SEED PROTEIN BODIES
IN THE
FAMILY UMBELLIFERAE

By

ERNEST SPITZER, B. Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

April 1981

MASTER OF SCIENCE (1981)
(Biology)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Seed Protein Bodies from Several Members of the Family Umbelliferae

AUTHOR: Ernest Spitzer, B. Sc. (McMaster University)

SUPERVISOR: Dr. J. N. A. Lott

NUMBER OF PAGES: xv, 117

ABSTRACT

Protein bodies of the seeds of the family Umbelliferae have not been studied extensively since late in the nineteenth century. Using a variety of recent technology and methodology certain aspects of the protein bodies of carrot (Daucus carota L. cv imperator 408), caraway (Carum carvi L.), anise (Pimpinella anisum L.), dill (Anethum graveolens L.), celery (Apium graveolens L. cv tall utah), fennel (Foeniculum vulgare Mill), parsnip (Pastinaca sativa L. cv hollow crown), parsley (Petroselinum sativum L. cv moss curled) and chervil (Anthriscus cerefolium L. cv curled) and wild carrot (Daucus carota L.) were studied. Structure of the protein bodies was determined using light and electron microscopy. Structurally, the protein bodies from all genera studied were similar in that two types of protein bodies were found. One protein body type consisted of a homogeneous proteinaceous matrix and a number of variously sized globoid crystal inclusions. The other protein body type consisted of a homogeneous proteinaceous matrix and either an individual or more commonly an aggregate of calcium-rich crystals,

commonly termed a druse crystal. Both protein body types were never found in the same cell. No calcium-rich crystals were found in the embryos. The elemental composition of the various components which compose these protein bodies including globoid crystals, calcium-rich crystals, and the proteinaceous matrix were determined using energy dispersive x-ray analysis. Globoid crystals in the endosperm usually contained either P, K and Mg or P, K, Mg and Ca while in the embryos of carrot and caraway P was always present with a combination of K, Mg and Ca. No elemental distribution pattern was found to explain variations in elemental content of globoid crystals. The calcium-rich crystals contained only calcium and the proteinaceous matrix always contained S and K. Quantitative elemental analyses were carried out and these revealed that most of the Ca was located in the endosperm. During germination and early seedling growth in carrot, few if any calcium-rich crystals were degraded. The chemical composition of the calcium-rich crystal inclusions was determined to be calcium oxalate using a number of methods including x-ray diffraction, infra-red spectrometry, microincineration, staining and solubility studies.

ACKNOWLEDGEMENTS

I wish to thank Dr. John N. A. Lott for his supervision and guidance over the past two and a half years. His patience, observations and suggestions as the research progressed were an enormous help to me.

I would like to thank Mrs. Cathy Vollmer for the technical assistance she provided during the research. Dr. H. D. Grundy, Romeo Faggiani, Steve Goudey, Helene Robotham and Michael White also aided in various aspects of this work.

I would also like to thank Miss Monika Pompetizky for typing portions of this thesis and Mrs. Cheryl Forde for typing the final draft.

Lastly, I would like to thank my wife, Mary Ann, for all her support.

Mary Ann and Michael

TABLE OF CONTENTS

	Page
Descriptive Note	ii
Abstract	iii
Acknowledgments	v
Dedication	vi
Table of Contents	vii
List of Figures	x
List of Tables	xv
INTRODUCTION	
I Seed Protein Bodies	1
II Previous Studies on Protein Bodies of Umbelliferous Seeds	9
III Members of the Umbelliferae Studied	15
IV Objectives of Present Study	18
V Choice of Procedures	
1) Structure	22
2) Elemental Analysis	
i) Energy Dispersive X-ray (EDX Analysis	24
ii) Quantitative Elemental Determinations	25
3) Chemical Composition of Crystal Inclusions	27
MATERIALS AND METHODS	
I Source of Materials	31
II Light Microscopy	31
III Fixed and Thin-sectioned Material	32
IV Freeze-fracturing	34
V Scanning Electron Microscopy	34
VI Energy Dispersive X-ray Analysis	36
VII Phosphorus, Potassium, Magnesium and Calcium Determinations	38
VIII Calcium Determinations	38

IX Determinations on the Solubility of the Calcium-rich Crystals 39
X Microincineration 40
XI Procedures used for Staining of Inclusions Found in the Protein Bodies
1) Globoid Crystals 41
2) Calcium-rich Crystals 41
XII Preparation for Obtaining Infra-red Spectra of the Calcium-rich Crystals 42
XIII Preparation for Obtaining X-ray Diffraction Patterns of the Calcium-rich Crystals 43

RESULTS
I Light Microscopy 45
II Thin-sectioned Material 46
III Freeze Fracture 50
IV Scanning Electron Microscopy
1) Protein Bodies 57
2) Inclusions 57
V Energy Dispersive X-ray Analysis
1) Globoid Crystals 61
2) Calcium-rich Crystals 66
VI Phosphorus, Potassium, Magnesium and Calcium Determinations 68
VII Calcium Determinations on Mericarps of all Species and during Early Seedling Growth in Carrot 68
VIII Solubility of the Calcium-rich Crystals 73
IX Staining of Inclusions Found in the Protein Bodies
1) Globoid Crystals 75
2) Calcium-rich Crystals 75
X Microincineration 76
XI Infra-red Spectra 76
XII X-ray Diffraction 79

DISCUSSION
I Structural Aspects 81
II Elemental Analyses
1) Energy Dispersive X-ray (EDX) Analysis 86
2) Quantitative Elemental Analysis . 90
III Calcium Oxalate Crystal Inclusions
1) Synthesis of Oxalates 93
2) Crystal Structure and Deposition in Cells 95

3) Function of Calcium Oxalate Crystals 97
4) Identification of Calcium Oxalate Crystals 101
IV. Summary of Findings 105

APPENDIX A 108

BIBLIOGRAPHY 110

LIST OF FIGURES

Figure		Page
1.	Light Micrograph of Carrot Endosperm Tissue	51
2.	Polarized Light Micrograph of Carrot Endosperm Tissue	51
3.	Protein Bodies with Globoid Crystal Inclusions from Carrot Endosperm	51
4.	Protein Body with Globoid Crystal Inclusions from Carrot Endosperm	51
5.	Freeze-fracture Replica of a Protein Body from Carrot Endosperm	51
6.	Carrot Endosperm Cell Containing Protein Bodies with Calcium-rich Crystal Inclusions	51
7.	Protein Body with a Calcium-rich Druse Crystal Inclusion from Carrot Endosperm	51
8.	Protein Body with Calcium-rich Crystal Inclusions from Carrot Endosperm	51
9.	Thin Section from Carrot Embryo Tissue	52
10.	Carrot Endosperm Cell Contents	52
11.	Carrot Mericarp Sectioned and Treated with Lipase and Protease	52
12.	Carrot Endosperm Tissue after Treatment with Chloroform and Propylene Oxide	52
13.	A Calcium-rich Druse Crystal from Carrot Endosperm	52
14.	A Calcium-rich Druse Crystal from Carrot Endosperm	52

15. A Calcium-rich Druse Crystal from Carrot Endosperm 52

16. An Individual Calcium-rich Crystal from Carrot Endosperm 52

17. Thin Section of Caraway Endosperm Tissue . 53

18. Protein Body with Globoid Crystal Inclusions from Caraway Endosperm 53

19. Protein Body with a Globoid Crystal Inclusion from Caraway Endosperm 53

20. Protein Body with a Calcium-rich Druse Crystal Inclusion from Caraway Endosperm . 53

21. Protein Body with a Calcium-rich Druse Crystal Inclusion from Caraway Endosperm . 53

22. Scanning Electron Micrograph of Protein Body Inclusions from Caraway Endosperm ... 53

23. Calcium-rich Crystals from Caraway Endosperm 53

24. Aggregate of Calcium-rich Crystals from Caraway Endosperm 53

25. Protein Body with Globoid Crystal Inclusions from Dill Endosperm 54

26. Protein Body with Two Calcium-rich Druse Crystal Inclusions from Dill Endosperm.... 54

27. Calcium-rich Druse Crystal from Dill Endosperm 54

28. Small Aggregates and Individual Calcium-rich Crystals from Dill Endosperm 54

29. Protein Body with Globoid Crystal Inclusions from Anise Endosperm 54

30. Protein Body with a Calcium-rich Druse Crystal Inclusion from Anise Endosperm ... 54

31. Calcium-rich Druse Crystal from Anise Endosperm 54

32. Aggregate of Calcium-rich Crystals from Anise Endosperm 54

33. Protein Body with a Globoid Crystal Inclusion from Parsley Endosperm 55

34. Protein Body with a Calcium-rich Druse Crystal Inclusion from Parsley Endosperm . 55

35. Protein Body with Globoid Crystal Inclusions from Parsnip Endosperm 55

36. Protein Body with a Calcium-rich Druse Crystal Inclusion from Parsnip Endosperm . 55

37. Protein Body with Globoid Crystal Inclusions from Chervil Endosperm 55

38. Protein Body with a Calcium-rich Druse Crystal Inclusion from Chervil Endosperm . 55

39. Protein Body with Globoid Crystal Inclusions from Wild Carrot Endosperm 55

40. Protein Body with a Calcium-rich Druse Crystal Inclusion from Wild Carrot Endosperm 55

41. Celery Endosperm Protein Body with Globoid Crystal Inclusions 56

42. Celery Endosperm Protein Body with Region which Contained a Calcium-rich Druse Crystal Inclusion 56

43. Cell from Fennel Endosperm Containing Protein Bodies with Globoid Crystal Inclusions 56

44. Fennel Endosperm Protein Body with Region which Contained a Calcium-rich Druse Crystal Inclusion........................ 56

45. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm 63

46. EDX Analysis Spectrum of a Globoid Crystal from a Section of Caraway Endosperm 63

47. EDX Analysis Spectrum of a Globoid Crystal from a Section of Celery Endosperm 63

48. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm 63

49. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm 63

50. EDX Analysis Spectrum of a Globoid Crystal from Carrot Endosperm Freeze-dried Powder 63

51. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm 63

52. EDX Analysis Spectrum of Protein from a Section of Globoid Crystal Containing Protein Body from Carrot Endosperm 63

53. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue 63

54. EDX Analysis Spectrum of a Globoid Crystal from the Same Carrot Ground Meristem Cell as Fig. 53 63

55. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue 63

56. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue.................................... 63

57. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Embryo Protodermal Tissue 64

58. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Embryo Protodermal Tissue 64

59. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Protodermal Tissue 64

60. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Provascular Tissue 64

61. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Provascular Tissue 64

62. EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Provascular Tissue 64

63. EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Carrot Endosperm 67

64. EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Caraway Endosperm 67

65. EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Celery Endosperm 67

66. EDX Analysis Spectrum of a Calcium-rich Crystal from Caraway Endosperm Freeze-dried Powder 67

67. EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Carrot Endosperm 67

68. EDX Analysis Spectrum of Protein from a Section of Calcium-rich Crystal Containing Protein Body from Carrot Endosperm 67

69. Infra-red Spectra of Calcium-rich Crystals from Carrot Endosperm and Reagent Grade Calcium Oxalate Monohydrate 78

LIST OF TABLES

Table Page

1. The Percentage by Dry Weight of Phosphorus, Potassium, Magnesium and Calcium in Whole Mericarps 69

2. Calcium Determinations of Whole and Dissected Resting Mericarps 70

3. Calcium Content Three and Five Days after Radicle Emergence (Carrot) 72

4. Calcium-rich Crystal Solubility Studies .. 74

5. Microincineration Studies on Calcium-rich Crystals, Reagent Grade Calcium Oxalate and Regrade Grade Calcium Carbonate 77

6. Comparision of X-ray Diffraction Data from Calcium-rich Crystals (Carrot) with Known X-ray Diffraction Data of Calcium Oxalate Monohydrate (Whewellite) . 80

INTRODUCTION

I Seed Protein Bodies

Storage tissues in seeds of the angiosperms and gymnosperms contain subcellular components called protein bodies. Most protein bodies are approximately spherical in shape and appear to be surrounded by a single unit membrane. Most of a seed's storage protein and mineral reserves are contained in the protein bodies. Protein bodies are important for the survival of many plant species since they provide the embryos of angiosperms and gymnosperms with nitrogenous compounds and minerals needed during germination and early seedling growth. Protein bodies in seeds of a number of crop plants are of great importance to the nutrition of humans and domestic animals.

Hartig (1855) studying seeds of several species first reported the presence of protein bodies. Hartig (1855) termed them klebermehl or aleurone grains. The term aleurone grain is still preferred by some workers (Altschul _et al_, 1966), however the term protein body has become the most commonly used term when referring to these subcellular components. The term aleurone grain is now being used less frequently because this term implies

incorrectly, that these subcellular components are found only in the aleurone layer of monocot seeds. Protein bodies occur not only in the aleurone layer but in other storage tissues in seeds. Also protein bodies consist primarily of protein (see reviews by Ashton 1976, Pernollet 1978, Lott 1980, and Webber and Neumann 1980).

Studies on seeds of numerous species have shown that protein bodies are variable and complex subcellular components. Protein bodies vary in occurrence, size, internal structure and chemical composition. Outlined below are a number of these variable parameters. Referenced examples are given, however the reader is referred to detailed reviews pertaining to protein bodies by Ashton (1976), Pernollet (1978), Lott (1980), and Webber and Neumann (1980).

Protein bodies occur in a number of seed tissues. The ploidy number of a given tissue appears to have no effect on the presence or absence of protein bodies (Lott, 1980). Protein bodies are found in the haploid megagametophyte tissue of some conifers. They are found occasionally in the diploid perisperm of certain angiosperms and are frequently found in the diploid embryo tissue of most seeds. Polyploid endosperm tissues also contain protein bodies.

In size, protein bodies vary in diameter from

0.1 µm to 22 µm with most protein bodies being between 1.5 µm and 8.0 µm in diameter (Ashton, 1976). Seeds of certain species contain uniformly sized protein bodies throughout (Craig et al, 1979) whereas seeds of other species such as those in tomato (Spitzer and Lott, 1980) have protein bodies that vary in size. Where variation occurs, protein bodies may vary in size from tissue-to-tissue as well as within the same cell. The size of the protein bodies appears to be influenced to some degree by the size of the cell in which the protein bodies are located. For example, the largest cells in a given tissue usually contain the largest protein bodies.

Protein bodies may show considerable structural diversity. This diversity may occur within a given cell, cell-to-cell or tissue-to-tissue. In their simplest form protein bodies may consist solely of structurally homogeneous proteinaceous matrix as observed in a number of legumes (see review by Ashton, 1976 and Pernollet, 1978). More recent studies and a critical evaluation of the previous research on the protein bodies of legumes indicates that inclusions may be present (Lott and Buttrose, 1978a). Thus the presence of such structurally simple protein bodies may be less frequent than initially indicated. Most frequently protein bodies contain inclusions. Several types of inclusions may be present.

Commonly occurring inclusions include protein crystalloids, globoid crystals, soft globoids and druse crystals. To date no protein body has been found that contains all four types of these inclusions, however protein bodies consisting of a proteinaceous matrix and one, two or three of these inclusions have been found. Examples of the different combinations of inclusions found in protein bodies are outlined below. Globoid crystals, soft globoids and protein crystalloids were found in the protein bodies of *Cucurbita maxima* (Lott *et al*, 1971; Lott and Vollmer, 1973). Globoid crystals and protein crystalloids occurred in the protein bodies of tomato (Spitzer and Lott, 1980). Only globoid crystal inclusions occurred in the protein bodies of sunflower (Buttrose and Lott, 1978a). In protein bodies of *Eucalyptus erythrocorys*, globoid crystals and druse crystals were found (Buttrose and Lott, 1978b). In certain protein bodies of *Aethusa cynapium* druse crystal and protein crystalloid inclusions occurred (Pfeffer, 1872) while only druse crystal inclusions were found in certain protein bodies of *Silybum marianum* (Pfeffer, 1872). A brief description of each type of inclusion is given below.

When present, one or more protein crystalloid inclusions may be found in a given protein body. The number of such inclusions per protein body may remain constant throughout a tissue or tissues, or it may vary, even with-

in the same cell. The number of protein crystalloids can often be revealed by viewing thin-sectioned material since areas of proteinaceous matrix are usually visible between the crystalloids. On occasion, studies using freeze-fracture have shown several protein crystalloids fused in such a manner that they appear as only one large protein crystalloid when viewed as thin-sectioned material (Lott and Buttrose, 1978b). Protein crystalloids vary in shape, but often have angular edges in a given plane of section. Less frequently protein crystalloids have irregular or rounded edges in a given plane of section. If globoid regions are also present the shape of the protein crystalloids appears unaffected in the majority of the seeds studied. In Brazil nut the globoid crystals, although surrounded by proteinaceous matrix, appear within "cavities" of the protein crystalloids in such a way as to suggest that they had influenced the shape of the protein crystalloid inclusions (Lott and Buttrose, 1978b). Structurally, protein crystalloids consist of numerous subunits arranged in a complex lattice network. The lattice and subunits are rarely seen in thin-sectioned material but they can often be seen in freeze-fractured material (Lott and Buttrose, 1978b ; Lott and Buttrose, 1978c; Spitzer and Lott, 1980). These inclusions are thought to consist primarily of storage

protein.

Globoid crystal inclusions are often approximately spherical in shape. They vary in size and number. Although most studies indicate that no membrane surrounds these inclusions, studies by Buttrose (1971), and Swift and Buttrose (1972) on barley and wheat respectively indicate that membranes may be present. Globoid crystals are naturally electron dense regions. The main function of globoid crystals is mineral storage. The globoid crystals in most species studied contain most of a protein body's phytin (Lui and Altschul, 1967; Ogawa et al, 1975; Sharma and Dieckert, 1975). Phytin is a cation salt of myo-inositol hexaphosphoric acid (Asada and Kasai, 1962; Ashton and Williams, 1958; Ergle and Guinn, 1959; Lui and Altschul, 1967). It is a store of phosphorus, cations and myo-inositol.

Studies have indicated that more than half of a seed's total phosphorus is in the form of phytin (Ashton and Williams, 1958; Lolas and Markakis, 1975; Matheson and Strother, 1969; Nahapetian and Bassiri, 1976). The most commonly occurring cations in globoid crystals are magnesium, potassium and calcium (see reviews by Pernollet, 1978; Lott, 1980). Iron, manganese, sulfur, silicon, sodium and barium have also been detected in varying amounts in globoid crystals of certain species. Sulfur

and chlorine have been reported in a number of studies (see reviews by Pernollet, 1978; Lott, 1980). Sulfur's presence may be in association with protein. Protein is present in the globoid crystals of peanut (Sharma and Dieckert, 1975) and thus may be present in the globoid crystals of other species. The presence of sulfur is difficult to interpret since isolated globoid crystals may have protein contamination and EDX analyses studies may have analysed adjacent protein regions. Chlorine, reported by Lott and Buttrose (1978a), van Staden and Comins (1976), may be present because epoxy resins may contain chlorine. Lott and Buttrose (1978b) reported the presence of barium in globoid crystals in Brazil nut. Sodium was present in globoid crystals of _Crambe abyssinica_ (Hofsten, 1973). Van Staden and Comins (1976) reported the presence of silicon in the protein bodies of _Protea compacta_. Manganese has been detected in oat phytin (Ashton and Williams, 1958) and in peanut globoids (Sharma and Dieckert, 1975). Both manganese and iron have been detected in the globoid crystals of _Avena sativa_ (Buttrose, 1978), several species of _Casuarina_ (Buttrose, 1978), tomato (Spitzer and Lott, 1980) and wheat (Lott and Spitzer, 1980). The phytic acid in globoid crystals

is a chelator of minerals. It's properties as a chelator can cause nutritional deficiencies in animals and humans. Phytic acid can chelate such minerals as calcium, magnesium, iron, zinc and molybdenum thus reducing the availability of these minerals in the digestive tract and causing mineral deficiencies (reviewed by Bassiri and Nahapetian, 1977; Lolas and Markakis, 1975). Compounds other than phytin and protein have been reported in globoid crystals. Lipids have been found in the globoids of barley (Jacobsen *et al*, 1971) and oxalic acid was found in the globoid crystals of peanut (Sharma and Dieckert, 1975).

