight other species of *Geastrum* showed no crystals, indicating that only *G. minus* forms crystals. Comparison of the five forms with crystals on old basidiocarps indicates Form Four is predominant. The functional value of the crystals is discussed and they are compared with crystal forms found in human and animal urinary tracts.

KEY WORDS: calcium oxalate crystals, whewellite, weddellite, fungus, Basidiomycete, Lycoperdales, *Geastrum minus*, basidiocarp, fruiting body

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Introduction

Calcium (Ca) oxalate crystals are associated with a wide variety of fungi (Pobequin, 1954). The crystals are present on either the vegetative or fruiting structures of some fungi (Horner et al., 1983) and they typically have a specific shape (habit) indicative of a given species.

Genesis of crystals has been described for only a few fungi (Arnott, 1982; Arnott and Webb, 1983; Horner et al., 1983; Arnott and Fryar, 1984; Powell and Arnott, 1985) and among these studies, it is agreed that crystal formation probably is an intracellular process similar to that found in higher plants (Arnott and Pautard, 1970; Franceschi and Horner, 1980). Even though many fungi and higher plants contain Ca oxalate crystals, little is known about their functional significance. This paucity of information is surprising since the presence of similar crystals in animal systems is well-documented (King, 1969), especially with regard to human urinary tracts (Hodgkinson, 1977; Kim, 1982).

It is important to extend these studies to crystals associated with a fungal system and to understand the implications of their genesis and final habit. The present study contributes to this theme by following the development of Ca oxalate crystals in the basidiocarps of *Geastrum minus* (Horner et al., 1985). The goals of this study, then, are to describe the development of the crystals; determine whether the crystals are of intra- or extracellular origin; ascribe a functional significance to the crystals; and ascertain whether any other species of *Geastrum* also have crystals.

Materials and Methods

Nebraska Collection - A single collection of *Geastrum* (= *Geaster*, small earthstar), consisting of many first-season, young to maturing basidiocarps with associated mycelia, was obtained under an old, well-established stand of *Juniperus virginiana* L. in the Nebraska National Forest near Halsey, Nebraska, Thomas County, on August 9, 1984 by two of the authors (L.H.T. and G.K.). The fungal material was collected in an area of about 1.2 square meters and was considered to be from one fungal source. The specimens were identified

as *Geastrum minus* (Persoon) Fischer (ISC 365530; Cunningham, 1942). The maturing, opened basidiocarps were compared with those from six collections of *G. minus* studied earlier (Horner et al., 1985) and similar bipyramidal crystals were found covering the outer surface of the endoperidium and the inner surface of the exoperidium (facing peridial surfaces). Younger, unopened basidiocarps with peridial layers still fused together or just separating from each other were also present in the Nebraska collection. Thirty-four opened basidiocarps as well as seven unopened basidiocarps in various stages of development were observed. A dissecting microscope was used in categorizing the general appearance of the visible crystals on the peridial surfaces.

Other Species of Geastrum Observed: Cunningham (1942) recognized 23 species of *Geastrum*, with only *G. minus* being described as having "glistening particles" on the facing peridial surfaces. We examined these surfaces of mature basidiocarps from eight other species of *Geastrum* for the presence of crystals. These eight species were listed as having endoperidia which are farinose or asperate. The remaining species, described as having smooth peridia, were not observed in this study. Peridia were mounted for SEM as described in the next subsection. The species studied were: *G. campestre* (ISC 365524), *G. triplex* (ISC 365528), *G. fimbriatum* (ISC 365533), *G. fenestriatum* (ISC 365526), *G. arenarius* (ISC 365525), *G. rufescens* (ISC 365527), *G. pectinatum* (ISC 365532), and *G. hygrometricus* (ISC 365529); this latter taxon is recognized as a separate genus = *Astraeus* because of its different global development (see Cunningham, 1942; Smith et al. 1981; Coker and Couch, 1928; Kambly and Lee, 1936). All of the collections mentioned here and in Horner et al. (1985) are permanently

stored in the Iowa State University Herbarium, Department of Botany (ISC herbarium reference numbers).

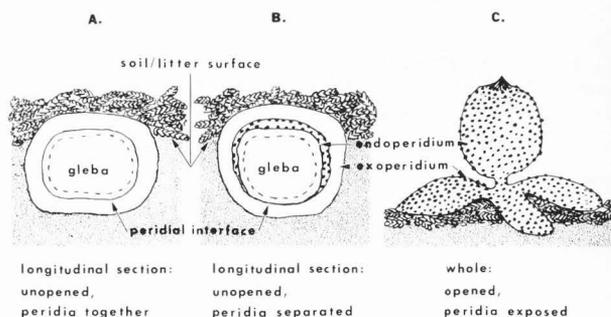
Microscopy - Portions of 13 endoperidia and one exoperidium of opened, unfixed basidiocarps of the Nebraska collection were mounted directly on brass discs using double-stick tape and silver paint. Four young, unopened basidiocarps were sectioned longitudinally with razor blades to expose their interiors. In one case, the exo- and endoperidia were still attached to each other, while in the three other unopened basidiocarps they were just separating. In two of these latter preparations, the peridia were mechanically separated from each other with tweezers and mounted on brass discs as previously described.

All preparations for SEM observation were coated with approximately 15 nm of gold: palladium (20:80) in a Polaron E5100 sputter coater and viewed in a JEOL JSM 35 SEM. Photographs were made using Polaroid type 665 film.

X-ray diffraction analyses - Crystals were removed from both mature peridia and younger peridia with developing crystals and were mounted on glass slides, and exposed to nickel-filtered  $\text{CuK}\alpha$  radiation generated at 20 mA and 40 kV. Peaks were obtained at a scanning speed of  $0.5^\circ/\text{min}$  between  $0^\circ$  and  $50^\circ 2\theta$ . American Society for Materials X-ray standard file cards for whewellite, weddellite, and cellulose II were used to identify X-ray patterns.