Soft globoid inclusions have been reported in the protein bodies of *Cucurbita maxima* (Lott *et al*, 1971; Lott and Vollmer, 1973) and *Ricinus* (Tully and Beevers, 1976). This inclusion surrounds globoid crystal inclusions and is electron transparent. The function and composition of these inclusions is not known. The presence of soft globoids may be hydration dependant, however further investigation on this inclusion is required.

Another type of inclusion which may be present consists of a crystal or aggregate of crystals. The aggregates are commonly termed druse or rosette crystals. Crystals occurring singly or in aggregates vary in size and shape but are usually 1 µm to 5 µm in their longest dimension. They are often prismatic in shape. There

appears to be no limiting membrane surrounding such crystals (Buttrose and Lott, 1978b; Lott and Buttrose, 1978c). Crystal inclusions are believed to be composed of calcium oxalate (see reviews by Ashton, 1976; Lott, 1980). The chemical composition of these crystal inclusions has not been rigorously determined with more advanced methods such as x-ray diffraction. The function(s) of these crystals remains unclear. Such crystals are perhaps the least understood inclusions found in protein bodies.

Other less frequently appearing inclusions have been reported. Jacobsen _et al_ (1971) found protein-carbohydrate inclusions within the protein bodies in the aleurone layer of barley. The type(s) of carbohydrate and protein remain unknown. Buttrose and Lott (1978c) in their study on _Eucalyptus_ reported an area surrounding druse crystals in several protein bodies. The composition and function of such an area also remains unknown. Neither inclusion has been reported elsewhere.

II Previous Studies on Protein Bodies of Umbelliferous Seeds

The number of previous studies which deal with protein bodies of umbelliferous seeds are few even though protein bodies from several umbelliferous species were among the earliest protein bodies studied. Many of these

studies were done in the latter part of the last century. Since then, the early work on these protein bodies has been assumed to be correct and little reinvestigation has been done.

The earliest study on the protein bodies of any umbelliferous seed was done by Pfeffer (1872), seventeen years after Hartig (1855) initially discovered protein bodies in the cells of seeds from several species. Pfeffer (1872) studied a number of seeds from various families and was the first researcher to understand many of the basics regarding protein body structure and composition. Pfeffer (1872) specifically mentions studying seeds of the umbelliferous species Coriander sativum (coriander) and Aethusa cynapium. Pfeffer (1872) neglected to mention the protein bodies of the embryos of the Umbelliferae. He indicated however, that two types of protein bodies were present in the endosperm. One type of protein body consisted of proteinaceous matrix and several variously sized globoid inclusions. The other type of protein body consisted of proteinaceous matrix and crystals. These crystals were considered to be calcium oxalate on the basis of their solubility. These crystals were generally arranged in the form of a druse around a centre composed of protein. The two types of protein bodies were never found in the same cell.

Pfeffer (1872) reported that only one member of the Umbelliferae, Aethusa cynapium, contained protein crystalloid inclusions along with either globoids or druse crystals. To date no other umbelliferous species has been found that contains either globoids or druses along with protein crystalloid inclusions. Upon germination in the seeds of the Umbelliferae the crystals thought to be chemically composed of calcium oxalate remained while other components of the protein bodies broke down and disappeared (Pfeffer, 1872). Pfeffer (1872) also reported that unlike other families he had studied, all members of the Umbelliferae had protein bodies which contained inclusions thought to be composed of calcium oxalate.

Kohl (1889), in an article on crystals in plants, mentioned Pfeffer's studies and similar studies of his own on the protein bodies of Coriander sativum (coriander) and Aethusa cynapium. He presented no new information about the protein bodies of these two species, or the Umbelliferae in general.

Studies of protein bodies made by Ludtke (1890) included seeds from three species of the Umbelliferae, namely Foeniculum vulgare (fennel), Carum carvi (caraway) and Daucus carota (carrot). Lüdtke (1890) found in fennel endosperm that two types of protein bodies

were present in separate cells. Some protein bodies contained globoid inclusions while others contained crystal inclusions in the form of a druse thought to be composed of calcium oxalate. Caraway endosperm was found to be similar to fennel, however, occasionally a given protein body had more than one druse crystal present. Lüdtke (1890) found three cell types in carrot endosperm based on the presence of subcellular components. One type of cell contained large and small aggregates of crystals thought to be composed of calcium oxalate. These aggregates were not contained in protein bodies. A second type of cell contained protein bodies with druse crystal inclusions. The third type of cell contained protein bodies with globoid inclusions.

Winton (1916) mentioned various aspects of the structure and composition of the seeds from several families including some members of the Umbelliferae. _Carum carvi_ (caraway), _Pimpinella anisum_ (anise), _Anethum graveolens_ (dill), _Foeniculum vulgare_ (fennel), _Apium graveolens_ (celery), _Cuminum cyminum_ (cumin), and _Coriander sativum_ (coriander) were specifically mentioned and compared in terms of various aspects of their taxonomy including the structure of their endosperms. Winton (1916) noted the following. The endosperm cells

of each species studied were quadrilateral or polygonal in shape in section and thick walled. The endosperm cells contained protein bodies and lipids. In all species studied, except cumin, the protein bodies were from 2.0 to 8.0 µm in diameter. In cumin the protein bodies were up to 15 µm in diameter. In all seed types mentioned two types of protein bodies existed. Some protein bodies contained one or two globoid inclusions. Other protein bodies contained rosettes of crystals thought to be composed of calcium oxalate. Winton (1916) noted that the embryos of each of the members mentioned were of little value in terms of taxonomy due to their small size and thus were not considered.

Some information on the protein bodies of six species including Carum carvi (caraway), Pimpinella anisum (anise), Anethum graveolens (dill), Apium graveolens (celery), Foeniculum vulgare (fennel), Daucus carota (carrot) which are to be considered in the present study are thus presented in the articles by Lüdtke (1890) and Winton (1916). Little study directly concerning the protein bodies of any member of the Umbelliferae has been done since Lüdtke (1890) and Winton (1916). Other researchers mentioned the protein bodies of Umbelliferae but the protein bodies were not the main concern of their studies. Rompel (1905) studying the presence of

calcium oxalate crystals in the pericarp of a large number of members of the Umbelliferae indicated that it was difficult to determine if these crystals were in the pericarp or were fragments from crystals located in the protein bodies of the endosperm. Rompel's study although not directly concerned with the crystals found in the protein bodies did indicate that the occurrence of such crystal inclusions were widespread in the Umbelliferae. Guilliermond (1941), in his studies on the cytoplasm of the plant cell, mentioned that protein bodies sometimes contain crystals of calcium oxalate. Guilliermond (1941) used Oenanthe phellandrium (now Oenanthe aquatica (L.) Poir.) a member of the Umbelliferae, as an example of plants which have such protein bodies present in their seeds. Jacobsen et al, (1976) and Jacobsen and Pressman, (1979) noted in studies on endosperm breakdown in Apium graveolens (celery) that some protein bodies had globoid inclusions while others had angular inclusions. Jacobsen and Pressman, (1979) noted that no protein bodies containing globoid inclusions were found in the outermost layer of the endosperm. Also all cellular material in the endosperm , including the angular inclusions, disappeared upon germination probably being used by the embryo. These studies were not directly concerned with the protein

bodies of celery, however.

Recent texts (ie. Cutter, 1978) and several reviews (Ashton, 1976; Lott, 1980; Webber and Neumann, 1980) mention the protein bodies of the Umbelliferae briefly. These protein bodies were mentioned primarily because some of them contain aggregates of crystals commonly termed druses or rosettes. These protein bodies thus typify a structural type of protein body which presently is considered rare based on the few species whose protein bodies have been studied. Pernollet (1978) in a recent review on protein bodies neglected to mention the existence of protein bodies containing such crystal aggregates.

Thus the protein bodies of the Umbelliferae as a whole have received little serious study for over sixty years and little new information has been added since Pfeffer's studies in 1872. Since Pfeffer reported his studies in 1872 there have been many major advances both in technology and methodology. With the use of electron microscopy and other highly advanced instrumentation the protein bodies of the Umbelliferae can be investigated in much greater detail than was previously possible.

III Members of the Umbelliferae Studied

In the studies described in this thesis the protein

bodies of nine species of the Umbelliferae were included. All nine species contain widely used cultivars and include Daucus carota (carrot), Carum carvi (caraway), Pimpinella anisum (anise), Anethum graveolens (dill), Apium graveolens (celery), Foeniculum vulgare (fennel), Pastinaca sativa (parsnip), Petroselinum sativum (parsley), and Anthriscus cerefolinum (chervil). Daucus carota (wild carrot) was the only native member of the Umbelliferae studied. It was included primarily to determine if any major differences in protein body structure or composition occurred between seeds of those members of a species that have been subject to extensive plant breeding for improved horticultural performance and those members of a species that have not been genetically manipulated. While all species were studied to some degree, certain tests were made only on carrot. Daucus carota (carrot) was chosen as the main species to be studied since it is a well known member of the Umbelliferae and is a most important species economically. Caraway, anise, dill, fennel and chervil were included in the present study since the fruits of these five members are frequently included in the preparation of a variety of foods and medicines. The residue remaining after extraction of the essential oils has been fed to cattle (Winton, 1916). Although the petioles of celery are the most important food source

from this species, the fruits are used as a spice and thus are eaten to a small extent. Because of this celery was included in the study. Parsnip and parsley were included since they are important crop plants even though the vegetative parts are used for food purposes.

The term seed is often incorrectly applied in the family Umbelliferae. This occurs primarily due to the nature of the fruit. The fruit of the Umbelliferae is classified as a schizocarp. At maturity a schizocarp splits in half into one-seeded carpels each of which is termed a mericarp. It is the individual mericarps that are often incorrectly termed seeds. Care has been taken in using the correct terminology in the present study.

In shape the mericarps of all species of the Umbelliferae studied in this thesis, except chervil, are plano-convex and have a number of ribs running the length of the entire mericarp. In appearance fennel and caraway are slender, dill and parsnip are broad and flat, while carrot, anise, parsley and celery are almost as broad as they are long but not flat. Chervil mericarps are linear and cylindric with no ribs. The length of the mericarps varied from species-to-species, from approximately 1.0 mm in celery to 6.0-8.0 mm in chervil with most of the others being between 2.0-4.0 mm. The

endosperm of these seeds is the major storage tissue. The seeds contain linear embryos which usually occupy a very small portion of the seed, however they may occasionally be almost the length of the entire seed. For a more complete description of the fruit and the seeds of the Umbelliferae the reader is referred to Gray's Manual of Botany (1970) and Martin (1946).

IV Objectives of Present Study

This thesis presents the results of the first detailed ultrastructural study of the protein bodies of nine species from the family Umbelliferae. It is the first electron microscopic study of the endosperm protein bodies and is the first detailed structural study of the protein bodies of the embryos in any member of the Umbelliferae. The structure of the globoid crystal and calcium-rich crystal inclusions, and the surrounding proteinaceous matrix were investigated. An attempt was made to retain the calcium-rich crystals in thin-sectioned material. In previous studies only the areas which contained such crystals were retained (Lott and Buttrose, 1978c; Buttrose and Lott, 1978b; Jacobsen _et al_, 1976).

An attempt was made using scanning electron microscopy to determine the three dimensional structure of the calcium-rich crystals. This was the first study

in which the structure of such crystal inclusions in protein bodies has been investigated with scanning electron microscopy. This procedure gave a more precise understanding of the crystal aggregates and thus permitted a consideration of the terminology that can best be used to describe the aggregates of the crystal inclusions found in protein bodies. Common terms previously used to describe crystal aggregates in protein bodies include druse and rosette crystals. Druse crystals are spherically shaped aggregates and consist of a variable number of component crystals which radiate outwards in all directions from a central core. Rosette crystals, illustrated in a book pertaining to the crystal structure of minerals edited by Prinz et al (1978), are shown to be composed of component crystals which vary in size, are arranged like petals of a rose, and do not radiate out in all directions from the centre. Cutter (1978) stated that the crystal aggregates found in the protein bodies of the Umbelliferae were rosette crystals, however her description of such crystals indicated that these crystal aggregates are really druses and not rosettes. In this thesis, aggregates with component crystals radiating out in all directions will be termed a druse crystal and any other collection of crystals will be termed an aggregate.

Energy dispersive x-ray analysis was used to determine for the first time in any umbelliferous seed, the elemental composition of the protein bodies in both endosperm and embryo. The determination of the elemental composition of the calcium-rich crystals aided in determining their chemical composition. The possibility of any variations in elemental composition between crystal aggregates of the different species, within the same species or crystals within a given aggregate were investigated. The elements stored in the globoid crystals of both endosperm and embryo and any variations were also determined. The elemental composition of the proteinaceous matrix of both types of protein bodies from each species were investigated and compared. Another objective was to investigate the effects of the various tissue preparation methods used to determine elemental composition.

For the first time with mericarps of several Umbelliferae, elemental analyses using atomic absorption spectrometry were carried out. Quantitative values for P, K, Mg, and Ca on a percentage dry weight basis were determined.

Calcium content of whole and selected portions of mericarp from all species studied were determined on a per mericarp basis. Dissection of entire mericarps

into pericarp + testa + endosperm, and embryo components of each of the species and calcium determinations of each of these components indicated the relative amounts of calcium contained in each. The most important objective involving calcium determinations in this thesis was the determination of calcium content in the pericarp + testa + endosperm and the embryo portions during early seedling growth in carrot. There are conflicting reports regarding the utilization of the calcium-rich crystal inclusions in the endosperm of the Umbelliferae. Pfeffer (1872) indicated such crystals are not degraded in the Umbelliferae during germination or afterward. Jacobsen et al (1976) stated that the inclusions in the protein bodies of celery endosperm are degraded. My study of calcium contents is the most extensive study concerned with determining the fate of calcium-rich crystal inclusions in protein bodies carried out to date.

In this thesis, one of the main objectives was to determine conclusively the chemical composition(s) of the calcium-rich crystal inclusions in the protein bodies of the Umbelliferae. In no plant species have such crystal inclusions within protein bodies been investigated as thoroughly as in this thesis. In the past such crystal inclusions in protein bodies have often been assumed to be calcium oxalate without testing

or have been tested only to the point where it is likely that they are calcium oxalate. Also, due to the number and variety of analytical methods employed here another objective was to determine which method or methods would be best suited to the characterization of such crystal inclusions in protein bodies in other species.

V Choice of Procedures

1) Structure

Early researchers such as Pfeffer (1872), Kohl (1889) and Lüdtke (1890) had only the light or polarizing light microscope with which to determine the structure of protein bodies. Details of their studies had to be presented either as hand drawn diagrams or as written observations. Pfeffer (1872) indicated several times that the presence of small inclusions was difficult to determine. Even at present, although both the light and polarizing light microscopes are useful, some structural details of protein bodies cannot be resolved with these instruments.

In this thesis the structure of the protein bodies, globoids and calcium-rich crystals was studied using light and electron microscopy. The structural study using light microscopy was limited due to reasons outlined above. The majority of the structural study involved electron microscopy since protein bodies and

their inclusions are easily resolved. The structural study followed a multifaceted approach similar to those of Lott and Buttrose (1978a, b, c), Buttrose and Lott (1978a, b) and Spitzer and Lott (1980). Thin-sectioned material and freeze-fractured replicas were viewed with a transmission electron microscope. The major advantages with thin-sectioned tissue were that usually the position of the cells being viewed could be determined and large areas of tissue could be studied. The major disadvantage of fixed and thin-sectioned tissue was that there may be artifacts due to the chemical processing. The major advantage with freeze-fracturing was that the tissue is kept close to its natural state. The disadvantages of freeze-fracturing were that usually only small areas of tissue could be examined and often the exact location from which the replica was obtained was not known.

In addition to transmission electron microscopy scanning electron microscopy was used to determine the structure of the protein bodies, globoid inclusions and especially the calcium-rich crystal inclusions. The depth of field obtained in a scanning electron microscope allows one to obtain an image that appears three dimensional. Scanning electron microscopy is thus an excellent method for determining the structure of the complex

crystal aggregates and individual crystals. Serial sections of seed tissue are difficult to obtain and serial sections of crystals are almost impossible to obtain. Thus, transmission electron microscopy could not be used to study the complex three dimensional arrangements of the crystal aggregates.

2) Elemental Analyses

i) Energy Dispersive X-ray (EDX) Analysis

EDX analysis is a fast and highly sensitive system for the detection of elements with atomic number 11 (Na) or heavier. This system is capable of detecting as little as 10^{-18} grams of an element (Russ, 1972) depending on the instrument. With an EDX analysis system mounted on a transmission electron microscope small areas of tissue can be spot analyzed <u>in situ</u> for elemental composition. With EDX analysis systems analyses of all elements occurs simultaneously. For biological studies, simultaneous analysis has an advantage over other methods that require sequential analysis since the intense electron beam gradually destroys a given tissue region.

A number of EDX analysis studies on the elemental composition of protein bodies have been done. These studies primarily present analyses of globoid crystal inclusions, since they contain stores of minerals. However, the proteinaceous regions and crystal inclu-

sions have also been analyzed (see review by Lott, 1980). Through its use in such studies EDX analysis has provided investigators with a more accurate understanding of the types of mineral stored, their specific localization if any, and a·.y elemental variations in the complex mineral storage system found in seeds. Most EDX analysis studies on seed tissue have been done with thin-sectioned tissue fixed only witn glutaraldehyde. Lott _et al_ (1978) reported that extraction of a number of minerals, especially potassium, from the globoid crystals occurred when seed tissue was fixed with glutaraldehyde followed by osmium tetroxide. Freeze-dried powders, prepared according to the method of Lott and Vollmer (1979), were used in conjunction with thin-sections as a check for possible extraction of elements during chemical processing. In the study described here a danger of elemental extraction does exist since tissues are processed with chemicals. Since both globoid crystal and druse crystal inclusions are naturally electron dense they can be identified even when the seed tissue is prepared as a freeze-dried powder.

ii) Quantitative Elemental Determinations

In all investigations concerning quantitative elemental determinations atomic absorption spectrometry was used. Atomic absorption spectrometry is a highly

sensitive quantitative method. A wide range of elements can be detected. Usually only one element at a time is determined. Tissue samples must be digested, usually in nitric followed by perchloric acid, prior to analysis.