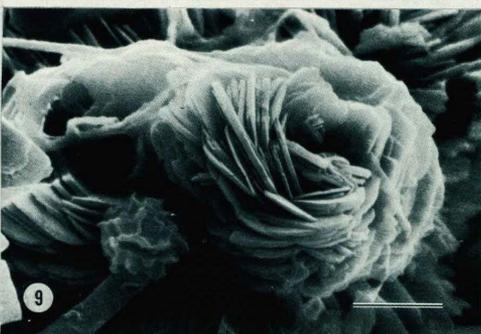
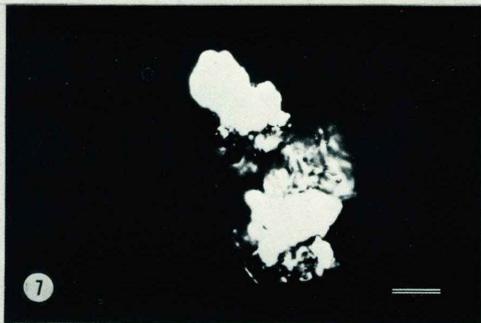
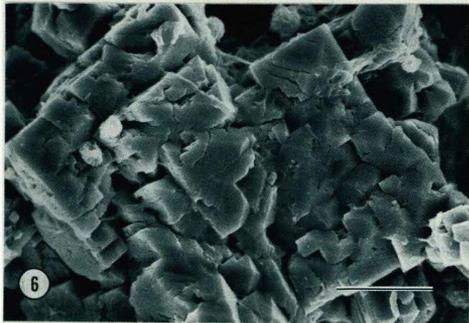
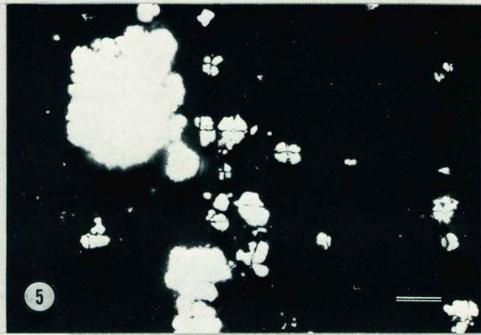
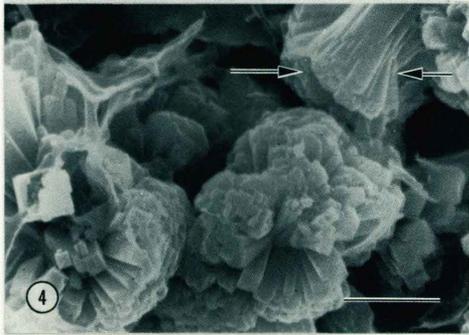
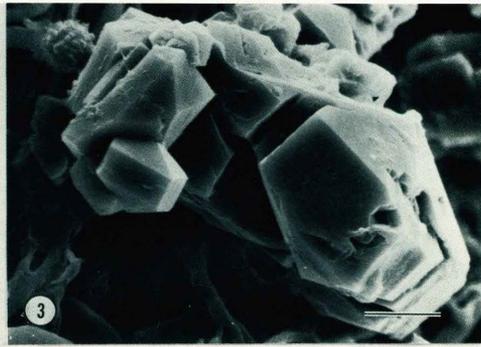
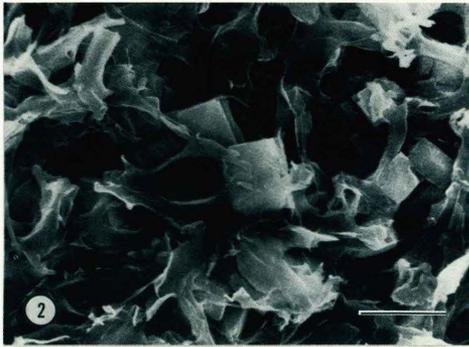
Light microscopy - Single crystals and crystal masses were scraped from the surfaces of the peridia and placed into drops of distilled water on microscope slides, coverslipped, and immediately viewed in white light and between crossed polarizers with a Leitz Orthoplan compound microscope with a rotating stage. Images were recorded on Kodak Techpan 35 mm film with an Orthomat camera.

Figure 1. Simplified diagrams depicting stages of basidiocarp development observed during this study. A. Longitudinal median section through unopened basidiocarp with exo- and endoperidia fused at a common interface. Gleba is region where spores will form. B. Longitudinal median section through underground, unopened basidiocarp with exo- and endoperidia separated. Crystals are present on facing peridial surfaces. C. Whole view of fully opened basidiocarp with exposed exo- and endoperidia. Crystals cover inner surface of exoperidium and outer surface of endoperidium.



Figures 2-9. Five forms of crystals observed on developing basidiocarps of *Geastrum minus*. Fig. 2. Form One COD small bipyramids in among hyphae at peridial interface of unopened basidiocarp; outer surface of endoperidium. Bar = 10  $\mu\text{m}$ . Fig. 3. Form One COD small bipyramids. Bar = 10  $\mu\text{m}$ . Fig. 4. Form Two COD crystals with split radiating tips with terminal tetragonal pyramids. Arrows indicate split tips emanating from single crystal body. Bar = 4  $\mu\text{m}$ . Fig. 5. Form Two crystals with split tips, polarized view. Crystals do not extinguish as a unit indicating subunits are oriented in a radial or spherical pattern. Bar = 25  $\mu\text{m}$ . Fig. 6. Form Three COD single crystal consisting of oriented subunits with square outline. Bar = 20  $\mu\text{m}$ . Fig. 7. Form Three crystals with oriented overgrowths, polarized views. All subunits extinguish at same angle of rotation. Bar = 25  $\mu\text{m}$ . Fig. 8. Form Four COD large bipyramidal crystal. Bar = 20  $\mu\text{m}$ . Fig. 9. Form Five COM rosette consisting of individual plate-like crystals. Bar = 20  $\mu\text{m}$ .

Fungal Calcium Oxalate Crystals



### Results

The Nebraska collection consisted of basidiocarps which were categorized arbitrarily into four stages of development, three of which are diagrammed in Fig. 1 A.-C.: unopened basidiocarps with peridia not separated; unopened basidiocarps with peridia separating; just opened basidiocarps with exo- and endoperidia exposed; and completely opened basidiocarps with exposed exo- and endoperidia. Four-unopened and thirteen-opened basidiocarps were examined for the presence of crystals. Crystals showing different crystal forms were analyzed by SEM (Figs. 2-4, 6, 8-25), X-ray diffraction (Tables 1, 2) and polarization optics (Figs. 5, 7).