In studies involving mineral storage of seeds, it is a valuable method for determining the quantitative amounts of such elements as P, K, Mg, and Ca. A large portion of these elements in the seed are usually found in the protein bodies. Although results are not specific to a given cell or subcellular region, atomic absorption spectrometry compliments the findings of the EDX analysis studies to a certain extent since strictly quantitative data is difficult to obtain with EDX analysis.

Since atomic absorption spectrometry is a very sensitive method, small portions of tissue such as the embryos of the umbelliferous seeds can be dissected out, digested and analysed separately. Thus in terms of determining if major amounts of calcium moved from the endosperm to the embryo during germination and early seedling growth this method could detect it. Since much of the calcium in the endosperm is believed to be in the calcium-rich crystals, any major movement of calcium detected using this method would indicate that some of these crystals have been used.

3) Chemical Composition of Crystal Inclusions

Pfeffer (1872) and other early researchers (Kohl, 1889; Lüdtke, 1890), determined the chemical composition of crystal inclusions in protein bodies using the polarizing light microscope and crystal solubility studies. The polarizing light microscope indicated that such crystals were birefringent, however numerous compounds are birefringent. Solubility tests were thus the most important method for identifying such crystals. Crystal solubility was tested with mineral acids, water, acetic acid and dilute sodium hydroxide. It was believed but not confirmed that these tests ruled out all but barium, strontium and calcium oxalate. Pfeffer (1872) assumed that since barium and strontium were rare in plants that such crystals were therefore composed of calcium oxalate. Such extensive solubility studies were limited to only a few species. Usually if crystals were insoluable in acetic acid, including those of the Umbelliferae, they were assumed by Pfeffer (1872) to be calcium oxalate crystals. It was not made clear in the reports by Kohl (1889) and Ludtke (1890) how extensively such crystals in the Umbelliferae were studied using solubility tests.

In this thesis a variety of methods are used to determine the chemical composition of the crystal inclusions, including the polarizing light microscope and

crystal solubility tests. Other methods used for determining chemical composition of crystals, included energy dispersive x-ray analysis, staining for calcium oxalate, microincineration, infra-red spectroscopy and x-ray diffraction. Each of these methods has certain advantages and are detailed below.

EDX analysis described earlier has recently been used on crystal inclusions in the protein bodies of Eucalyptus erythrocorys and Simmondsia chinensis (Buttrose and Lott, 1978b) and Corylus avellana (Lott and Buttrose, 1978c). EDX analysis in both studies indicate the crystals contained only calcium which is consistent with their being composed of calcium oxalate. Although the actual chemical composition of the crystals is not determined with EDX analysis any chemical compound which contains elements with atomic number 11 or higher; other than calcium, can be ruled out. Also, the small tissue area which can be analyzed allows for specific individual crystals of an aggregate or portions of an individual crystal to be analyzed for their chemical content. This is a valuable method for determining whether aggregate crystals or individual crystals are composed solely of one chemical compound or several. With other methods, EDX analyses is of value in determining the composition of crystal inclusions.

Staining of crystals is a method used extensively on calcified deposits in animal and human tissues. A relatively quick method to perform, it can be used *in situ*. One study has been done on specific staining of crystals thought to be calcium oxalate in plant tissue (Silver and Price, 1969). The method of Silver and Price (1979) is essentially the silver nitrate-hydrogen peroxide method of Pizzolato (1964) applied to plant not animal tissues. A second successful method is the silver nitrate-rubeanic acid method of Yasue (1969). Both these methods are used in the present study and are modified from the von Kossa method (von Kossa, 1901). Both rely on metal substitution. The calcium at the surface of the crystal is replaced with silver and thus when viewed under the light microscope the crystals, which are clear and colourless when unstained, are stained a dark brown or black.

Introduced by Johnson (1956) and developed further by Johnson (1958) and Johnson and Pani (1962), microincineration is a fast and accurate method used for the *in situ* identification of crystals thought to be calcium oxalate. Briefly, tissue sections containing such crystals are heated either at 450° C which converts the crystals to calcium carbonate, or at 600° C which converts them to calcium oxide. Subjecting the sections to 2.0 M acetic acid prior to heating removes

any other calcium salts of other dicarboxylic acids (Johnson, 1958). Microincineration however, had only been used on animal and human tissue prior to its use in this study.

Infra-red spectroscopy and x-ray diffraction of calcium-rich crystals in protein bodies have not been tried previously. Similar crystals in other areas of the plant have been identified using one or both of these methods (see review by Franceschi and Horner, 1980). Both methods are used in the present study. Both methods are highly sensitive and can indicate the exact chemical composition of a substance provided enough sample is present and the sample is relatively pure. Since the crystals from the protein bodies are small, these methods require that the crystals be removed, purified and concentrated prior to analysis.

MATERIALS AND METHODS

I Source of Material

Seeds of 9 species from 9 genera of the family Umbelliferae were studied. Mericarps of the 9 cultivated plants studied, including carrot (*Daucus carota* L. cv imperator 408), caraway (*Carum carvi* L.), anise (*Pimpinella anisum* L.), dill (*Anethum graveolens* L.), celery (*Apium graveolens* L. cv tall Utah), fennel (*Foeniculum vulgare* Mill), parsley (*Petroselinum sativum* L. cv moss curled), parsnip (Pastinaca sativa L. cv hollow crown) and chervil (*Anthriscus cerefolium* L. cv curled), were obtained from Tregunno Seeds Limited, Hamilton, Ontario. Mericarps of the one native member studied, wild carrot (*Daucus carota* L.) were collected at McMaster University, Hamilton, Ontario.

II Light Microscopy

Seeds of all 9 species were studied using the brightfield and polarizing modes of a Leitz Diolux-Pol polarizing light microscope. Seed tissues were either hand sectioned without fixation, or fixed with 5% glutaraldehyde then embedded in Spurr's epoxy resin as outlined under "Fixed and Thin-sectioned Material" and sectioned 1.0 to 2.0 μm thick with a Porter-Blum MT-1 microtome. Sections were air dried onto glass slides,

stained with either 0.05% toluidine blue(Jacobsen et al, 1976) or safranin and viewed. Also, calcium-rich crystals are highly birefringent under polarized light while globoid crystals are not. Thus it was possible to determine if any distribution patterns existed between globoid crystals and calcium-rich crystals.

III Fixed and Thin-sectioned Material

Several different fixations were tried to determine which one was most satisfactory. The pH was varied as was the combination of fixatives used. In all trials 6 dry mericarps from all 9 species were used. The pericarp and seed coats were removed and small cubes of endosperm tissue were dissected.

In the first trial all samples were fixed on ice in 5% glutaraldehyde in distilled H_2O pH 7.1 (pH adjusted with NaOH) for 1.5 hr. After washing, three seeds from each species were then post-fixed on ice in 2% OsO_4 in distilled H_2O for 8 hr. Both these fixations were poor since globoid crystals and calcium-rich crystals were extracted. A second fixation series consisted of placing samples on ice in 5% glutaraldehyde and 1.5% acrolein in distilled H_2O pH 7.0 (pH adjusted with NaOH) for 8 hr. After washing, three seeds from each species were then post-fixed on ice in 2% OsO_4 in distilled water for 6 hr. Both these fixations produced results similar to those

mentioned above. A third set of fixations similar to the first set was carried out. Differences included a pH increase to 7.5 from 7.1 and only 4 hr. post-fixation in 2% OsO_4 in distilled water. In samples fixed in glutaraldehyde only, the globoid crystals and calcium-rich crystals were retained while glutaraldehyde-OsO_4 tissue often had their globoid crystals and calcium-rich crystals extracted. Thus for the bulk of any studies requiring thin-sectioned material, samples were cold fixed for 1.5 hr. in 5% glutaraldehyde in H_2O (pH 7.5) with no post-fixation in OsO_4.

Samples from all fixations were dehydrated through an ethanol series consisting of 10%, 20%, 30%, 50%, 70%, 80%, and 95% ethanol on ice for 30 min. per step. Samples were then placed in 100% ethanol for 2 changes of 1 hr. each at 20° C, followed by 100% propylene oxide for 14 hr. at 20° C. Samples were placed into 10%, 25%, 50%, 75% and 100% Spurr's epoxy resin for 90 min. per step. Samples in 100% Spurr's epoxy resin were then heated at 60° C for 15 hr. to polymerize the resin. For structural studies using the TEM, ultra-thin sections (70 - 120 nm) were cut on a Porter-Blum MT-1 microtome using either glass or diamond microtome knives. Sections were picked up onto Formvar coated copper grids prior to viewing in a Philips EM 300.

Staining with uranyl acetate (25 min.) and lead citrate (1 min.) led to extraction of calcium-rich crystals and thus was discontinued. The protein bodies and their internal components were distinguishable without post-staining.

IV Freeze-fracturing

Only carrot was used. Small cubes of endosperm were cut to fit the specimen holders. The tissue samples were frozen in liquid Freon 22 after immersion in 20% glycerol for 5 to 10 min. Freeze-fracturing using standard procedures was done on an Edwards unit. After fracturing the samples were coated with Pt and C respectively. Upon removal from the freeze-fracturing device the samples were thawed, placed into 20% glycerol, 100% glycerol, or distilled water and replicas of fractured tissue were selected. The replicas were placed either into chromic acid for 2 to 3 weeks or were placed into 80% sulfuric acid at 60° C for 3 hr. to remove any tissue present. Replicas were then washed with distilled water, placed into sodium hypochlorite for 30 min. washed in distilled water, and picked up on parlodion coated copper grids prior to viewing in a Philips EM 300.

V Scanning Electron Microscopy

Several procedures were undertaken to obtain structural details of the protein bodies and their

globoid crystal and calcium-rich crystal inclusions. To obtain structural details of the entire protein body, whole dry carrot mericarps were broken in half with forceps. The tissue pieces were placed into 100% chloroform for 2 hr. at 20° C followed by 100% propylene oxide for 18 hr. at 20° C then air dried prior to mounting. Methods were attempted to obtain structural details of both globoid crystals and especially calcium-rich crystals. In one method carrot mericarps were placed onto filter paper in petri dishes, imbibed and germinated. The endosperm remains, which still contained many calcium-rich crystals, were squeezed from the mericarps and collected. This material was either left untreated, treated with 1% aqueous triton for 15 min. or treated with 100% chloroform for 1 hr. followed by 1% aqueous triton for 15 min. prior to mounting. Other procedures were attempted with ungerminated carrot. Whole dry carrot mericarps were cut in section and several treatments using combinations of lipase, protease, chloroform and triton were attempted. Only the lipase-protease treatment outlined as follows was successful. Tissue samples were placed into 2% lipase (Sigma type II) in distilled H_2O (pH 7.5) for 2 hr. at 20° C, rinsed twice with distilled H_2O (pH 7.5) placed into 1% protease (Papya) in distilled H_2O (pH 7.4) for 2 hr. at 37° C, rinsed twice

in distilled H_2O (pH 7.5) and allowed to air dry. Once results from the procedures using carrot mericarps were assessed other species including caraway, anise and dill were prepared using the lipase-protease method.

All dried tissue samples were mounted onto SEM stubs using metallic tape. Endosperm remains from germinated carrot were spread and dried down onto the SEM stubs. All samples were coated with about 480 Å of gold using a Polaron Instruments SEM coating Unit E5100 and viewed on a Philips 501B scanning electron microscope.

VI Energy Dispersive X-ray Analysis

Endosperm tissue from all 9 species and embryo tissues including radicle and cotyledon were dissected from dry mericarps. Three preparative methods were employed although the bulk of the study involved material fixed only in glutaraldehyde as described previously under the heading "Fixed and Thin-sectioned Material". Several seeds from each species were used to test for seed-to-seed variation. As a check on possible extraction of elements during fixation, dehydration and embedding, endosperm from a single seed of each species was quick frozen, crushed, low temperature freeze-dried and mounted according to the procedures of Lott and Vollmer (1979). Globoid crystals and calcium-rich crystals were detected in freeze-dried powders by their electron density. Proteinaceous areas

were less readily detectable but were believed to be adjacent to globoid crystals or calcium-rich crystals. Endosperm samples from carrot, fixed with 5% glutaraldehyde (pH 7.5) and 2% OsO_4 (4 hrs.) were prepared as described previously under the heading "Fixed and Thin-sectioned Material".

Fixed and embedded material was sectioned 150-200 nm thick. Sections and freeze-dried powders were placed onto Formvar coated grids and carbon coated. EDX analyses were carried out with an EDAX model 606 x-ray spectrometer mounted on a Philips EM300 transmission electron microscope, a model 707A detector, and and EDIT data improvement system (EDAX International Inc.). An accelerating voltage of 80 kV was used for all analyses. All analyses were 60 sec. in length except for a few proteinaceous regions which were analysed for 120 sec. For material fixed only in glutaraldehyde at least 5 globoid crystals, 5 calcium-rich crystals, and 2 proteinaceous regions were analysed from endosperm and from each cell type encountered in the embryo tissues of carrot and caraway. At least 5 globoid crystals and 5 calcium-rich crystals were analyzed from the freeze-dried powders of endosperm of each of the species and from glutaraldehyde-OsO_4 fixed endosperm of carrot.

VII Phosphorus, Potassium, Magnesium and Calcium Determinations

The percentage by dry weight of phosphorus, potassium, magnesium, and calcium were determined for carrot, caraway, anise, dill, celery, parsley and parsnip mericarps. Samples were prepared in the following manner. Several thousand whole dry mericarps of each species were ball milled in a Prolabo ball mill with liquid nitrogen for 2 min. This material was then sent to the Australian Mineral Development Laboratories, Frewville, South Australia. There the material was put into total solution by digestion with nitric and perchloric acids prior to determining the percentages of the above mentioned minerals using atomic absorption spectrometry.

VIII Calcium Determinations

The amount of calcium per dry mericarp was determined for each of the 9 species. Carrot mericarps were studied more extensively than any other species. Unimbibed carrot mericarps were dissected into pericarp + testa, endosperm, and embryo regions while the other species were dissected into pericarp + testa + endosperm, and embryo regions. Also carrot grown for 3 and 5 days after radicle emergence were analysed whole, or dissected into seedling, and endosperm + testa + pericarp. Each whole mericarp or part was analysed separately. Whole mericarp or dissected parts were digested

in concentrated nitric acid (HNO_3) using low heat until the solutions became clear and no mericarp remnants remained. Samples were allowed to cool to room temperature and 1 to 2 mls. of perchloric acid ($HClO_4$) were added. Samples were diluted with distilled water until the final concentration of the nitric acid (HNO_3) was 5%. Samples were analysed using a Perkin-Elmer Model 603 Atomic Absorption Spectrophotometer using a hollow cathode calcium lamp at lamp current 18 mA, slit setting 4 (1.7 nm) using the spectral line for calcium at 211 nm (visible), an air-acetylene flame, and a 4 inch burner slot. A 0.25 ppm standard gave an absorption of 0.012 units. Samples were aspirated and the concentration of calcium per sample was determined from a calcium standard calibration curve. Calcium standards were run each time a series of samples was analyzed.

IX Determinations on the Solubility of the Calcium-rich Crystals

All 9 species were studied and compared with reagent grade calcium oxalate and calcium carbonate. The tissue was fixed in 5% glutaraldehyde in distilled water (pH 7.5) and embedded in Spurr's epoxy resin as outlined previously. Sections of endosperm tissue 1 µm in thickness were cut on a Porter-Blum MT-1 microtome and dried onto glass slides. Samples of calcium oxalate and calcium carbonate were also placed onto glass slides.

Samples were covered with cover slips and then treated with each of the following: 12.0 M and 1.0 M HCl; 17.0 M, 2.0 M and 1.0 M acetic acid; 16.0 M and very dilute nitric acid; distilled H_2O (pH 6.5); 1.0 M sodium hydroxide; 2.0 M and 1.0 M ammonium chloride; chloroform (100%); ethanol (100%); propylene oxide (100%). For a period of 48 hr. samples were periodically placed under brightfield or polarizing modes to determine crystal solubility with each solution used. The entire solubility study was done at approximately 20° C.

X Microincineration

The microincineration method used to determine if the calcium-rich crystals were composed of calcium oxalate was modified from that of Johnson (1956). Samples of endosperm from each of the 9 species were used. Samples were fixed in 5% glutaraldehyde in distilled H_2O (pH 7.5) and embedded as detailed earlier. Sets of three serial sections, approximately 1 µm thick, were cut and then each individual section was dried onto a glass slide. One section from each set of serial sections was left untreated and was used as the control. A second section of each set of serial sections was heated at 600° C in a muffle furnace for 30 min. The third section from each set was treated with 2 M acetic acid prior to being treated at 600° C

for 30 min. in the muffle furnace. After removing the sections from the furnace and allowing them to cool, 2.0 M acetic acid was placed onto each of the three sections from each set. Observations were made using the polarizing light microscope. As a further check, samples of calcium oxalate and calcium carbonate were placed separately onto several slides and treated like the seed tissue sections.

XI Procedures used for Staining of Inclusions Found in the Protein Bodies

1) Globoid Crystals

Endosperm from all 9 species was fixed with 5% glutaraldehyde in distilled H_2O (pH 7.5) and embedded as detailed previously. Sections were cut 2 µm thick and dried onto glass slides. A solution of 0.05% toluidine blue(pH 5.0) in water (Jacobsen et al, 1976) was placed onto the slides for 2 min. then rinsed using distilled H_2O (pH 7.5). Globoid crystals stain red when observed under the light microscope.

2) Calcium-rich crystals

Two methods which specifically stain for calcium oxalate crystals were used. For both methods either 2 µm thick sections of carrot previously fixed in 5% glutaraldehyde in H_2O (pH 7.5) and embedded in Spurr's epoxy resin, or hand cut sections of fresh carrot mericarps were made and dried down onto glass slides.

The first method was that of Silver and Price (1969) which was essentially that of Pizzolato (1964). One ml. each of 30% aqueous hydrogen peroxide and 5% aqueous silver nitrate was mixed (pH 6.0) and placed onto a slide containing a section. The section was exposed to light from a 60 watt incandescent lamp at a distance of 15 cm for 30 min., rinsed with distilled H_2O (pH 7.5) and counterstained with safranin for 5 min. The sections were dehydrated through 10%, 30%, 50%, 80% and 100% ethanol prior to air drying. The calcium-rich crystals should stain as intense black when viewed under a light microscope.

The second method was the silver nitrate-rubeanic acid (dithiooxamide) method of Yasue (1969). Sections were immersed in 5% aqueous silver nitrate for 30 min., rinsed with distilled H_2O (pH 7.5) and immersed for 1 min. in a 100 ml solution containing saturated rubeanic acid in 70% alcohol and 2 drops of concentrated ammonium hydroxide. Sections were rinsed with 50% ethanol in H_2O, dehydrated through 80% and 100% ethanol and air dried. Calcium oxalate crystals are stained dark brown or black when viewed under a light microscope.

XII Preparation for Obtaining Infra-red Spectra of the Calcium-rich Crystals

Only carrot was tested using infra-red spectroscopy. Carrot mericarps were placed onto filter paper

in petri dishes, imbibed with distilled H_2O (pH 7.5) and germinated. Five days after germinating the embryos were removed from the rest of the mericarp. The remaining endosperm tissue, which still contained many calcium-rich crystals (Fig. 10), was squeezed from the mericarp and collected. This endosperm tissue was suspended in distilled water (pH 7.5) and dried down onto a AgCl disc. The sample was scanned using a Perkin-Elmer 283 Spectrophotometer. As a check reagent grade calcium oxalate monohydrate was dried down onto another AgCl disc and scanned using the same instrument. The two spectra were compared with spectra obtained from spectra files (The Sadtler Standard Spectra Files) to determine the chemical composition of the crystals.