Five different forms of crystals were identified with certain stages of basidiocarp development. A description of each form follows:

Form One (small bipyramidal crystals)- Small, elongate or stubby single crystals consisted of two tetragonal pyramids separated by a short four-sided prism (Figs. 2, 3). These crystals typically did not show any oriented overgrowths. X-ray diffraction analysis showed they were Ca oxalate dihydrate (COD) or weddellite (Tables 1, 2).

Form Two (crystals with split tips)- These crystals appeared as small clusters of radiating crystals; each crystal terminated with a tetragonal pyramid (Fig. 4). The clusters of crystals did not extinguish as a unit when rotated between crossed polarizers (Fig. 5) indicating that the individual crystal subunits making up a cluster were oriented in a radial or spherical fashion. Crystallographic literature (Grigor'ev, 1965 refers to crystal clusters such as the one in the upper right of Fig. 4 (arrows) as single crystals with split tips, or split crystals. X-ray diffraction analysis of these crystals showed they were Ca oxalate dihydrate (COD) or weddellite (Tables 1, 2).

Form Three (crystals with oriented overgrowths)- These are single crystals that are square in outline and consist of oriented subunits (Fig. 6). When these crystals were rotated between crossed polarizers, all subunits within the square outline extinguished at approximately the same angle of rotation (Fig. 7), indicating that their lattices were in approximately the same orientation and that the mass was actually a single crystal with a rough surface referred to in crystallographic literature (Grigor'ev, 1965) as "oriented overgrowths." Most subunits ended in tetragonal pyramids. X-ray diffraction analysis of these crystals showed they were Ca oxalate

dihydrate (COD) or weddellite (Tables 1, 2).

Form Four (large bipyramidal crystals)- These large crystals consisted of two partially or completely formed tetragonal pyramids usually separated by a short four-sided prism (Fig. 8) making them twelve-sided. Some bipyramids displayed distinct growth steps whereas others had pitted surfaces. Quite often two or more bipyramids were interpenetrant or intergrown. When viewed between crossed polarizers, the bipyramids exhibited extinction identical to that of crystals with oriented overgrowths (Form Three; Fig. 7). X-ray diffraction analysis of the bipyramids from an Iowa collection (Horner et al., 1985) identified them as Ca oxalate dihydrate (COD) or weddellite (Tables 1, 2).

Form Five (rosettes of plate-like crystals)- Masses of plate-like crystals appeared as rosettes (Fig. 9). Individual plate-like crystals had distinct edges. X-ray diffraction analysis of the crystals indicated that they were Ca oxalate monohydrate (COM) or whewellite (Tables 1, 2).

The five forms of crystals were present on the peridial surfaces at different stages of basidiocarp development (Table 2). A more detailed account of these stages and the crystal forms follows.

Unopened basidiocarps with exo- and endoperidia not separated- The youngest basidiocarp collected displayed two peridia that had not yet separated from each other (Fig. 1A.). The peridial interface between them was intact and there were no visible crystals present. In a second basidiocarp the two peridia were still joined except in a small region; again, no crystals were evident (Fig. 10; arrow).

Unopened basidiocarps with exo- and endoperidia separating- Two unopened basidiocarps, when longitudinally sectioned, displayed exo- and endoperidia that either had separated (Figs. 1B, 11) or were just separating. These peridia were mechanically separated with tweezers and both exhibited similar Form One and Form Two crystals (Figs. 2-4; Table 2). Figure 12 represents a field of crystals typical of either peridial surface at this stage. In some regions Form One bipyramidal crystals were closely packed (Fig. 13) and displayed subunits similar to those of Form Two. Form Two was the most common and each crystal seemed attached to a hyphal surface with its ends flaying out as many small crystal subunits (Figs. 14, 15); each subunit end was capped with a tetragonal pyramid.

Just opened basidiocarp with exo- and endoperidia exposed- One basidiocarp was collected in

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Figures 10-17. Early stages of basidiocarp development of *Geastrum minus* prior to and after crystal formation. Fig. 10. Longitudinal section through upper portion of unopened basidiocarp; both exo- and endoperidia are still joined except in small region (arrow). See Fig. 1A. Bar = 2 mm. Fig. 11. Portion of section through unopened basidiocarp showing separated exo- (X) and endoperidia (N). Material (M) above exoperidium is litter. Note crystals on surface of endoperidium. Bar = 4 mm. Fig. 12. Crystals from endoperidial surface of unopened basidiocarp. This region is similar to exoperidial surface. Bar = 100  $\mu$ m. Fig. 13. Form One bipyramidal crystals from unopened basidiocarp that are closely packed together and are similar to Form One crystals. Bar = 5  $\mu$ m. Fig. 14. Form Two crystals associated with hyphae of outer surface of endoperidium of unopened basidiocarp. Bar = 5  $\mu$ m. Fig. 15. Same as Fig. 14. Bar = 6  $\mu$ m. Fig. 16. Portion of inner surface of exoperidium (one arm of star) covered with Form Two crystals from a just-opened basidiocarp. Bar = 4 mm. Fig. 17. Form Two crystals associated with hyphae from exoperidium shown in Fig. 16. Bar = 6  $\mu$ m.

Fungal Calcium Oxalate Crystals

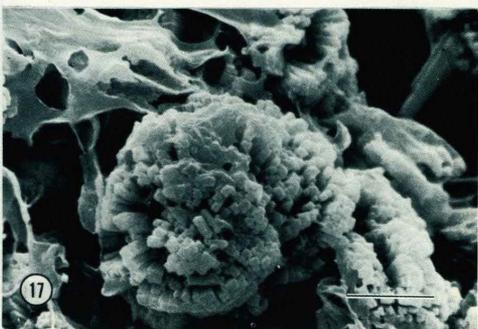
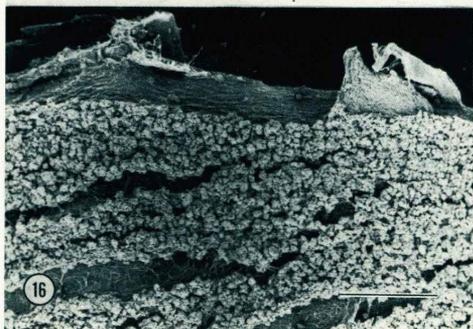
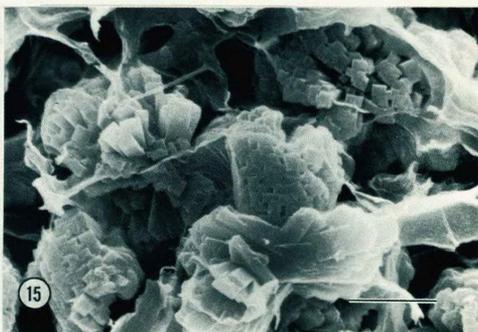
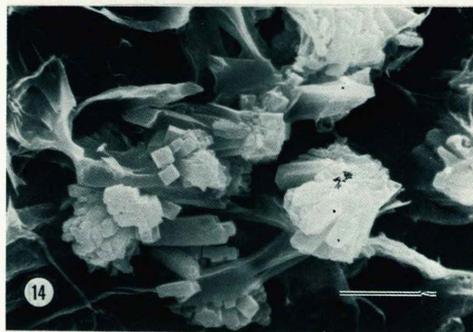
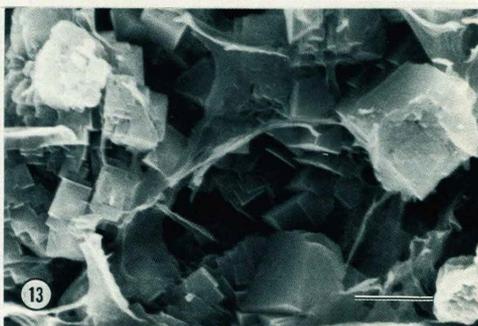
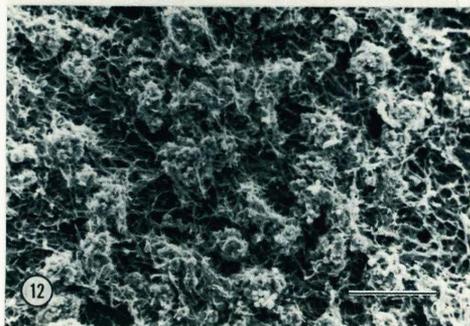
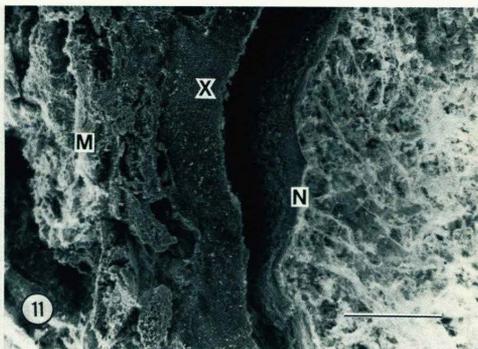
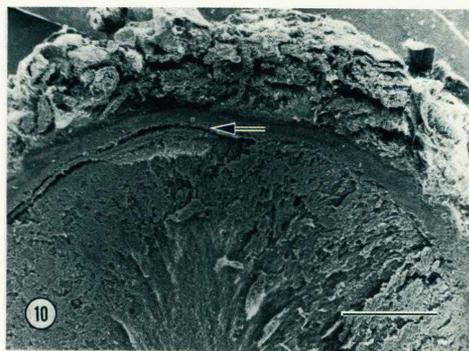


Table 1. X-ray analyses of *Geastrum minus* crystal forms.  $d\text{\AA}$ (ASTM I/I<sub>0</sub>)

A. <u>Form One</u> (small bipyramids), <u>Form Two</u> (split crystals) and <u>Form Three</u> (crystals with oriented overgrowths)
<u>Weddellite</u> (ASTM #17-541): 8.73(4), 6.32(6), 6.18(100), 4.42(30), 4.37(2), 3.68(12), 3.91(8), 3.16(4), 3.09(10), 2.815(14), 2.775(65), 2.762(4), 2.422(8), 2.408(16), 2.367(2), 2.342(4), 2.243(25), 2.210(6), 2.189(2), 2.118(8), 2.024(6), 1.996(4), 1.957(10), 1.953(4), 1.899(16).
<u>Whewellite</u> (ASTM #20-231) - small peaks indicating trace quantities: 5.93(100), 5.79(30), 2.966(45).
<u>Cellulose II</u> (ASTM #3-0226): 7.55(80), 5.14(90), 4.30(100).
B. <u>Form Four</u> (large bipyramids) and <u>Form Five</u> (rosettes)
<u>Weddellite</u> (ASTM #17-541): 6.32(6), 6.18(100), 4.42(30), 3.91(8), 3.68(12), 3.39(4), 3.09(10), 2.815(14), 2.775(65), 2.762(4), 2.679(2), 2.422(8), 2.408(16), 2.367(2), 2.342(4), 2.243(25), 2.210(6), 1.996(4), 1.899(16), 1.836(10).
<u>Whewellite</u> (ASTM #20-231): 5.93(100), 5.79(30), 4.77(2), 3.65(70), 3.01(10), 2.966(45), 2.915(10), 2.897(8), 2.840(10), 2.494(18), 2.417(6), 2.355(30), 2.347(12), 2.263(8), 2.254(6), 2.075(14), 1.933(8), 1.859(4).
C. <u>Form Four</u> (large bipyramids)
<u>Weddellite</u> (ASTM #17-541) - Horner <i>et al.</i> (1985)

which the exoperidium had split and was bending away from the endoperidium. Both facing peridial surfaces were covered with Form Two crystals as represented by the exoperidium in Fig. 16. The radiating (split) ends were more distinctive (Figs. 17,18). Sometimes fragments of what appeared to be a layer or membrane covered portions of the crystals (Fig. 18).

Completely opened basidiocarps with exposed exo- and endoperidia - These basidiocarps apparently were developmentally older than any of those previously described. The endoperidium surrounding the spore sac was bottle-shaped and the exoperidium was split and spread out on the soil/litter substrate to form the characteristic arms that give this organism and related taxa the common name "earthstar." The outer surfaces of 13 endoperidia and inner surface of one exoperidium were examined with a light microscope. Both peridia were covered with many crystals, some of

which could be seen as distinct bipyramids (Form Four. Figs. 1C., 8; Table 2).