XIII Preparation for Obtaining X-ray Diffraction Patterns of the Calcium-rich Crystals

Only carrot was used. The calcium-rich crystals, which were obtained with the same method used to obtain crystals for the infra-red spectroscopy, were placed into a glass test tube and air dried. The dried endosperm tissue was then ground with a mortar and pestle. The resulting powder was placed onto a 2 cm^2 glass microscope slide and placed into a Philips powder diffractometer. The diffractometer was set at 30 kV, 20 mA, and a scale of 4×10^2 was used. Angles through which the

sample was analyzed were 5° to 78°. As the sample was rotated from 5° to 78°, peaks of various intensity were recorded. Each of the peaks occurred at a specific angle. By using a standard table of interplanar spacings, the set of interplanar distances ("d" spaces) measured in Å, within the crystals was found using the set of specific angles at which peaks were present. The intensities of the peaks were also recorded, with the largest peak being arbitrarily set at 100. When the "d" spaces and the corresponding intensities were found, the identity of the calcium-rich crystals was found by matching the data obtained with the "d" spaces and intensities of known compounds listed in a powder diffraction file.

RESULTS

I Light Microscopy

Cells from the endosperm of all species studied were irregular when viewed in section and approximately 20 to 40 µm in their greatest dimension, as shown in carrot (Fig. 1). The cell walls of the endosperm cells were thick. Two cell types appeared to be distinguishable. One cell type contained a number of protein bodies with small transparent circular inclusions believed to be globoid regions while the other cell type contained one or a few large transparent crystalline inclusions (Fig. 1). With the light microscope it was not possible to determine however, if these crystals were always inclusions of the protein bodies. Under polarized light the crystals were birefringent while the globoid inclusions were not (cf. Figs. 1 and 2) as shown in carrot.

The embryo cells of all species studied were often irregular in shape when viewed in section. Their size varied from 8 to 15 µm in their greatest dimension and their cell walls were much thinner than cell walls in the endosperm. Embryo cells all appeared to contain protein bodies, some of which contained small inclusions

thought to be globoids since the inclusions were not birefringent. No birefringent crystals were found in any embryo region in all the species studied.

II Thin-sectioned Material

In all species, both the endosperm and the embryo were studied using thin-sectioned material. In section, endosperm cells contained numerous lipid vesicles, several protein bodies and a centrally located nucleus (Fig. 43). In section embryo cells contained numerous lipid vesicles, a larger number of protein bodies than found in endosperm cells and a centrally located nucleus (Fig. 9).

All species studied were similar in their protein body structure. Two cell types were present with regard to protein body structure. One cell type contained protein bodies consisting of proteinaceous matrix and a number of globoid inclusions (Figs. 3, 17 and 43). The other cell type contained protein bodies consisting of proteinaceous matrix and calcium-rich crystal inclusions (Fig. 6). Both protein body types were never found in the same cell. No cells containing protein bodies with calcium-rich crystal inclusions were found in the embryo of any species studied. Also, in celery, chervil and parsley

but not in carrot, wild carrot, anise, dill, fennel, parsnip or caraway, the outermost layer of endosperm cells always contained protein bodies with calcium-rich crystal inclusions. Generally cells which contained protein bodies with globoid crystals appeared to have more and smaller protein bodies than cells which contained protein bodies with calcium-rich crystals, however no quantitative study was done. Both protein body types were approximately circular in section. Carrot protein bodies contained the largest globoid and calcium-rich crystal inclusions of all the species studied. Both globoid and calcium-rich crystals were naturally electron dense. In section globoid crystals were generally circular (Figs. 3, 4, 17, 18, 19, 25, 33 and 37), while calcium-rich crystals were angular often having saw toothed edges (Figs. 6, 7, 20, 21, 26, 30, 34, 36, 38, 40, 42 and 44). No membrane surrounding either type of inclusion was present.

In the endosperm, globoid crystal containing protein bodies consisted of structurally homogeneous proteinaceous matrix and globoid regions consisting solely of globoid crystal. The electron transparent gaps present between the globoid crystals and the proteinaceous matrix as shown in Fig. 18 were artifacts. Penetration of fixatives into globoid crystals is poor

and differential heating of the section under the electron beam are the probable causes of such gaps. Electron transparent areas in protein bodies were due either to chemical extraction of globoid crystals during fixation and embedding or physical extraction during sectioning when the brittle poorly fixed globoids are chipped out. The globoid crystals varied in size, even within the same protein body (Fig. 3) and in number (cf. Figs. 4 and 17). Illustrations showing protein bodies of this type include Figs. 4 (carrot), 18 and 19 (caraway), 25 (dill), 29 (anise), 33 (parsley), 35 (parsnip), 37 (chervil), 39 (wild carrot), 41 (celery), and 43 (fennel). Rarely in very thin sections of endosperm tissue, globoid crystals containing a pattern of less electron dense concentric rings were found (Fig. 19). This may indicate the manner in which globoid crystals form. It may also be an extraction effect, however.

In the embryo, protein bodies were often smaller and more numberous than in the endosperm. Often the proteinaceous matrix appeared flocculent (Fig. 9). In a given section of embryo tissue none or only one globoid crystal per protein body was found (Fig. 9).

The other type of protein body consisted of a structurally homogeneous proteinaceous matrix and a number of calcium-rich crystals. The calcium-rich

crystals occurred most commonly as large aggregates termed druse crystals. Illustrations showing protein bodies of this type include Figs. 7 (carrot), 20 and 21 (caraway), 26 (dill), 32 (anise), 34 (parsley), 36 (parsnip), 38 (chervil), 40 (wild carrot), 42 (celery) and 44 (fennel). In some protein bodies the druse crystal appeared to fill most of the protein body (Fig. 7) while in others the druse crystal appeared to occupy a small portion of the protein body (Fig. 21). This difference may be a sectioning effect, however. If a protein body such as that in Fig. 21 were sectioned in the plane perpendicular to the present plane of section the druse crystal might appear to occupy much of the protein body. Usually only one druse crystal per protein body was found in a given section, however, occasionally in all species studied, two or more druse crystals were found in a given protein body (Fig. 26).

Although most commonly calcium-rich crystal containing protein bodies contained large aggregates termed druse crystals, some protein bodies contained small aggregates of a few individual calcium-rich crystals as shown in Fig. 8. Rarely, individual crystals occurred. Limited serial sectioned material revealed that individual crystals do exist and are not always a sectioning effect.

The shape of the individual calcium-rich crystals and the aggregates they composed was difficult to determine. In section, individual crystals often appeared polygonal (Fig. 8). Druse crystals generally appeared circular with distinct saw toothed edges (Figs. 7, 20, 21, 26, 30, 34, 36, 38, 40, 42 and 44). Commonly, portions of druses and individual crystals were no longer present in the thin-sectioned material due to being chipped out during sectioning (Figs. 7 and 8). In other instances the calcium-rich crystals were removed during fixation and/or embedding (Figs. 42 and 44). Occasionally some central material of the druses was observed (Fig. 20). Often this central material appeared much like the proteinaceous matrix (Fig. 20). Rarely, a faint concentric, banding appeared to be present (Fig. 20). The banding effect may have reflected the arrangement of individual calcium-rich crystals in the druse or may be an extraction and/or sectioning effect.

III Freeze Fracture

Freeze-fracture replicas of the protein bodies of carrot seed endosperm were difficult to obtain, since cleansing treatments cracked most of the replicas into tiny pieces. The only protein body found consisted of a structurally homogeneous proteinaceous matrix and

Figures 1 - 8 The bar at the bottom of each micrograph represents 1.0 µm unless labeled otherwise.

Figure 1 Light Micrograph of Carrot Endosperm Tissue.

Fixed and sectioned carrot endosperm tissue with cells containing protein bodies with globoid regions (double arrows) and other cells containing calcium-rich crystals (single arrows).

Figure 2 Polarized Light Micrograph of Carrot Endosperm Tissue.

The same area as Fig. 1 viewed with polarized light. The birefringent areas (single arrows) are calcium-rich crystals. Globoid regions (double arrows) are not birefringent and hence are not seen.

Figure 3 Protein Bodies with Globoid Crystal Inclusions from Carrot Endosperm.

Thin-section showing a portion of a cell which contains protein bodies with globoid crystal (GC) inclusions and proteinaceous matrix (PM). The electron transparent gap between the globoid crystals and the proteinaceous matrix is a preparation artifact.

Figure 4 Protein Body with Globoid Crystal Inclusions from Carrot Endosperm.

Thin-section showing a protein body which consists of a proteinaceous matrix (PM) and a number of globoid crystals (GC) of various size; Lipid vesicle (L).

Figure 5 Freeze-fracture Replica of a Protein Body from Carrot Endosperm.

The replica shows a protein body consisting of a proteinaceous matrix (PM) and a globoid crystal (GC); Lipid vesicle (L).

Figure 6 Carrot Endosperm Cell Containing Protein Bodies with Calcium-rich Crystal Inclusions.

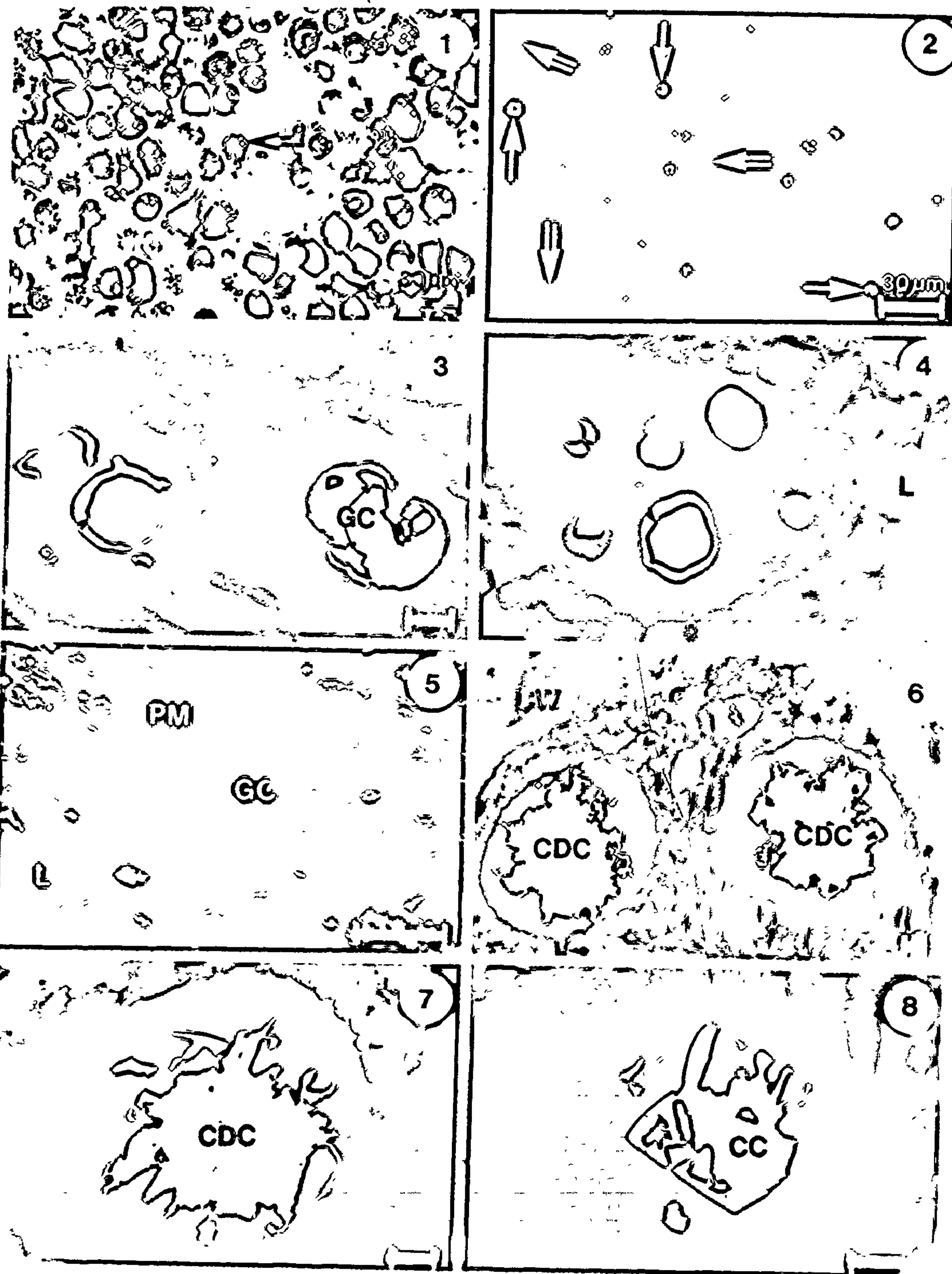
Thin-section showing a cell which contains protein bodies with only calcium-rich crystal inclusions, in the form of calcium-rich druse crystals (CDC) or as single calcium-rich crystals (CC) and proteinaceous matrix (PM); Cell wall (CW).

Figure 7 Protein Body with a Calcium-rich Druse Crystal Inclusion from Carrot Endosperm.

Thin-section showing a protein body which consists of a proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

Figure 8 Protein Body with Calcium-rich Crystal Inclusions from Carrot Endosperm.

Thin-section showing a protein body which consists of a proteinaceous matrix (PM) and a few calcium-rich crystals (CC).


1
2
30 µm
3
GC
4
L
5
PM
GC
L
6
CDC
CDC
7
CDC
8
CC

Figures 9 - 16 The bar at the bottom of each micrograph represents 1.0 μm unless labeled otherwise.

Figure 9 Thin Section from Carrot Embryo Tissue.

Protein bodies in the embryo consist of a proteinaceous matrix (PM) and usually none or only one globoid globoid (GC) per protein body in a given section; Nucleus (N); Lipid vesicle (L); Cell wall (CW).

Figure 10 Carrot Endosperm Cell Contents.

Scanning electron micrograph of carrot endosperm cell contents five days after radicle emergence showing the presence of numerous calcium-rich druse crystals (arrows) covered with cellular material.

Figure 11 Carrot Mericarp Sectioned and Treated with Lipase and Protease.

Scanning electron micrograph of a mericarp cut through an area consisting of only endosperm (E); Testa (T); Pericarp (P).

Figure 12 Carrot Endosperm Tissue after Treatment with Chloroform and Propylene Oxide.

Scanning electron micrograph showing protein bodies (PB); Cell wall (CW).

Figure 13 A Calcium-rich Druse Crystal from Carrot Endosperm.

Scanning electron micrograph showing the structure of a calcium-rich druse crystal (CDC) consisting of individual crystals of various shapes and sizes.

Figure 14 A Calcium-rich Druse Crystal from Carrot Endosperm.

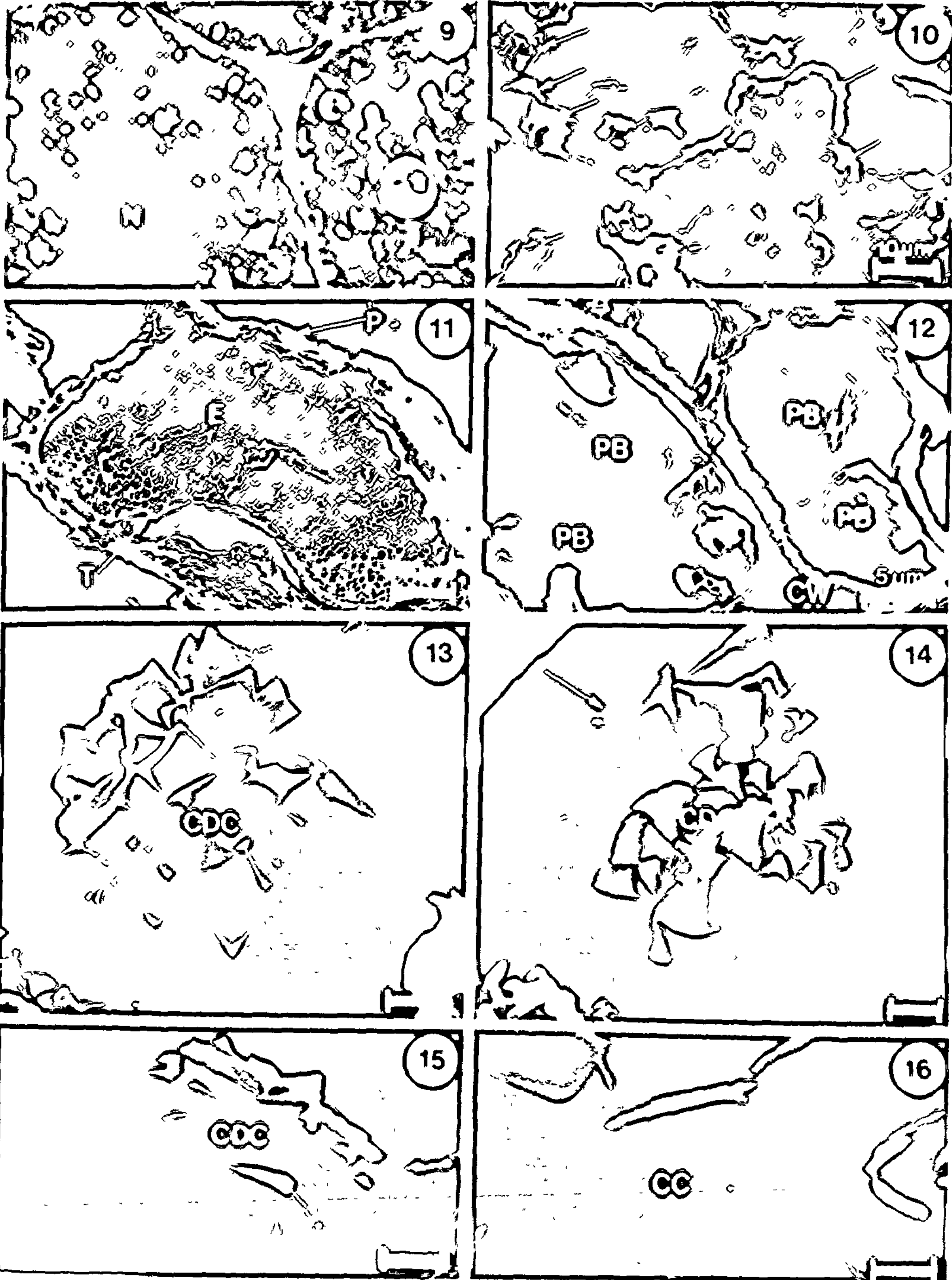
Scanning electron micrograph of a calcium-rich druse crystal (CDC). Note the twinned crystal (arrow) and the other crystals which form this druse.

Figure 15 <u>A Calcium-rich Druse Crystal from Carrot Endosperm</u>.

Scanning electron micrograph of a calcium-rich druse crystal (CDC) having a rarer layered arrangement of individual crystals.

Figure 16 <u>An Individual Calcium-rich Crystal from Carrot Endosperm</u>.

Scanning electron micrograph of a rare large individual calcium-rich crystal (CC).