Observations of all exposed peridial surfaces indicated that collectively four of the five forms of crystals were present (Table 1). The presence of any of these forms on a single peridium falls into three categories, with Form Four being consistently present.

Category 1 - The majority of the peridia displayed Forms One, Three, and Four (Figs. 19-21; Table 2). The crystals often appeared as incomplete bipyramids (Fig. 19) with major faces showing overgrowths (Fig. 20). At higher magnification, individual subunits of each forming face could be seen (Fig. 21). More nearly complete bipyramids were commonly found among the two other forms.

Category 2 - Some peridia were predominantly covered with Form Four bipyramid crystals. These crystals were usually densely packed and either

Figures 18-25. Later stages of crystal formation in opened basidiocarps of *Geastrum minus* including surrounding mycelia with crystals. Fig. 18. End view of Form Two crystal with tetragonal pyramid ends. Note fragment of a layer or membrane covering a portion of crystal. Bar = 1  $\mu\text{m}$ . Fig. 19. Forms One and Three crystals on outer surface of endoperidium of opened basidiocarp, Category 1. Bar = 25  $\mu\text{m}$ . Fig. 20. An incompletely formed Form Four bipyramid with major faces showing overgrowths on outer surface of endoperidium of opened basidiocarp, Category 1. Bar = 25  $\mu\text{m}$ . Fig. 21. Enlarged view of portion of Form Four bipyramid showing overgrowths, growth steps, and subunits oriented in same direction; from outer surface of endoperidium of opened basidiocarp, Category 1. Bar = 20  $\mu\text{m}$ . Fig. 22. Form Four bipyramidal crystal with pitted surfaces, Category 2. Bar = 20  $\mu\text{m}$ . Fig. 23. Portion of exposed outer surface of endoperidium displaying both Form Four and Form Five crystals, Category 3. Bar = 40  $\mu\text{m}$ . Fig. 24. Portion of mycelium from soil/litter region around basidiocarps. Individual hyphae display clusters of crystals. Bar = 20  $\mu\text{m}$ . Fig. 25. Portion of Fig. 24 showing three crystal clusters (druses) on a hypha. Bar = 2  $\mu\text{m}$ .

Fungal Calcium Oxalate Crystals

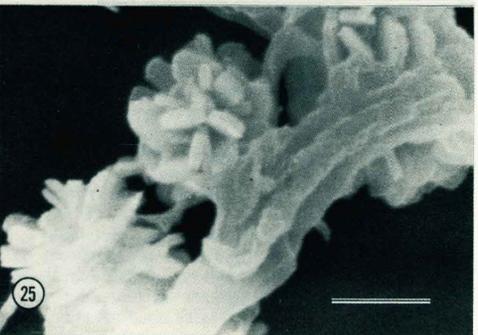
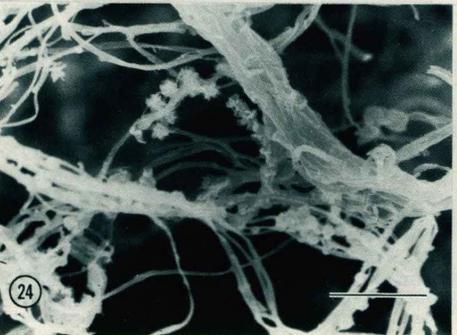
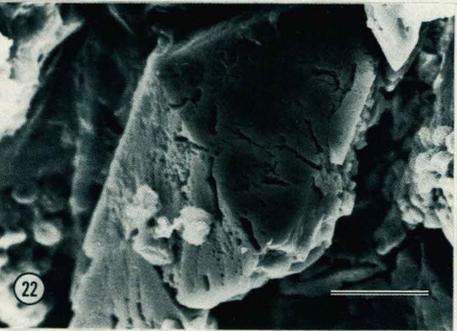
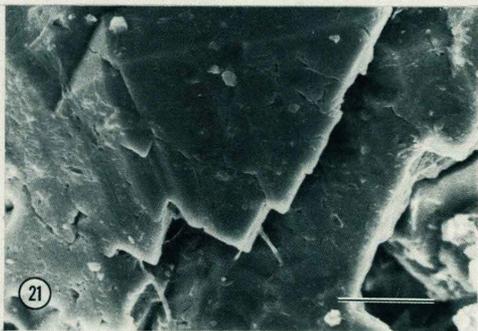
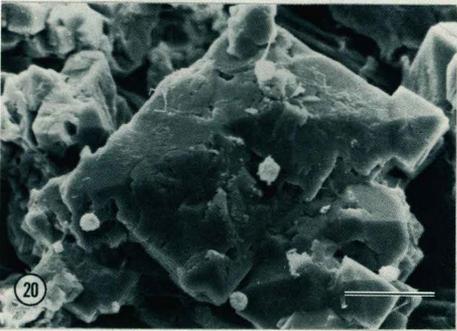
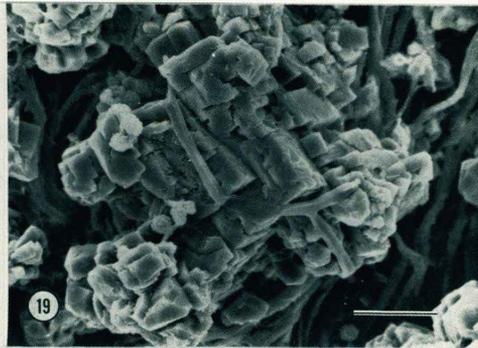
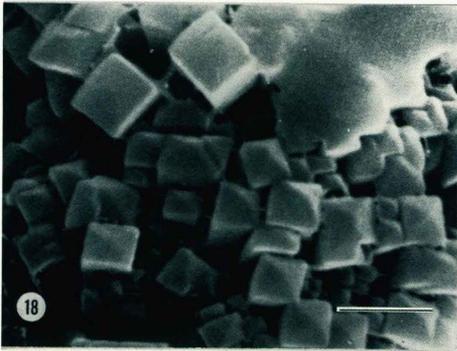


Table 2. Five forms of crystals found on *Geastrum minus* basidiocarp peridia at different stages of basidiocarp development. Composition of each crystal form is indicated.