9
10
N
10 um
11
P
E
T
12
PB
PB
PB
PB
CW
5 um
13
CDC
14
15
CDC
16
CC

Figures 17 - 24 The bar at the bottom of each micrograph represents 1.0 µm.

Figure 17 Thin Section of Caraway Endosperm Tissue.

Portion of a cell containing protein bodies which consist of a proteinaceous matrix (PM) and a number of globoid crystals (GC) of various sizes.

Figure 18 Protein Body with Globoid Crystal Inclusions from Caraway Endosperm.

Thin section showing a protein body consisting of a proteinaceous matrix (PM) and globoid crystals (GC) of various sizes.

Figure 19 Protein Body with a Globoid Crystal Inclusion from Caraway Endosperm.

Thin section showing a protein body containing a globoid crystal (GC) which displays concentric rings (R); Proteinaceous matrix (PM).

Figure 20 Protein Body with a Calcium-rich Druse Crystal Inclusion from Caraway Endosperm.

Thin section showing a protein body consisting of a proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC) which appears to contain a proteinaceous centre (arrow).

Figure 21 Protein Body with a Calcium-rich Druse Crystal Inclusion from Caraway Endosperm.

Thin section showing a protein body consisting of a calcium-rich druse crystal (CDC) surrounded by proteinaceous matrix (PM).

Figure 22 — Scanning Electron Micrograph of Protein Body Inclusions from Caraway Endosperm.

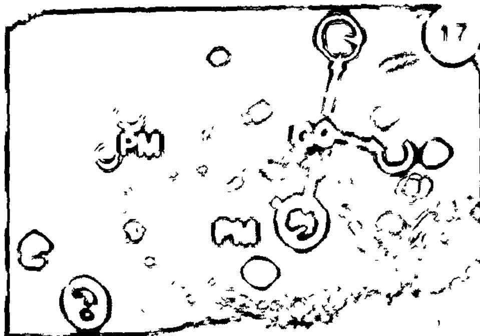
Several calcium-rich druse crystals (CDC) and globoid crystals (GC).

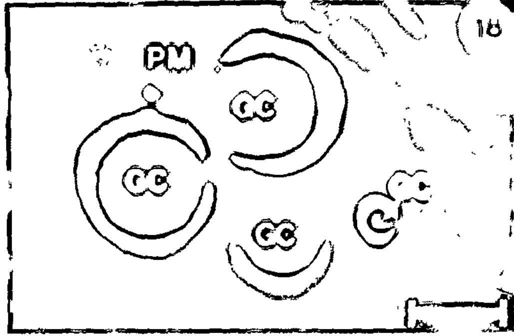
Figure 23 — Calcium-rich Crystals from Caraway Endosperm.

Scanning electron micrograph showing a calcium-rich druse crystal (CDC) and two rare large single calcium-rich crystals (CC) which are pyramidal in shape.

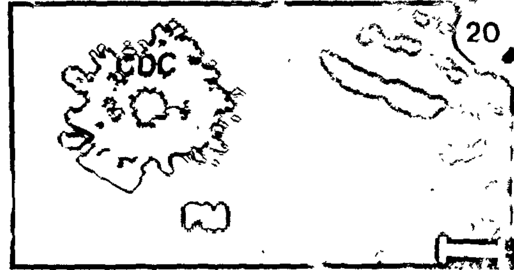
Figure 24 — Aggregate of Calcium-rich Crystals from Caraway Endosperm.

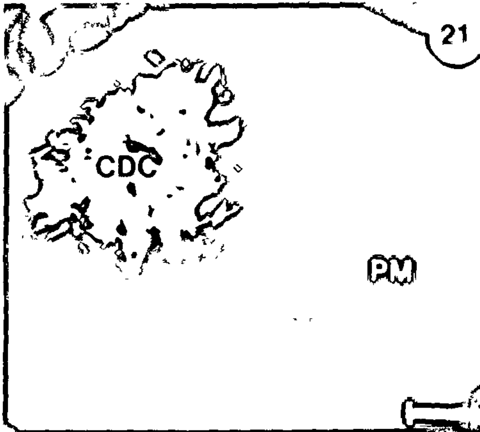
Scanning electron micrograph showing a small aggregate of calcium-rich crystals (CCA).

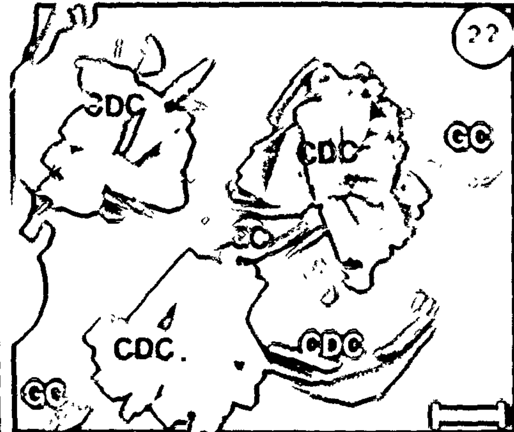

17
PM
PM

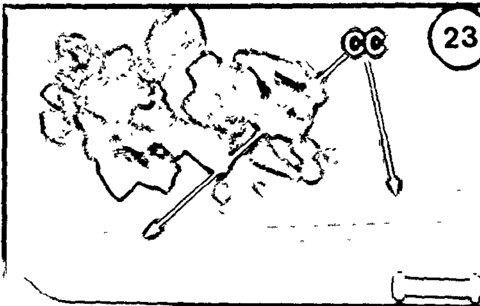

18
PM
GC
GC
GC

19
PM
GC


20
CDC
PM


21
CDC
PM


CDC
CDC
GC
CDC.
CDC
GC


23
GC

24

Figures 25 - 32 The bar at the bottom of each micrograph represents 1.0 µm.

Figure 25 Protein Body with Globoid Crystal Inclusions from Dill Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and globoid crystals (GC).

Figure 26 Protein Body with Two Calcium-rich Druse Crystal Inclusions from Dill Endosperm.

Thin section of a rarer protein body which consists of a proteinaceous matrix (PM) and two calcium-rich druse crystals (CDC).

Figure 27 Calcium-rich Druse Crystal from Dill Endosperm.

Scanning electron micrograph showing the structure of a calcium-rich druse crystal (CDC).

Figure 28 Calcium-rich Crystals from Dill Endosperm.

Scanning electron micrograph showing two small aggregates of calcium-rich crystals (CCA) and a single calcium-rich crystal (CC).

Figure 29 Protein Body with Globoid Crystal Inclusions from Anise Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and several globoid crystals (GC).

Figure 30 Protein Body with a Calcium-rich Druse Crystal Inclusions from Anise Endosperm.

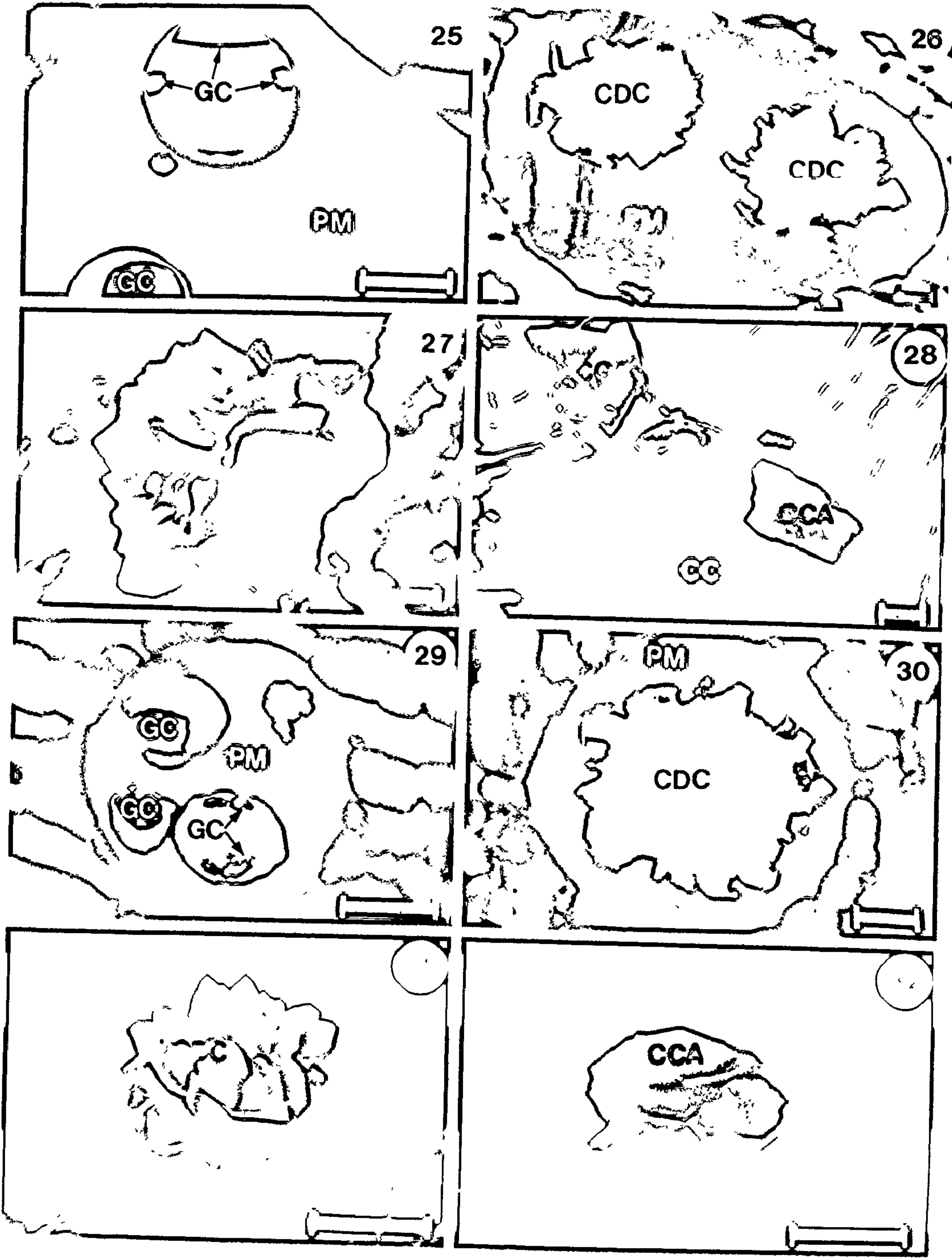
Thin section showing a protein body consisting of proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

Figure 31 — Calcium-rich Druse Crystal from Anise Endosperm.

Scanning electron micrograph showing the structure of a calcium-rich druse crystal (CDC).

Figure 32 — Aggregate of Calcium-rich Crystals from Anise Endosperm.

Scanning electron micrograph showing the layered structure of an aggregate of calcium-rich crystals (CCA).


25
GC
PM
GC
26
CDC
CDC
27
28
CCA
GC
29
GC
PM
GC
GC
30
PM
CDC
CCA

Figures 33 - 40 The bar at the bottom of each micrograph represents 1.0 μm

Figure 33 Protein Body with a Globoid Crystal Inclusion from Parsley Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and a globoid crystal (GC).

Figure 34 Protein Body with a Calcium-rich Druse Crystal Inclusion from Parsley Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

Figure 35 Protein Body with Globoid Crystal Inclusions from Parsnip Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and several globoid crystals (GC).

Figure 36 Protein Body with a Calcium-rich Druse Crystal Inclusion from Parsnip Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

Figure 37 Protein Body with Globoid Crystal Inclusions from Chervil Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and several globoid crystals (GC).

Figure 38 Protein Body with a Calcium-rich Druse Crystal Inclusion from Chervil Endosperm.

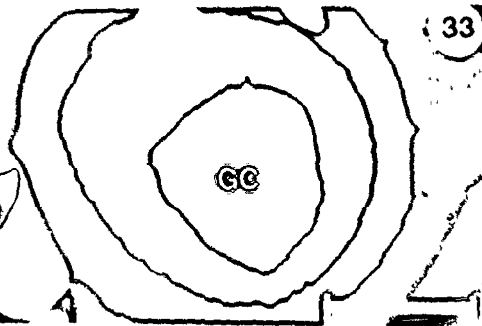
Thin section showing a protein body consisting of proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

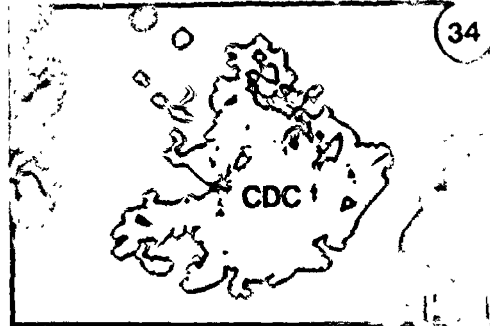
Figure 39 — Protein Body with Globoid Crystal Inclusions from Wild Carrot Endosperm.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and several globoid crystals (GC) of various sizes.

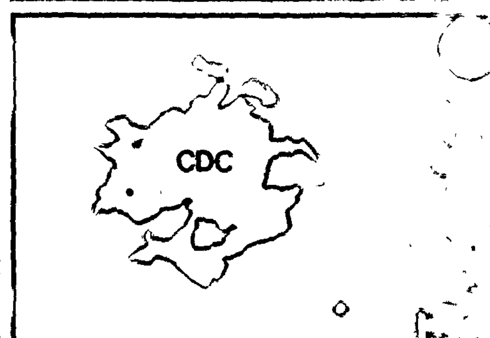
Figure 40 — Wild Carrot Endosperm Protein Body with a Calcium-rich Druse Crystal Inclusion.

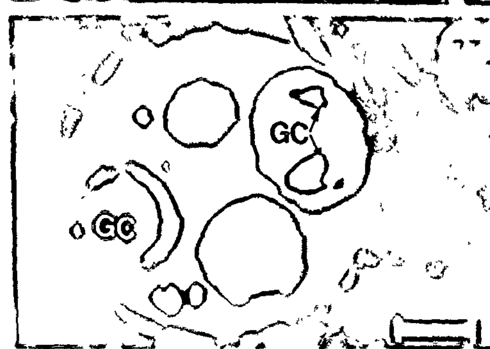
Thin section showing a protein body consisting of proteinaceous matrix (PM) and a calcium-rich druse crystal (CDC).

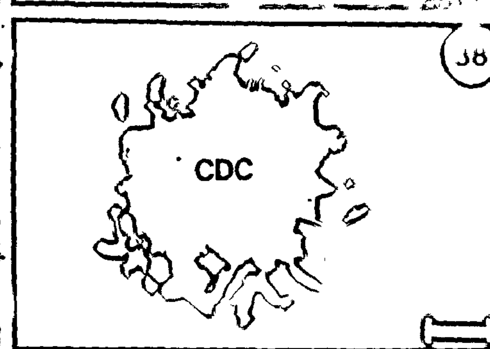

33
GC


34
CDC

GC


CDC


GC
GC


CDC

GC
GC

Figures 41 - 44 The bar at the bottom of each micrograph represents 1.0 μm.

Figure 41 <u>Celery Endosperm Protein Body with Globoid Crystal Inclusions</u>

Thin section showing a protein body consisting of proteinaceous matrix (PM) and numerous globoid crystals (GC) of various sizes.

Figure 42 <u>Celery Endosperm Protein Body with Region which Contained a Calcium-rich Druse Crystal</u>.

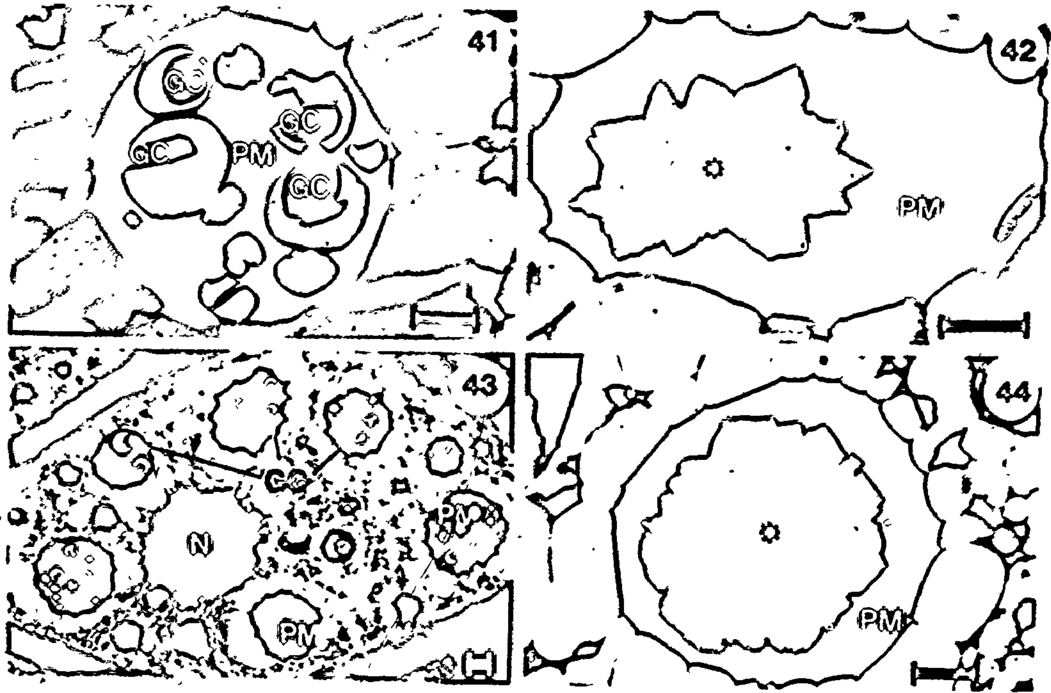
Thin section showing a protein body with proteinaceous matrix (PM) and an area which contained a calcium-rich druse crystal (star). The crystal was removed through chemical processing.

Figure 43 <u>Cell from Fennel Endosperm Containing Protein Bodies with Globoid Crystal Inclusions</u>.

Thin section of a cell containing protein bodies consisting of proteinaceous matrix (PM) and globoid crystals (GC); Nucleus (N).

Figure 44 <u>Fennel Endosperm Protein Body with Region which Contained a Calcium-rich Druse Crystal Inclusion</u>.

Thin section showing a protein body consisting of proteinaceous matrix (PM) and an area which contained a calcium-rich druse crystal (star). The crystal was removed during chemical processing.


41
GC
GC
GC
PM
GC
42
PM
43
GC
N
PM
PM
44
PM

a globoid region which probably consisted solely of a homogeneous globoid crystal (Fig. 5). No soft globoid region was present.

IV Scanning Electron Microscopy

1) Protein Bodies

The large amount of lipid present in these seeds made studying the external structure of the protein bodies difficult. In carrot mericarps that were broken or cut in section and treated with chloroform and propylene oxide to remove the lipid, protein bodies were found. Most protein bodies were roughly spherical in shape (Fig. 12) and varied in size from 2.0 to 10.0 μm in diameter.

2) Inclusions

To show the three dimensional structure of the inclusions found in the protein bodies, several methods were attempted. Initially only carrot was used. Numerous calcium-rich crystals, most in the aggregate form termed druses, were found in endosperm material removed from mericarps which had been germinated 5 days previously (Fig. 10). However, the crystals were coated with cellular material making it impossible to obtain a clear image of the aggregates or individual crystals (Fig. 10). The globoid crystal inclusions were

no longer present in the endosperm material of the five day old seedlings. No calcium-rich crystals were found when this endosperm material was treated with either 1% triton, or 100% chloroform and 1% triton to remove the remaining cellular material covering the crystals.