Basidiocarp stage and crystal form and composition	Form One	Form Two	Form Three	Form Four	Form Five
Unopened, peridia fused	absent	absent	absent	absent	absent
Unopened, peridia separated	<u>present</u>	<u>present</u>	absent	absent	absent
Just opened, peridia exposed	<u>present</u>	<u>present</u>	absent	absent	absent
Fully opened, peridia exposed					
Category 1	<u>present</u>	absent	<u>present</u>	<u>present</u>	absent
Category 2	absent	absent	absent	<u>present</u>	absent
Category 3	absent	absent	absent	<u>present</u>	<u>present</u>
Crystal composition COD/COM	COD <sup>1/</sup>	COD <sup>1/</sup>	COD <sup>1/</sup>	COD <sup>1/</sup>	COM <sup>2/</sup>

<sup>1</sup>COD = Ca oxalate dihydrate, weddellite

<sup>2</sup>COM = Ca oxalate monohydrate, whewellite

displayed growth steps at their edges or pitted surfaces (Fig. 22). All of the opened peridia were from basidiocarps that had mature spores which sometimes covered the peridial surfaces.

Category 3 - Two peridia displayed a dense covering of Forms Four and Five (Fig. 23). The majority of the crystals were rosettes (Fig. 9). Bipyramids were interspersed among the latter. No intermediate forms were visible. Because X-ray diffraction analysis of this sample gave strong COD and COM peaks, while *Geastrum* samples containing only bipyramids gave only COD peaks (Horner *et al.*, 1985), we conclude that Form Five crystals are Ca oxalate monohydrate (COM) and the bipyramids are Ca oxalate dihydrate (COD).

Other X-ray data - Besides obtaining distinct, identifying peaks for Ca oxalate dihydrate and monohydrate, peaks for cellulose II were present. Cellulose is a major constituent of the fungal hyphal walls making up the peridia.

Mycelia associated with soil/litter and basidiocarps - Pale white mycelia permeated the soil/litter region around the basidiocarps. Individual hyphae displayed many small clusters of crystals on their surface (Fig. 24). Each cluster, termed a druse, consisted of many individual crystals (Fig. 25). No X-ray diffraction analysis was conducted on the mycelia and associated crystals because of the small amount collected.

#### Discussion

Cunningham (1942) is the most recent, complete taxonomic source of information about *Geastrum*. He identifies 23 species and describes only one of them (*Geastrum minus*) as having "glistening particles" on the surfaces of the

peridia. Horner *et al.* (1985) studied six geographically diverse collections of *G. minus* and concluded that opened, mature basidiocarps typically exhibit bipyramidal crystals of Ca oxalate dihydrate on both the outer surface of the endoperidium and inner surface of the exoperidium. Absence or paucity of crystals on the peridial surfaces of some of the basidiocarps was interpreted as a consequence of extreme weathering and age.

In this study, single collections of 8 other species of *Geastrum*, with farinose- to asperate-appearing peridial surfaces, showed no indications of any crystalline structures. These results, when considered with the descriptions of Cunningham (1942), suggest that *G. minus* is the only species of the 23 that is capable of producing Ca oxalate crystals in association with its basidiocarps. The significance of this observation is difficult to assess because some of the *Geastrum* species are broadly distributed geographically and exist, in some cases, in similar environmental niches. The phylogeny of the species within *Geastrum* is unknown, so nothing can be said regarding whether crystal formation is a primitive or advanced trait.

Ca oxalate crystals occur in a diversity of organisms throughout the plant and animal kingdoms (Hodgkinson, 1977; Arnott and Pautard, 1970; Franceschi and Horner, 1980). Their habits (external forms) as well as their locations are specific for a given taxon. This also seems to hold true for *G. minus*. One additional fact is that crystals were found both on the basidiocarp (fruiting body) surfaces and the intimately associated vegetative hyphae. We were unable to identify the crystal-bearing hyphae by culturing

fruiting bodies from them in the laboratory. Because the crystal-bearing hyphae were the only ones associated with the basidiocarps, it is most probable that they belong to *G. minus*. If so, this may be the first report of the presence of crystals on both the fruiting and vegetative parts of the same fungus (Pobequin, 1954; Horner *et al.*, 1985).

Based on the methods used, it was impossible to determine whether any of the crystals had an intracellular origin. The youngest crystals observed associated with the peridia of unopened basidiocarps were definitely in contact with the peridial hyphae, and split crystals (Form Two crystals) always occurred on the hyphal surfaces. Initial crystal formation must occur very rapidly as the peridia separate from each other. As the exoperidium splits open and the facing peridial surfaces are exposed to the outside environment, more crystal forms appear.

For physiological reasons that are yet unclear, *G. minus* seems to produce oxalate in both its vegetative and reproductive stages. Internal and external calcium ions provide a source of calcium for calcium oxalate crystal formation. If the chemical and physical conditions during crystal growth remained constant, we would expect all of the crystals formed to have the same outward appearance. The presence of five different forms of crystals at various stages of peridial development (Table 1) suggests that a variety of factors controlling crystal form are operational at different stages.

Previous studies (Cody *et al.*, 1982; Cody and Horner, 1984) show that the presence of trace ions and molecules, certain physical parameters, and rate of growth of the biological system and/or the crystal are effective in controlling crystal form. An understanding of the factors that produce the different forms on *G. minus* should aid in understanding changes that occur during basidiocarp development.

Small bipyramids (Form One) were common in unopened basidiocarps and throughout the period of exoperidial evagination. They are probably one of the earliest crystal forms and it is possible that the other forms may result from an alteration of this one.

Crystals with split tips (Form Two) were also observed in unopened basidiocarps. Crystals with this morphology are common in the mineral world and have been found to result from various conditions during crystal growth. Grigor'ev (1965) cites nonuniform sectional adsorption of impurities which may cause expansion of crystal planes and subsequent splitting of the crystal during growth. According to Grigor'ev (1965) and Shubnikov (1957), the split parts at first resemble an aggregate of crystals, then a sheaf and finally a spherulite as the split segments multiply and are offset away from each other during growth. Manganese has been shown to be responsible for the split growth of calcium carbonate (Grigor'ev, 1965). Various chemicals produced during basidiocarp development may act as the impurity that causes splitting of COD crystals in *G. minus*. *In vitro* crystal growth experiments are necessary to determine the effective chemicals. Clontz and McCabe (1971) have shown experimentally

that rapid crystal growth in supersaturated solutions may also produce split crystals of magnesium sulfate heptahydrate.