Dry mericarps of carrot cut in section, as shown in Fig. 11, then treated with lipase and protease respectively showed both the calcium-rich crystals and globoid crystal inclusions free of other cellular material. Other combinations of lipase, protease, triton and chloroform, either contained no globoid crystal or calcium-rich crystal inclusions, or contained both types of inclusions however other cellular material covered them. Thus, the lipase – protease treatment which gave the best results on carrot was used on the dry mericarps of caraway, anise and dill.

The calcium-rich crystals and the globoid crystals in all four species studied in this manner showed similar features. Globoid crystals were spherical in shape and varied in size as shown in caraway (Fig. 22). The calcium-rich crystals were much more variable in their shape and size. Calcium-rich crystals were found

either individually or more commonly as aggregates. Aggregates varied in size, shape and the number of individual calcium-rich crystals present. Most commonly, aggregates were approximately spherical in shape, and composed of numerous individual crystals. These aggregates were termed druse crystals. Such druse crystals were often composed of 30 to 60 individual crystals (Figs. 15, 22, 27 and 31) and rarely over 100 (Figs. 13 and 14). Examples of such aggregates of crystals include Figs. 13, 14 and 15 (carrot), 22 (caraway), 27 (dill), and 31 (anise). The largest druse crystals were found in carrot. Druse crystals in carrot were from 6.0 to 10.0 µm in diameter. Druse crystals from caraway, dill and anise were 2.0 to 5.0 µm in diameter. Structurally most druse crystals consisted of individual crystals which radiated out from the centre of the aggregate as shown in Figs. 13(carrot), 22 (caraway), 27 (dill) and 31 (anise). Less frequently, druse crystals appeared layered due to the orientation of the individual crystals which composed them as shown in Figs.15 (carrot), and 22 (caraway). Other druse crystals found appeared to have a random arrangement of their individual crystals (Fig. 14).

Other smaller aggregates of calcium-rich

crystals were also found in all four species. These aggregates were much smaller in size and the number of individual crystals composing them was less than found in the larger aggregates. The individual crystals in most of these smaller aggregates were oriented randomly as shown in caraway (Fig. 24) and dill (Fig. 28). Rarely however, these smaller aggregates would appear layered as shown in anise (Fig. 32). Infrequently, in all four species studied in this manner single calcium-rich crystals were found (Figs. 16, 23 and 28). A number of these single calcium-rich crystals may have been incorporated in a small aggregate or druse prior to the lipase-protease treatment.

Individual calcium-rich crystals varied in size and shape whether they were found as single crystals or as part of an aggregate. The size of the individual calcium-rich crystals ranged from 1.0 to 10.0 µm in their longest dimension. Generally individual crystals were from 1.0 to 3.0 µm however rarely such crystals occurring singly were as large as 10.0 µm in their longest dimension in carrot (Fig. 16). The shape of the crystals varied immensely and could not be pinned down to a few forms. Most crystals which occurred as single crystals were rhomboid or prismatic

(Figs. 16 and 28). In one instance in caraway two pyramidal crystals were found (Fig. 23). Most crystals forming aggregates were rhomboid or prismatic (Figs. 13 and 14). Twinned crystals occurred commonly in aggregates of all four species. Twinning of crystals occurs when two crystals fuse along a plane common to both. Twinned crystals can best be seen in Fig. 14, however other twinned crystals occur in Figs. 13 and 15.

V Energy Dispersive X-ray Analysis

1) Globoid Crystals

The results of the energy dispersive x-ray analysis studies on the globoid crystals from the endosperm of all species studied were similar in their elemental composition. Most commonly the globoid crystals in the endosperm contained Mg, P and K as shown in Figs. 45 (carrot), 46 (caraway) and 47 (celery). Infrequently, the globoid crystals of the endosperm of all species studied also contained small amounts of Ca as shown in a spectrum from a carrot globoid crystal (Fig. 48). The amount of Ca present varied from a trace to quite a distinguishable peak. Rarely, globoid crystals in the endosperm also were found to contain traces of Fe as shown in a spectrum from a carrot globoid crystal (Fig. 49). Any

differences in the elemental composition of the globoid crystals in the endosperm of all species studied were intercellular. Freeze-dried powders of endosperm from all species studied, done as a check against differential extraction during fixation, dehydration and embedding, showed little difference from the glutaraldehyde fixed material. The K and Ca peaks tended to be higher in the freeze-dried powder samples as shown in a spectrum from a freeze-dried carrot endosperm globoid crystal (Fig. 50). Samples of carrot endosperm fixed in glutaraldehyde followed by osmium tetroxide, done as a further check of the work of Lott <u>et al</u> (1978) on the differential extraction caused by fixation with osmium tetroxide, showed that the globoid crystals lost most of their K (Fig. 51). The proteinaceous matrix found in a globoid crystal containing protein body from glutaraldehyde fixed material was also analyzed. The proteinaceous matrix contained S and K as shown in a spectrum from a carrot protein body (Fig. 52).

Embryo globoid crystals from various tissues of the embryo from both carrot and caraway were analysed. Although all the spectrum shown are from carrot embryo, caraway embryo globoid crystals were generally similar in their elemental composition. In both carrot and caraway, elemental composition of globoid crystals in

Figures 45 - 68 Endosperm tissues from all species and embryo tissues from carrot and caraway were fixed in glutaraldehyde alone or in glutaraldehyde followed by osmium tetroxide, dehydrated, embedded in Spurr's epoxy resin, and sectioned (150-200 nm). Endosperm tissue from all species was also quick frozen, ground, low-temperature freeze-dried and the resulting powder dusted on grids. All analyses were 60s and an accelerating voltage of 80 KV was used. Energy levels are shown on each abscissa. The vertical scale (VS) is shown above each spectrum. Elements present, energy levels in kiloelectron-volts, and principal emission lines are as follows: calcium 3.690, $K_{\alpha 1,2}$ and 4.012, K_{β} (10% of $K_{\alpha 1,2}$); chlorine 2.621, $K_{\alpha 1,2}$; copper 0.930, L_{α} and 8.040, $K_{\alpha 1,2}$; iron 6.398, $K_{\alpha 1,2}$; magnesium 1.253, K_{α}; manganese 5.894, $K_{\alpha 1,2}$; and 6.489 $K_{\beta 1}$ (13% of $K_{\alpha 1,2}$); phosphorus 2.013, $K_{\alpha 1,2}$ and 2.028, $K_{\alpha 4}$ (10% of $K_{\alpha 1,2}$ peak) and 2.137 K_{β} (4% of $K_{\alpha 1,2}$ peak); potassium 3.312, $K_{\alpha 1,2}$ and 3.589, K_{β} (10% of $K_{\alpha 1,2}$

peak); sulfur 2.307 $K_{\alpha 1,2}$ and 2.322, $K_{\alpha 4}$ (50% of $K_{\alpha 1,2}$). Note that the K_{α} peak for calcium at 3.690 keV is overlapped by the K_{β} peak of potassium at 3.589 keV. Since the minor potassium K_{β} peak is 10% of the major K_{α} peak for potassium at 3.312 keV, subtraction will reveal the true calcium value. Copper peaks, when present on the spectra are not identified since they are an artifact of copper grid usage. Note: unless stated all sectioned material was fixed only with glutaraldehyde.

Figure 45 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm.

Figure 46 EDX Analysis Spectrum of a Globoid Crystal from a Section of Caraway Endosperm.

Figure 47 EDX Analysis Spectrum of a Globoid Crystal from a Section of Celery Endosperm.

Figure 48 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm.

Figure 49 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm.

Figure 50 — *EDX Analysis Spectrum of a Globoid Crystal from Carrot Endosperm Freeze-dried Powder.*

Figure 51 — *EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Endosperm.*

Tissue had been fixed with glutaraldehyde and osmium tetroxide. Note the low K peak.

Figure 52 — *EDX Analysis Spectrum of Protein from a Section of Globoid Crystal Containing Protein Body from Carrot Endosperm.*

Figure 53 — *EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue.*

Figure 54 — *EDX Analysis Spectrum of a Globoid Crystal from the Same Carrot Ground Meristem Cell as Fig. 53.*

Figure 55 — *EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue.*

Figure 56 — *EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Ground Meristem Tissue.*

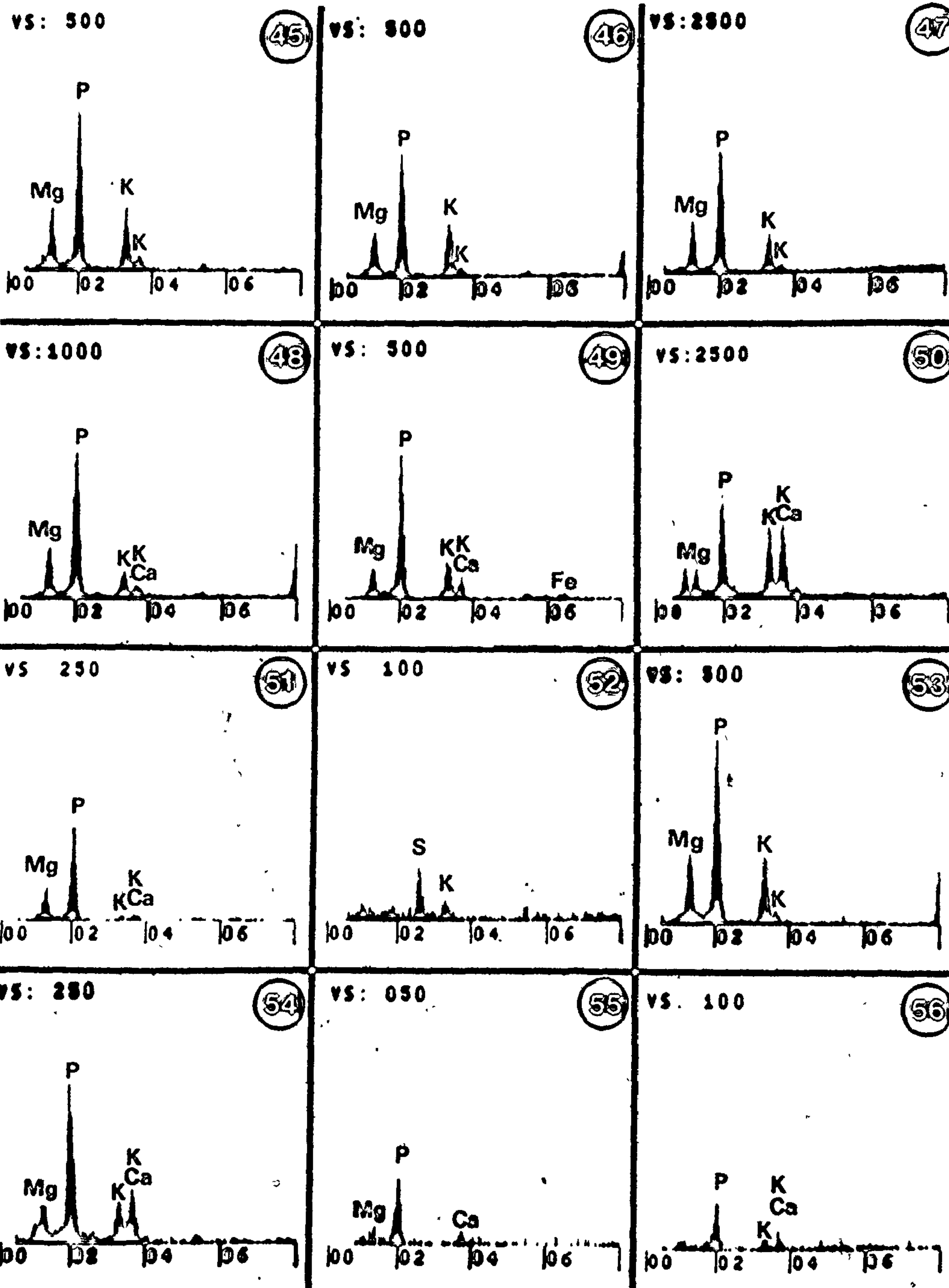

VS: 500
45
P
Mg
K
K
VS: 500
46
P
Mg
K
K
VS:2500
47
P
Mg
K
K
VS:1000
48
P
Mg
K
K
Ca
VS: 500
49
P
Mg
K
K
Ca
Fe
VS:2500
50
P
K
K
Ca
Mg
VS 250
51
P
Mg
K
K
Ca
VS 100
52
S
K
VS: 500
53
P
Mg
K
K
VS: 250
54
P
Mg
K
K
Ca
VS: 050
55
P
Mg
Ca
VS. 100
56
P
K
Ca
K

Figure 57 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Protodermal Tissue.

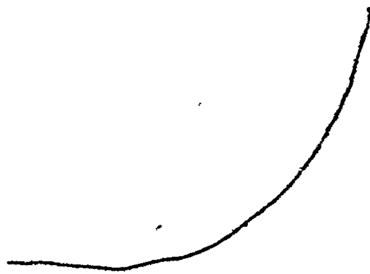
Figure 58 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Protodermal Tissue.

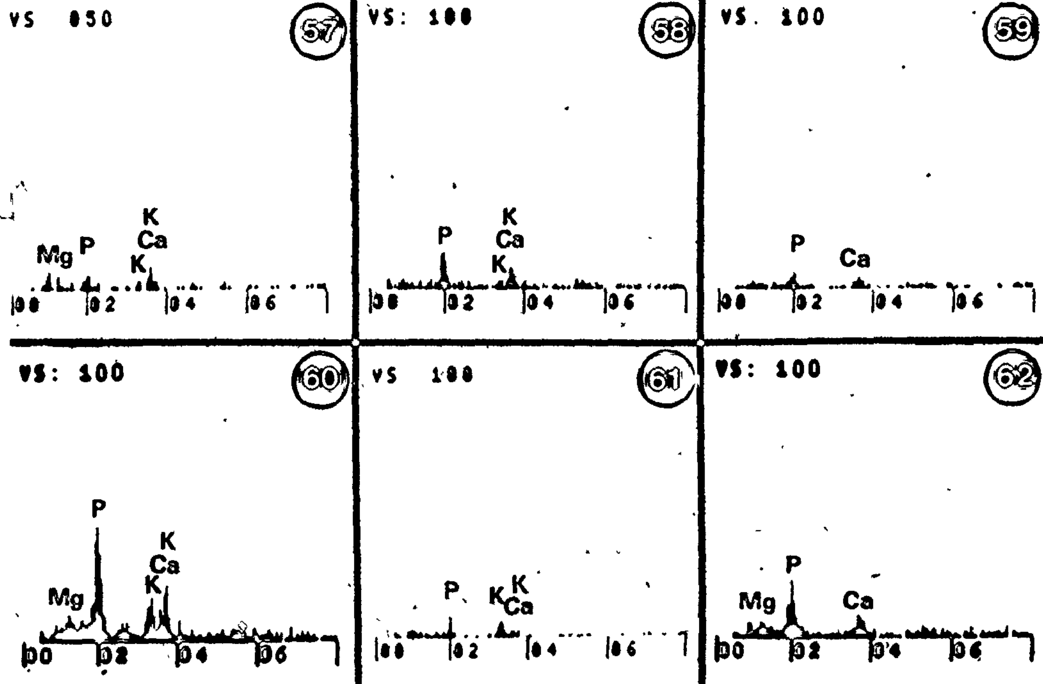
Figure 59 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Protodermal Tissue.

Figure 60 EDX Analysis Spectrum of a Section of Carrot Provascular Tissue.

Figure 61 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Provascular Tissue.

Figure 62 EDX Analysis Spectrum of a Globoid Crystal from a Section of Carrot Provascular Tissue.




VS 050
57
Mg
P
K
Ca
K
VS: 100
58
P
K
Ca
K
VS. 100
59
P
Ca
VS: 100
60
P
K
Ca
K
Mg
VS 100
61
P
K
K
Ca
VS: 100
62
P
Mg
Ca

protodermal, provascular and ground meristem regions in the radicle were similar to those of the cotyledons.

The globoid crystals in the ground meristem cells of both carrot and caraway commonly contained either Mg, P and K (Fig. 53), or Mg, P, K and Ca (Fig. 54). In caraway ground meristem cells, the globoid crystals also commonly contained only Mg, P and Ca. This elemental composition of the globoid crystals occurred only infrequently in carrot (Fig. 55). Rarely, both carrot and caraway ground meristem cell globoid crystals containing P, K, and Ca were found (Fig. 56). In protoderm cells the globoid crystals of carrot commonly contained P, K, and Ca (Fig. 58) or only P and Ca (Fig. 59). Less frequently globoid crystals of protodermal cells from carrot contained Mg, P, K and Ca (Fig. 57). Protodermal cell globoid crystals in caraway commonly contained Mg, P and Ca. Rarely, caraway protodermal cell globoid crystals contained only P and Ca, or Mg and Ca. The globoid crystals in the provascular cells of both carrot and caraway commonly contained Mg, P, K and Ca (Fig. 60), P, K and Ca (Fig. 61), or Mg, P and Ca (Fig. 62).

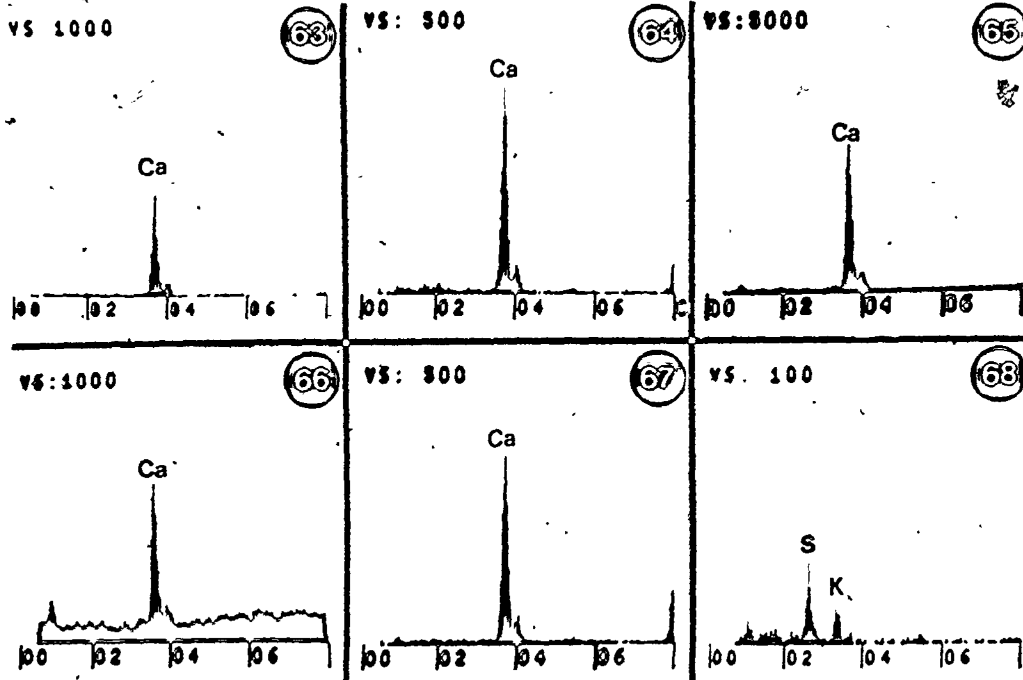
Generally differences in the elemental composition of the globoid crystals in both carrot and caraway embryos were intercellular. Rarely, however, an intracellular difference was noted in the ground meristem cells of both

carrot and caraway. The larger globoid crystals in a given ground meristem cell contained only Mg, P and K (Fig. 53) while smaller globoid crystals in other protein bodies in the same cell would also contain Ca (Fig. 54).

2) Calcium-rich Crystals

The calcium-rich crystals in tissue fixed with glutaraldehyde only from all species studied were EDX analysed. In all species studied the calcium-rich crystals contained only Ca as shown in Fig. 63 (carrot), 64 (caraway), and 65 (celery). No elemental differences were detected within or between individual crystals whether they occurred singly or as aggregates. Freeze-dried powder samples, EDX analysed to determine if large amounts of any elements were removed from the calcium-rich crystals during fixation, dehydration and embedding, showed that some Ca may have been lost and no other element was present (Fig. 66). The same result was found when samples of calcium-rich crystals from carrot which had been fixed in glutaraldehyde followed by osmium tetroxide were analysed (Fig. 67). The proteinaceous matrix surrounding a calcium-rich crystal was EDX analysed and found to be similar in elemental composition to the proteinaceous matrix found in protein bodies with globoid crystal inclusions in that it con-

Figures 63-67	No barium (4.465) or strontium (1.806) peaks were present.
Figure 63	EDX Analysis Spectrum of a Calcium-rich Crystal from a section of Carrot Endosperm.
Figure 64	EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Caraway Endosperm.
Figure 65	EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Celery Endosperm.
Figure 66	EDX Analysis Spectrum of a Calcium-rich Crystal from Caraway Endosperm Freeze-dried Powder.
Figure 67	EDX Analysis Spectrum of a Calcium-rich Crystal from a Section of Carrot Endosperm. Tissue was fixed with glutaraldehyde followed by osmium tetroxide.
Figure 68	EDX Analysis Spectrum of Protein from a Section of Calcium-rich Crystal Containing Protein Body from Carrot Endosperm.


VS 1000
63
Ca
VS: 500
64
Ca
VS:5000
65
Ca
VS:1000
66
Ca
VS: 500
67
Ca
VS. 100
68
S
K

tained only S and K (Fig. 68).

VI Phosphorus, Potassium, Magnesium and Calcium Determinations

Of the four elements analysed, K was present in the largest amounts in caraway, anise, celery, dill and parsnip mericarps followed in decreasing order by Ca, P and Mg (Table 1). In both carrot and parsley mericarps Ca was present in the largest amounts followed in decreasing order by K, P and Mg (Table 1).

VII Calcium Determinations of Mericarps from all Species and during Early Seedling Growth in Carrot

Whole mericarps of parsnip contained the most Ca on a per mericarp basis followed by parsley, carrot, chervil, fennel, dill, caraway, anise, celery and wild carrot (Table 2). The majority of the Ca was located in the pericarp plus testa plus endosperm component of the mericarp in each of the species analysed (Table 2). When pericarp plus testa and endosperm from carrot were analysed separately, 79.4% of the total Ca was in the endosperm and 18.3% in the pericarp plus testa. The remaining 2.3% was located in the embryo. In caraway, carrot, dill, fennel and parsnip the embryo contained a small amount of Ca when compared to the total Ca present in the entire mericarp whereas in anise, celery, chervil, parsley and wild carrot the Ca content in the embryo was higher relative to the total Ca present in the

Table 1

The Percentage by Dry Weight of Phosphorus, Potassium, Magnesium and Calcium in Whole Mericarps

	PHOSPHORUS	POTASSIUM	MAGNESIUM	CALCIUM
Anise	0.47	1.29	0.22	0.98
Caraway	0.51	1.21	0.26	0.65
Carrot	0.68	0.85	0.37	1.40
Dill	0.36	1.39	0.24	1.34
Parsley	0.53	1.00	0.27	1.12
Parsnip	0.55	1.30	0.24	0.65

Samples were analysed by The Australian Mineral Development Laboratories, Frewville, South Australia.

Table 2

Calcium Determination of Whole and Dissected Resting Mericarps

	Pericarp plus Testa plus Endosperm µg/pericarp + testa + endosperm		Embryo µg/embryo		Whole Mericarp[a] µg/mericarp	
	$\bar{x}$[b]	range	$\bar{x}$[b]	range	$\bar{x}$[b]	range
Anise	11.5	9.8-12.8	2.7	2.1-3.5	14.2	11.9-15.3
Caraway	13.2	12.0-15.0	1.1	0.8-1.5	14.3	13.0-16.0
Carrot[c]	21.3	16.8-26.5	0.5	0.1-1.1	21.8	17.1-27.1
Celery	8.4	6.3- 9.5	5.8	4.5-7.0	14.2	12.1-16.3
Chervil	20.8	18.0-24.8	3.8	2.5-6.4	24.6	21.5-28.6
Dill	12.7	9.5-15.5	1.4	1.0-1.9	14.1	11.4-16.6
Fennel	16.9	13.0-21.3	1.3	1.1-1.9	16.9	14.1-22.6
Parsley	17.0	14.0-18.3	5.9	5.4-6.3	22.9	20.3-23.9
Parsnip	29.5	15.5-49.5	1.1	0.8-1.5	30.4	17.0-50.3
Wild Carrot	7.8	5.8-10.5	2.8	2.6-2.9	10.6	8.7-13.1

a) Whole mericarp values were obtained by adding pericarp + testa + endosperm and embryo values together.

b) Values are an average of five mericarps.

c) Carrot mericarps were dissected into pericarp + testa, endosperm and embryo. Average Ca content followed in brackets by the range (in µg/seed portion); endosperm 17.3 (11.5-24.0), testa plus pericarp 4.0 (2.5-5.3), embryo 0.5 (0.1-1.1).

mericarp (Table 2).

In the study designed to determine if Ca moved from the endosperm to the embryo during early seedling growth the following was found. Carrot mericarps imbibed, germinated and analysed whole contained more Ca than when the two portions (1. pericarp + testa + endosperm and 2. embryo) were dissected and analysed separately (Table 3). Although the ranges do overlap, it appears that some Ca was lost. That some of the Ca was lost onto the filter paper on which the mericarps were placed was considered possible, however when the filter paper was analysed no elevated amounts of Ca were found. Three and five days after radicle emergence the endosperm nearest the young seedling was degraded to such an extent that some of it flowed onto the microscope stage and the instruments during dissection. This was probably the most likely cause for the lower Ca values. When dissecting dry mericarps of carrot this problem did not occur and total Ca values were similar to undissected germinated carrot Ca values (cf. Tables 2 and 3). Thus unlike Table 2 which whole mericarp values could be obtained by adding the two dissected portions together, in Table 3 an undissected, germinated Ca value was included to indicate the Ca lost due to dissection. It was found that little if

Table 3

Calcium Content Three and Five Days after Radicle Emergence (Carrot)

	Pericarp Plus[a] Testa Plus Endosperm µg/non seedling tissues	Seedling Plant[a] µg/seedling	Seedling Plus[a] other Tissues Undissected µg/seedling plus other tissues
3 days after radicle emergence (seedling length 5.8 cm)[b]	16.0 (11.8-21.3)	0.2 (0-1.5)	21.3 (10.8-28.0)
5 days after radicle emergence (seedling length 9.7 cm)[b]	16.2 (13.5-21.3)	0.2 (0-1.8)	23.2 (19.8-30.0)

a) Values are an average of 10 samples with the range bracketed.

b) Lengths are an average of 10 samples.

any Ca moved from the pericarp, testa or endosperm to the seedling plant by three and five days after radicle emergence (cf. carrot Table 2 with Table 3). Actually the seedling plants contained slightly less Ca but this was not a dissection artifact since the embryos were removed intact. Also, most of the Ca was still present in the pericarp + testa + endosperm portion even five days after radicle emergence when often this portion fell off. Even taking into account the Ca lost through dissection only a maximum of 30% of the Ca contained in the pericarp + testa + endosperm portion of the mericarp was lost. Thus these results indicated the calcium-rich crystals were not used in supplying Ca to the young seedling in major amounts.

VIII Solubility of the Calcium-rich Crystals

Calcium-rich crystals from all species studied were similar in terms of their solubility (Table 4). In all cases the results from the calcium-rich crystals were similar to those found using reagent grade calcium oxalate crystals. The calcium-rich crystals and the reagent grade calcium oxalate crystals were found to be soluble in hydrochloric acid (12.0 M and 1.0 M), and very dilute nitric acid (Table 4). The calcium-rich crystals and the reagent grade calcium oxalate were not soluble in nitric acid (16.0 M), acetic acid

Table 4

Calcium-rich Crystal Solubility Studies

Soluble (S); Not Soluble (NS)

	Hydrochloric Acid		Nitric Acid		Acetic Acid			Distilled Water	Sodium Hydroxide	Ammonium Chloride		Chloro-form	Ethanol	Propylene Oxide
	12M	1M	16M	Dilute	17M	2M	1M	(pH 6.5)	1M	2M	1M	100%	100%	100%
Anise	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Caraway	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Carrot	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Celery	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Chervil	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Dill	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Fennel	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Parsley	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Parsnip	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Wild Carrot	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Calcium Oxalate	S	S	NS	S	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Calcium Carbonate	S	S	NS	S	S	S	S	NS	NS	NS	NS	NS	NS	NS

(17.0 M, 2.0 M and 1.0 M), distilled water (pH 6.5), sodium hydroxide (1.0 M), ammonium chloride (2.0 M and 1.0 M), chloroform, ethanol and propylene oxide (Table 4). The reagent grade calcium carbonate, unlike the calcium-rich crystals and the reagent grade calcium oxalate, was soluble in acetic acid (17.0 M, 2.0 M and 1.0 M).

IX Staining of Inclusions found in the Protein Bodies

1) Globoid Crystals

Globoid crystals from all species studied stained red when viewed with a light microscope after being treated with a solution of 0.05% toluidin blue in water.

2) Calcium-rich Crystals

While both methods used in staining the calcium-rich crystals were supposed to be specific stains for calcium oxalate crystals, the results were inconclusive. Fixed and fresh material reacted similarily using both methods. In all cases when sections were viewed with the light microscope some crystals stained dark brown or black indicating that they were composed of calcium oxalate. Other crystals within the same section would be stained only a light brown while still other crystals did not stain at all. Usually those crystals that did stain were near the edge of the section and may have been more readily exposed to the stains. When a stained

section was viewed with polarized light, crystals which were stained no longer displayed birefringence while those crystals which were unstained remained birefringent.

X Microincineration

After heating, the calcium-rich crystals in sections of endosperm from all species studied, dissolved in 2.0 M acetic acid with no gas released (Table 5). Prior to heating none of the calcium-rich crystals dissolved when treated with 2.0 M acetic acid. The reagent grade calcium oxalate sample was similar to the calcium-rich crystals (Table 5). The reagent grade calcium carbonate dissolved when treated with the 2.0 M acetic acid prior to heating, releasing gas as it dissolved. When samples of calcium carbonate were heated and then treated with 2.0 M acetic acid the samples dissolved releasing no gas (Table 5). These results indicate that the calcium-rich crystals converted to calcium oxide during the heat treatment and were initially composed of calcium oxalate.

XI Infra-red Spectra

The infra-red spectra of the calcium-rich crystals obtained from the endosperm of carrot was virtually identical to the infra-red spectra obtained from a sample of reagent grade calcium oxalate mono-

Table 5

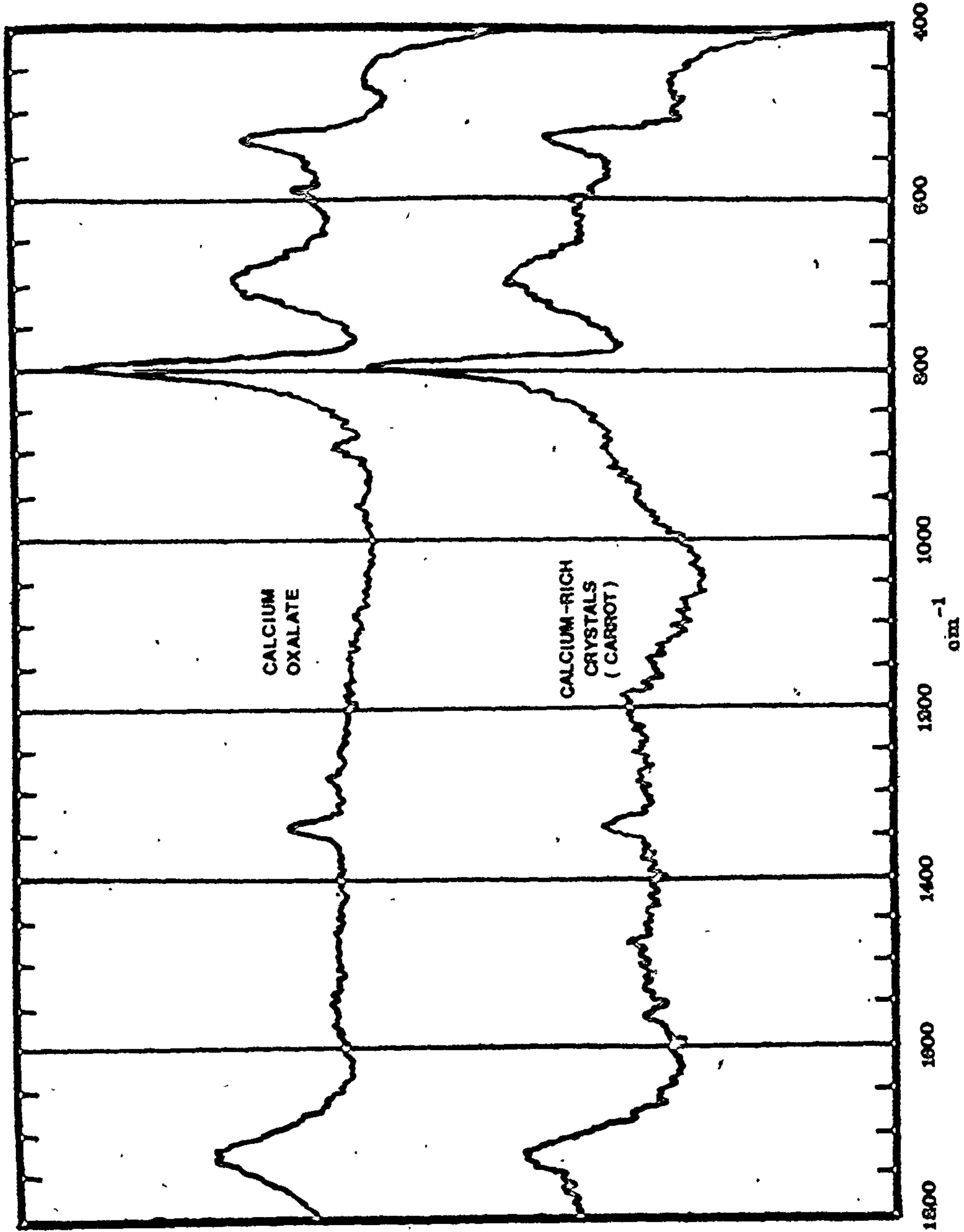
Microincineration Studies on Calcium-rich Crystals, Reagent Grade Calcium Oxalate and Reagent Grade Calcium Carbonate

soluble(S); not soluble (NS)

	Crystals Treated with 2M acetic acid prior to heating	Crystals Heated at at 600° C then treated with 2M acetic acid
Anise	NS	S
Caraway	NS	S
Carrot	NS	S
Celery	NS	S
Chervil	NS	S
Dill	NS	S
Fennel	NS	S
Parsley	NS	S
Parsnip	NS	S
Wild Carrot	NS	S
Calcium Oxalate	NS	S
Calcium Carbonate	S	S

Crystals dissolved in 2M acetic acid with no release of gas, indicating that the oxalate converted to oxide during the heating.

Figure 69 Infra-red Spectra of Calcium-rich Crystals from Carrot Endosperm and Reagent Grade Calcium Oxalate Monohydrate.


CALCIUM
OXALATE
CALCIUM-RICH
CRYSTALS
(CARROT)
400
600
800
1000
1200
1400
1600
1800
cm-1

hydrate (Fig. 69). The only difference between the two spectra was that the spectrum of the reagent grade calcium oxalate was smoother in appearance than the spectrum of the calcium-rich crystals. This difference would be expected since the calcium-rich crystals were not nearly as pure as reagent grade calcium oxalate.

XII X-ray Diffraction

An x-ray diffraction scan was obtained from the calcium-rich crystals from carrot endosperm. Calculations were done to obtain the set of "d" spaces and corresponding intensities of the calcium-rich crystals and these were matched against sets of "d" spaces and corresponding intensities of known compounds contained in a powder diffraction file. It was found that the "d" spaces and corresponding intensities of the calcium-rich crystals matched almost identically with those of a form of calcium oxalate monohydrate known as whewellite (Table 6). A number of less important, low intensity peaks, were not included in Table 6, since such peaks approached background levels.

Table 6

Comparison of X-ray Diffraction Data from Calcium-rich Crystals (Carrot) with Known X-ray Diffraction Data of Calcium Oxalate Monohydrate (Whewellite)

Calcium-rich Crystals (Carrot)		Calcium Oxalate Monohydrate (Whewellite)	
d spaces	intensity I/I_o	d spaces	intensity I/I_o
5.94	100	5.93	100
5.78	33	5.79	30
--	--	3.78	6
3.63	73	3.65	70
3.14	26	3.12	2
3.01	5	3.01	10
2.966	45	2.966	45
2.909	12	2.915	10
2.891	9	2.897	8
2.830	19	2.840	10
2.490	21	2.494	18
2.450	12	2.447	4
2.420	7	2.417	6
2.350	40	2.355	32
2.348	16	2.347	12
2.257	9	2.263	8
2.247	7	2.254	6
--	--	2.210	6
2.070	16	2.075	14
1.967	7	1.978	10
1.950	7	1.950	10
1.939	6	1.933	8
--	--	1.890	6
1.843	4	1.846	6
--	--	1.823	6

Data of Calcium Oxalate Monohydrate (Whewellite) obtained from a Powder Diffraction File.

DISCUSSION

Protein bodies of umbelliferous plants have received little study in the last one hundred years even though the Umbelliferae are an economically important family of plants. The fruits of a number of species are used in the preparation of a number of medicines and foods. An important component of their fruits, the seed protein bodies, were investigated in the studies reported here.

The protein bodies in the 9 species studied were similar in most aspects of their structure, chemical composition of their calcium-rich crystal inclusions and their elemental composition. Quantitative elemental determinations revealed the greatest variation between the species studied.

I Structural Aspects

Primarily due to the use of electron microscopy this thesis presents the most detailed study of seed protein bodies in the family Umbelliferae that has been carried out to date. Light microscopy provided little new structural information when compared

to the findings of the early investigators. The most striking feature found in all species studied was the presence of two structurally different types of protein bodies, those with globoid crystals and those with calcium-rich crystals. These were never found in the same cell. Other than the findings outlined below, the two cell types appeared to be randomly distributed. Structurally, the globoid crystal and calcium-rich crystal inclusions found in all species were similar. The inclusions in carrot were the largest of all species. They were noticeably larger than the inclusions found in wild carrot.