Form Three crystals with oriented overgrowths were observed in opened basidiocarps. The mosaic block crystals were shown to be subunits of a single crystal because the subunits all extinguished at approximately the same angle when the crystal was rotated between crossed polarizers. The term syntaxial growth is used to describe the overgrowths of a mineral by crystalline material of the same composition (Weller, 1960). Khaimov-Mal'kov *et al.* (1962) produced this surface structure on alum crystals by adding 0.2% dye #518 to the growth solution. Silicon crystals grown in the presence of iodine impurities show epitaxial oriented overgrowths of SiI (Glang and Wajda, 1963). Bisailon and Tawashi (1975) found that COD crystals grown in calf skin gelatin showed identical features. Our own experiments (unpublished) show ferric ions to produce oriented overgrowths of COD, verifying that impurities present in the crystal growth environment are responsible for this growth modification. When crystals from *G. minus* were observed in transmitted light, a brown stain was seen between the subunits.

Grigor'ev (1965) described Khingansk quartz crystals in which each face of the crystal consisted of several subunits which grew at a rate that retained the surface of the crystal faces. It is most probable that Form Three crystals with oriented overgrowths grow into large bipyramids (Form Four) in the same manner. The pits on the surfaces of large bipyramids appear to parallel the directions of fissures in crystals with oriented overgrowths.

Horner *et al.* (1985) observed pitted surfaces on bipyramids from mature basidiocarps that were more than a season old. They interpreted the pitting to be at least partly due to extreme weathering. Khan *et al.* (1979) in their animal experimental system, interpreted etched crystal surfaces and eroded edges as dihydrate dissolutions. Because COD crystals with oriented overgrowths are found in both plant and animal systems, it is possible that surface textures attributed to dissolution may actually be a result of growth mechanism.

Forms One, Two, Three and Four, are all calcium oxalate dihydrate (COD), the mineral hydration form identified in other collections of *Geastrum minus* and in nearly all other fungal deposits (Horner *et al.*, 1985). X-ray diffraction analysis indicated that Form Five, rosettes of plate-like crystals, were calcium oxalate monohydrate (COM), a hydration form different from all other *Geastrum* crystals, and previously described only on one other fungus, *Hysterangium crassum* (Graustein *et al.*, 1977). Rosettes were infrequently observed among bipyramids on mature weathered peridia (Horner *et al.*, 1985, their Fig. 13).

Under certain conditions, COM is considered to be more stable than COD. Franchini-Angela and Aquilano (1979) and Heijnen and Duijneveldt (1984) pointed out that hydration form and morphology of calcium oxalate crystals depend on pH, temperature, degree of saturation, ratio of Ca/oxalate, and

presence of crystal poisons. For COD, high pH and a high Ca/oxalate ratio are generally necessary conditions. Under conditions of low pH, COD becomes unstable, dissolves, and is reprecipitated as COM (Frey, 1925; Hammarsten, 1929; Cody and Horner, 1985). Because COM rosettes are found only on some fully-opened basidiocarps, it is possible that they are formed through the dissolution of preexisting COD. Furthermore, since a small percentage of basidiocarps displayed a predominance of COM crystals, this could indicate intermittent environmental conditions that favored COD dissolution and COM recrystallization on exposed basidiocarps.

Crystal Forms One, Three, Four and Five, but not Two, have been found in human and animal urinary tracts under both natural and experimental conditions (Khan et al., 1979; Kim, 1982; Kim and Johnson, 1981; Rushton et al., 1980). These similar occurrences are most curious since the biological systems and environments are uniquely different.

The function of calcium oxalate crystals in green plants and in fungi is still unclear. Horner et al. (1985) suggested that formation of the crystals at the peridial interface of G. minus before the basidiocarp opens, may facilitate separation of the peridia by utilizing cell wall calcium released during separation. The present study indicates that no other Gaeastrum species observed forms crystals and that all of the species, as well as other earthstar taxa, have the same peridial arrangement and open in a similar way without the formation of crystals. Therefore, it seems that crystal formation may coincide with peridial separation but it is not necessary for this process to occur.

Other researchers working with fungal systems suggest several other functions for the crystals. Jennings (1984) hypothesized that because the mycelium is in contact with soil water containing Ca ions, if these ions enter the mycelial cytoplasm and cannot be removed by outward translocation, the fungus must somehow bind the excess Ca ions in a nondestructive way. The production of oxalate by the fungus would accomplish the feat by precipitating the Ca as insoluble calcium oxalate.

Another hypothesis is that oxalate is formed to maintain or regulate concentrations of cytoplasmic ions other than Ca. This could be important in maintaining a physiological balance in both the vegetative and reproductive stages of a fungus.

Thompson (1984) suggested that the Ca oxalate crystals, along with a dense mycelium of cords could serve as protection against predatory microorganisms in the immediate environment by aiding the lysing of mycelia of competitive fungi and by lowering pH and thereby inhibiting bacterial growth.

Graustein et al. (1977), Arnott (1982), Arnott and Webb (1983), and Horner et al. (1985) have discussed the importance of large amounts of oxalate and Ca oxalate crystals in soil genesis, soil fertility, and retention or cycling of elements important for establishing and maintaining plant growth.

All of these hypotheses suggest an important role for crystal formation in maintaining a physical-chemical balance within plants and within the soil environment. Therefore, we believe it is important to document the presence and location of Ca oxalate crystals associated with fungi because these observations, coupled with those from higher plant and animal systems provide a greater awareness of crystals associated with biological systems and may help to elucidate their functional significance.

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#### Discussion with Reviewers

H. J. Arnott: What do you think is the explanation for your observation that of the species you investigated only *Geastrum minus* produces crystals on the basidiocarp?

Authors: We do not have a specific answer to your question. The eight other species of *Geastrum* that we observed represented the spectrum of 23 species recognized by Cunningham (1942) as having farinose or asperate endoperidia. None of the eight species displayed any crystals. Our results, therefore, suggest that only *G. minus* produces crystals. A mutation could have occurred to cause oxalate to be produced in relatively large quantities which, in turn, results in crystals. This trait is consistent for *G. minus* since collections studied from the U.S.A. and Europe all display the same crystal form. Obviously, the lack of crystals does not adversely affect the other species of *Geastrum* in terms of normal development.

S. R. Khan: Did you detect any fluid in the peridial interface of unopened basidiocarps?

Authors: When the unopened basidiocarps were sliced open with a razor blade there was no detectable fluid visible either in the global region or in association with the peridia. This was also true in unopened basidiocarps at the time when the two peridia separated from each other and crystals first appeared.

Z. K. Punja: As the authors have stated, a variety of factors controlling crystal form are operational at different stages of basidiocarp development. Since the influence of these factors may change with time, are the associations between crystal forms and stage of development reported here consistent and reproducible?

Authors: Our first paper dealing with six collections of *G. minus* from the U.S.A. and Europe (Horner et al., 1985) demonstrated that the bipyramids were the dominant crystal form. The present study clearly indicates these latter results are, in fact, correct. However, it is clear that between crystal initiation and the mature to old basidiocarps, there are at least three and possibly four intermediate forms. Based on development of the basidiocarps, it seems that the intermediate forms are a result of influences from both the fungus and the environment. It was impossible to ascertain what the influences were.

As to consistency and reproducibility, the various stages of basidiocarp development suggest that there is a pattern of crystal development beginning with Form One and culminating in Form Four. The only way we know of demonstrating that the five forms are consistent is to find another collection of *G. minus* exactly at the same stages of development.

H. J. Arnott: What is your evidence that Form Five as seen in Figures 9 and 23 is the result of either dissolution and/or recrystallization?

Authors: We believe Form Five crystals are the secondary crystals because: they are not present during the early stages of crystal formation when the basidiocarps are still closed but the peridia have separated; they are only present in small numbers, if at all, on old basidiocarps; and they occurred on only a few of the developing basidiocarps we observed in this study. We believe that certain physical/chemical (environmental) conditions existed during the time the basidiocarps having Form Five crystals had just opened. We think these basidiocarps had Forms One, Two, and Three but that possibly pH, rate of growth of the basidiocarp, availability of Ca, or other unknown factors, caused these COD forms to be dissolved and recrystallized as COM crystals. The fact that there also were Form Five crystals present suggests that some of the COD crystals (maybe Form Three) were not affected by these conditions and continued their development to the bipyramids prevalent on mature, old basidiocarps.

S. R. Khan: In human urinary stones calcium oxalate dihydrate crystals show only {011} faces. In urinary crystals {100} face is sometimes visible. In your micrographs predominance of {100} face is very striking. Is this feature common throughout the plant systems or is it restricted to this particular fungus?

Authors: The {100} faces are common throughout plant systems. Heijnen and Duijneveldt (1984) give several references to the occurrence of the {100} face on both plant and stone crystals.

Z. K. Punja: Is there any evidence in the literature that *G. minus* produces oxalic acid during either the vegetative or reproductive phase?

Authors: To our knowledge there are no studies in which analysis for oxalic acid was made during any phase of the *G. minus* life cycle. There are reports, however, of oxalic acid being produced

by other fungi (see Hodgkinson, 1977). The fact that Ca oxalate crystals are first evident on peridial surfaces inside the basidiocarp before it opens strongly supports the contention that both the oxalate and Ca are derived directly from the fungus and not its immediate environment.

H. J. Arnott: In your discussion of the development of the five crystal forms as recognized in your paper you refer to "impurities" being important elements in the process. Your table indicates that the crystal forms are closely related both in time and in distribution on the series of basidiocarp you examined and that their genesis may be under fungal control. Perhaps the continued use of the word "impurity" should be considered in light of the above. "Impurity" seems to have a "random" connotation which does not seem to be the case described. What do you think?

Authors: With regard to crystal formation, the term "impurity" is used as any substance, poison, or additive other than Ca or oxalate ions that affect crystal habit. Impurities can either be ionically active or inert. We do not have any results which suggest that an impurity or impurities were present at any or all stages of crystal development. However, the changes which we observed occurring from Form One to Form Four crystals indicate something (possibly an impurity) was affecting change in crystal form during basidiocarp development. Based on our definition of the term impurity, we believe it is appropriate and should neither connote a random nor non-random event.

S. R. Khan: Would you please elaborate on the mechanism of spore dispersal in *Geastrum*? It is my understanding that once the basidiospores are mature there is autodigestion of the hymenium and some of the peridial layers. A viscid material is formed inside the basidiocarp. At the time of their release spores are actually surrounded by water. An exposure to air results in drying and spores are dispersed by air currents. If that is the sequence of events, the environment that crystals are forming in undergoes drastic changes. Do you think that it is these changes that a basidiocarp goes through during spore dispersal, that result in various habits of the calcium oxalate crystals?

Authors: Basidiospore dispersal in *Geastrum* involves a bellows mechanism. After the exoperidium splits along lines of weakness extending from the apex toward the base, the "rays" bend backwards exposing the thin papery endoperidium. The endoperidium opens by an apical ostiole. At maturity, the glebal region has become a mass of dry basidiospores and a capillitium of dry hyphae. A raindrop striking the papery endoperidium depresses it and a cloud of spores is released through the ostiole. The endoperidium then resumes its original shape.

Z. K. Punja: Is it possible that formation of calcium oxalate crystals, particularly in fungi that produce oxalic acid, also acts as a mechanism to detoxify the acid rendering it insoluble?

## Fungal Calcium Oxalate Crystals

Authors: It is a commonly expressed idea that oxalic acid is toxic to biological systems and, therefore, it must either be eliminated from the system by excretion or precipitated as an insoluble, nontoxic crystal. This idea also has been suggested for many higher plants and animals containing calcium oxalate crystals. On the other hand, there are reports of oxalic acid apparently acting to maintain an ion balance or pH level. We have observed crystals being re-absorbed during normal development as well as in calluses grown on media minus Ca. These examples suggest other functions of oxalic acid beyond detoxification. In fact, detoxification may be only one of a wide variety of functions that oxalate plays in biological systems.