In celery, parsley and chervil no globoid crystal containing protein bodies were found in the outermost layer of the endosperm. Although evaluated on a strictly qualitative basis, it appeared that there were more numerous and generally smaller protein bodies present in the endosperm cells that contained globoid crystal inclusions compared to endosperm cells containing calcium-rich inclusions. Globoid crystal size and number varied even within the same cell. Protein bodies containing calcium-rich crystal inclusions were often larger and as a result there were fewer of them per cell. The calcium-rich crystal inclusions were often druse crystals with varying numbers of component crystals.

Occasionally either layered aggregates or individually occurring calcium-rich crystals were found. In previous investigations only the druse crystals were mentioned.

In all species studied, no calcium-rich crystals were found in the embryos. In the embryo, protein bodies were more numerous per cell and smaller in size than in the endosperm. Unlike the endosperm, the proteinaceous matrix of the protein bodies in the embryo often appeared flocculent.

Few investigations regarding the development of protein bodies in general and no such studies on protein bodies containing calcium oxalate inclusions have been carried out. Not until such developmental studies are undertaken will answers pertaining to the formation and arrangement of the protein bodies be found so at present one can only speculate. It is probable that in the endosperm of umbelliferous seeds, cell wall formation occurs prior to the formation of the protein body inclusions since the two types of inclusions are never found in the same cell. In the embryo, protein bodies may be smaller and more numerous than in the endosperm to allow for quick utilization of stores contained within them during germination and early seedling growth. Also, in the embryo the proteinaceous matrix often has a flocculent appearance in fixed and thin-sectioned

material. Flocculent proteinaceous matrix regions in protein bodies may be due to poor fixation (Mollenhauer and Totten,1971) or due to protein degradation by endogenous proteolytic enzymes (Ashton, 1976). Both are possible. The fixation of embryo tissue was often poor. Also, the fixative was in an aqueous solution and this may have initiated imbibition prior to fixing the tissue. That different storage proteins, which are affected differently by the fixatives, occur in embryo and endosperm regions is also another possibility.

Both Pfeffer (1872) and Kohl (1889) indicated that the central region of druse crystals was composed of protein. Buttrose and Lott (1978b) revealed a central region in similar inclusions in the protein bodies of Eucalyptus, however they only speculated as to this central region's composition. Although further investigation is required, in the present study it appears from viewing thin-sectioned material that this central core is not crystalline and may be similar in composition to the proteinaceous matrix.

Often micrographs of tissue that contained calcium-rich crystal inclusions showed only the area where the crystals were located prior to their being removed during processing. Examples can be seen in Lott and Buttrose (1978c), Buttrose and Lott (1978b), Jacobsen et al

(1976) and Arnott (1973). To retain such crystals I found that tissue fixed only in 5% glutaraldehyde in distilled water (pH 7.5) with no post fixation in OsO_4 was the most satisfactory method of preparation. The higher pH and use of only glutaraldehyde were perhaps the most important parameters for retaining these crystals. Conventional post-staining with uranyl acetate and lead citrate was not advisable since I found that such crystals were frequently removed during post-staining. Fortunately, the tissues studied here had enough natural contrast that I could view the subcellular components without post-staining.

Both transmission and scanning electron microscopy were valuable methods for determining structural aspects of the protein bodies studied. Transmission electron microscopy was particularly useful for studying the globoid crystals. Most globoid crystals were not visible under the light microscope due to their small size. This was especially true in embryo tissues. Scanning electron microscopy was an excellent method for obtaining structural information of the calcium-rich crystal inclusions. Not only was the structure of individual crystals obtained by using scanning electron microscopy but also the structure

of the complex aggregates could be determined. Information regarding the size, number and arrangements of the component crystals was thus found. Based upon my findings it is clear that, the aggregates with component crystals radiating outwards in all directions should be termed druse crystals and not rosette crystals. Although crystals composing aggregates and individual crystals vary in size and shape they are all consistent with their being composed of calcium oxalate.

At present, protein bodies with calcium-rich crystal inclusions often are considered a rare structural type of protein body and are often ignored in treatises on protein bodies. However, the articles by early investigators such as Pfeffer (1872) and Kohl (1889) and some more recent studies suggest that a number of species apparently have protein bodies with calcium-rich crystal inclusions in their seeds (see Appendix A). While many of the species in Appendix A have not been investigated using the sophisticated methodology now available, it is likely that protein bodies with calcium-rich crystal inclusions are more common than is generally believed.

II Elemental Analyses

1) Energy Dispersive X-ray (EDX) Analysis

Globoid crystals from each species studied in

detail with EDX analysis were much alike. EDX analysis of globoid crystals in endosperm tissue generally showed the presence of P, K and Mg. Infrequently small amounts of Ca were also found. In the embryo the globoid crystals always contained P and a combination of K, Mg and Ca. Rarely, Fe was present in trace amounts. The elemental composition of the globoid crystals is consistent with their being mainly phytin. Variations in elemental composition of the globoid crystals were generally intercellular. Rarely however, in some cells in the embryos of both carrot and caraway, larger globoid crystals lacked Ca while smaller globoid crystals in other protein bodies in the same cell contained some Ca.

Unlike EDX analysis studies of globoid crystals in other species, no minor element detected in this study was found to have a specific distribution pattern. Their distribution in the endosperm and embryo appeared random. Lott _et al_ (1979), in a study of globoid crystals of several species of _Cucurbita_, showed that only certain cells had globoid crystals which contained Ca. Spitzer and Lott (1980) and Lott and Spitzer (1980) in studies on tomato and wheat respectively showed that the globoid crystals that contained Mn and/or Fe were located in specific cell types even though Ca appeared

randomly throughout. Why certain elements found in globoid crystals occur in very specific cell types in some species and randomly in other species remains unknown.

Calcium-rich crystals always revealed only Ca when EDX analysed. This is consistent with their being chemically composed of calcium oxalate. It rules out the possibility that these crystals are composed of barium or strontium oxalate. EDX analysis also indicated that individual crystals of aggregates or singly occurring crystals were all calcium-rich. EDX spectra of these crystals obtained from all 9 species studied here were like spectra obtained from similar crystals in protein bodies of hazel nut (Lott and Buttrose, 1978c), jojoba and Eucalyptus (Buttrose and Lott, 1978b).

EDX analysis of the proteinaceous matrix in all protein bodies revealed the presence of S and K regardless of the inclusion present in the protein body.

Freeze-dried powders were prepared from all species studied and used as a check for elemental extraction from globoid crystal and calcium-rich crystal inclusions during glutaraldehyde fixation, dehydration and embedding. Freeze-dried powders revealed that some differential extraction of globoid crystals may have

occurred. Potassium peaks on EDX spectra of globoid crystals obtained from tissue prepared as a freeze-dried powder often were proportionally larger than those obtained from glutaraldehyde fixed and thin-sectioned tissue. Also, when Ca was present in globoid crystals, the Ca peaks appeared proportionally larger when tissue was prepared as a freeze-dried powder. No large amounts of Ca appeared to be extracted from the calcium-rich crystal inclusions, however this was not a quantitative study and it is difficult to compare whole crystals with sectioned pieces of crystals. Carrot tissue fixed with glutaraldehyde followed by OsO_4 was also checked to determine if any OsO_4 induced elemental extraction had occurred as previously reported for globoid crystals in a range of oil seeds that were studied by Lott et al (1978). I found that K was almost totally removed and some Ca may also have been removed from the globoid crystals. No large amounts of Ca appeared to have been extracted from the calcium-rich crystals. Elemental extraction from inclusions of protein bodies is dependent on a number of factors including the type and strength of fixative used, the times used for fixation, dehydration and embedding, the temperature used, the tissue block size, the type and composition of the tissue, and seed-to-seed variations.

Thus with all these possible factors leading to elemental extraction there is a definite need to use freeze-dried powders as a check on possible extraction from chemically prepared tissue. Unlike most biological tissue, seed tissue permits this check to be made because some components are naturally electron dense.

2) Quantitative Elemental Analysis

Quantitative elemental determinations of P, K, Mg and Ca on a percentage dry weight basis showed that Ca followed by K, P and Mg was the most abundant in carrot and parsley while K followed by Ca, P, and Mg was most abundant in anise, caraway, celery, dill and parsnip. The Ca content in the mericarps determined in this manner were all higher than values obtained for any other seed studies to date (compare with Webber and Neumann, 1980). However, since the mericarp consists of not only the seed but also the pericarp it was necessary to determine where most of the Ca was located in order to obtain a fair comparison with other seeds. Rompel (1905) had shown that species of the family Umbelliferae contained calcium oxalate crystals in their pericarps but the amount was not determined. Carrot mericarps dissected into pericarp + testa, endosperm, and embryo showed that most of the Ca was in fact located in the endosperm, a much smaller amount was located in the

pericarp + testa, and the smallest amount was located in the embryo region. Thus from this study and less detailed calcium localization studies of the other species studied, most of the Ca was in the seeds. Thus Ca content in these mericarps is the highest determined to date on a % dry weight basis. Calcium determinations and localization in wild carrot and the other species studied, revealed that the endosperm + testa + pericarp Ca content was always higher than the embryo Ca content. Variations of Ca content on a whole mericarp basis, and between embryos and the rest of the mericarps occurred between the species studied. These differences could largely be explained because different genera were investigated. However large differences between carrot and wild carrot, which are both Daucus carota L., also occurred. These differences may have occurred as a result of extensive selective breeding of carrot as opposed to no such breeding for wild carrot, however other factors such as growth conditions may also be involved.

The quantitative study to determine if there is movement of Ca from the endosperm, testa or pericarp to the young seedling during early seedling growth revealed that little Ca moved. The pericarp, testa and remaining endosperm often became separated from the

embryo 5 days after germination with large numbers of calcium-rich crystals still present in the endosperm. Although there was a maximum 30% loss in Ca content after germination most of the loss could be attributed to loss during dissection. The filter paper on which the mericarps were germinated was also analysed however no elevated Ca content was found. Thus few if any calcium-rich crystals were used directly. Jacobsen et al (1976) working with celery endosperm indicated that these crystals are degraded during germination and early seedling growth, however judging from their illustrations it is likely that calcium-rich crystals were extracted during chemical preparation or staining. Recent investigations of celery endosperm breakdown during germination also suggest that these crystals are degraded however quantitative proof is lacking (Dwarte and Ashford, per. com.). Pfeffer (1872) stated that in the Umbelliferae these crystals are not degraded during or after germination however the species studied were not mentioned. In the present study this has only been shown for carrot and more investigation with the other species is required before any family wide generalizations can be made. Since these crystals were not used directly, their function remains unclear. They may be degraded in the soil and the calcium

absorbed by the young seedling at a later time. Other possible functions of calcium oxalate crystals are detailed in the next section. To determine the role of such crystals in these seeds more investigation is required.

III Calcium Oxalate Crystal Inclusions

The calcium-rich crystal inclusions, found in some of the protein bodies of the 9 species of Umbelliferae studied, were determined to be composed of calcium oxalate. This is the first time such crystal inclusions occurring in protein bodies have been positively identified. Outlined below are the formation, function and identification of calcium oxalate crystals in plants.

1) Synthesis of Oxalates

The formation of calcium oxalate crystals in seed protein bodies has not been investigated, however the formation of such crystals in other plant tissues has been studied and is known to require the synthesis of oxalic acid. Oxalic acid synthesis has been investigated extensively and several possible pathways have been determined. The major pathways are outlined below. The reader is referred to Hodgkinson (1978) or Franceschi and Horner (1980) for detailed accounts of oxalic acid synthesis in plants.

One of the major pathways of oxalic acid

synthesis uses photosynthetically formed glycolate. The glycolate formed is then oxidized to glyoxylate which is then oxidized to oxalic acid. Glycolate oxidase is the enzyme responsible for both these oxidations. This pathway was proposed by Seal and Sen (1970) and a number of investigations using radioactive tracers have provided evidence for this pathway (Chang and Beevers, 1968; Richardson and Tolbert, 1961). Another pathway with glyoxylate as the immediate precursor to oxalic acid involves the Kreb cycle and isocitrate. Isocitrate formed in the Kreb cycle can be converted to glyoxylate and succinate by the enzyme isocitrate lyase. The glyoxylate formed in this manner can then be converted to oxalic acid (Kornberg and Kreb, 1957). L-ascorbic acid has also been indicated as a precursor to oxalic acid (Wagner and Loewus, 1973). Yang and Loewus (1975) have shown using labeled L-ascorbic acid that plants which accumulate oxalate formed oxalate while non-accumulating plants did not. This suggests that only in accumulating plants is the L-ascorbic acid to oxalic acid pathway functioning. Other pathways of oxalic acid synthesis have been indicated including direct oxidation of malic acid (Gentile, 1954) and enzymatic cleavage of oxaloacetate into acetate and oxalic acid (Hayaishi et al, 1956). Once the oxalate is present

some of it can bind with free Ca ions to form calcium oxalate. How large amounts of free Ca ions required in plants which accumulate large amounts of calcium oxalate occurs is not known. De Kock et al (1973) in studies on Lemna indicated that oxalate content and calcium absorption were related.

Which of these pathways of oxalic acid synthesis occurs in these seeds remains unclear. The developing seed has no apparent photosynthetic system, thus a pathway which includes photosynthetically derived glycolate may not be involved. However, it is possible that the oxalic acid or any precursor may have been transported to the seed from a region which produces photosynthetically derived glycolate. The L-ascorbic acid pathway has been shown in oxalate accumulating plants thus remains a possibility as does the isocitrate pathway. Further study is required however before a clearer understanding is obtained as to where and how the oxalate in such protein bodies is formed.

2) Crystal Structure and Deposition in Cells

Two principal forms of calcium oxalate, based on their hydration states, occur in plants (Franceschi and Horner, 1980). These are calcium oxalate monohydrate ($CaC_2O_4 . H_2O$) known as whewellite and calcium oxalate dihydrate ($CaC_2O_4 . 2H_2O$) known as weddelite.

Crystals of the monohydrate belong to the monoclinic system of crystalization. In this system all three of the crystallographic axes are of unequal length. The angles formed by the intersection of these crystallographic axes include one oblique intersection with the other two intersections equal to 90° (Franceschi and Horner, 1980). The crystals found using scanning electron microscopy in carrot, caraway, anise and dill all appeared to conform to the monoclinic system. Carrot crystals were confirmed to be calcium oxalate monohydrate by x-ray diffraction and infra-red spectrometry. The crystals of the dihydrate belong to the tetragonal system of crystalization. In the tetragonal system all three crystallographic axes are at right angles. The two lateral axes are of equal length while the third axis can be longer or shorter (Franceschi and Horner, 1980). In plants both dihydrate and monohydrate calcium oxalate have variously shaped crystals, all of which are consistent with their respective system of crystalization.

In plants the formation of such crystals is always associated with a membrane (Arnott, 1973; Franceschi and Horner 1980). At present their formation is believed to be a cell controlled process, however the role of such physical parameters as temperature must also be

considered as possible influences on crystal growth. The evidence obtained on crystal formation suggests that the formation of calcium oxalate crystals in plant cells does not involve simple precipitation but a complex cellular specialization (Arnott, 1973; Franceschi and Horner, 1980). No membrane directly surrounding such crystals in protein bodies has been found. During development in such protein bodies however, the crystal(s) may form in the vacuole which eventually becomes the protein body prior to protein accumulation. Thus since they are found in membrane bound structures (protein bodies) and may be influenced by membranes during their development, these crystals may also turn out to have a membrane directed deposition. However detailed structural investigation is required to prove or disprove this point.

Crystals of calcium oxalate occur in virtually all angiosperm plants (Arnott, 1973; Franceschi and Horner, 1980). Usually such crystals are found in specific tissues. They may be arranged randomly or very specifically. Why random or specific localization of calcium oxalate crystals occurs is not known. Crystal function may be partially responsible.

3) Function of Calcium Oxalate Crystals

The function(s) of calcium oxalate crystals in

protein bodies remains unknown. No quantitative evidence suggests that the Ca contained in such crystals in seeds of the Umbelliferae are used during germination and early seedling growth. During early seedling growth the crystals of carrot appear to be discarded with the testa and pericarp. If these crystals are then degraded in the soil and the Ca taken up by the seedling at a latter time has not been investigated but may occur. Possible functions of calcium oxalate crystals in other areas of various species of plants have been reported and are summarized in a review on calcium oxalate in plants by Franceschi and Horner (1980).

It is believed by many that calcium oxalate is an end product of metabolism and its formation aids in isolating oxalic acid and/or excess Ca ions which may be toxic to the plant at higher levels. Relationships between Ca uptake and oxalate formation (De Kock et al, 1973; Rasmussen and Smith, 1961), and between oxalate and Ca content of tissues and the number of crystals present (Frank, 1972) have been shown in a variety of plants. However, since other plants such as Begonia (Crombie, 1954) contain high levels of free oxalic acid doubt does exist that calcium oxalate functions in alleviating toxic effects. In some instances calcium

oxalate is metabolized (Franceschi and Horner, 1980).

Another proposed function of calcium oxalate in plants is related to nitrate assimilation. Clark (1936) found in tomato that the total organic acid and oxalate contents were higher when the nitrogen source was nitrate than when it was ammonium. Other investigations have been done in this area with a variety of plants (see Franceschi and Horner, 1980). A number of these investigations (Joy, 1964; Osmond, 1967; Raven and Smith, 1976) favour a relationship between nitrate assimilation and oxalate which involves ion balance. Raven and Smith (1974) indicated that nitrate assimilation leads to the formation of hydroxyl ions which if not removed, could lead to a large pH increase in the cell. Raven and Smith (1976) proposed that these hydroxyl ions could be neutralized by the formation of strong organic acids such as oxalic acid. When oxalic acid dissociates hydrogen can bind with the hydroxyl ions to form water and the oxalate could bind with Ca ions forming the insoluble salt, calcium oxalate. In this manner, with the formation of oxalic acid and subsequent precipitation of calcium oxalate there is a method of osmoregulation. While this function of calcium oxalate might occur in relation to protein body formation since nitrogen is important

in amino acid synthesis and thus protein formation, the fact that most species do not have such crystals in their protein bodies makes this possibility doubtful.

Storage of Ca and/or oxalate has been suggested as a function of calcium oxalate crystals in plants. There is some evidence detailed by Franceschi and Horner (1980) that in some instances, especially the dihydrate form of calcium oxalate, may have a storage function. Considering the number of other compounds stored in seeds and the general shortage of Ca storage in most seeds one might assume that calcium oxalate crystals found in protein bodies might function in a storage capacity. However, these crystals were not used appreciably during germination and early seedling growth in carrot.

Calcium oxalate crystals also function as a form of protection against foraging animals. Black (1918) indicated that such crystals were the sole cause of irritation when daskeen was eaten. Irritation and a burning sensation of the mouth after eating crystal containing plants are well known (Kingsbury, 1964). Such crystals are often in the form of raphides or styloids.

It is possible that none of the functions outlined above or only some pertain to calcium oxalate

crystals in protein bodies. A major storage function has seemingly been ruled out in carrot while Dwarte and Ashford (per. com.) suggest that in celery Ca storage is a possible function of such crystals. A protective function is possible since large numbers of crystals are present and in celery, parsley and chervil the peripheral endosperm cells contain only such inclusions in their protein bodies. Ion balance may be a function, however why some cells contain such crystals in their protein bodies and others do not remains unclear.

4) Identification of Calcium Oxalate Crystals

The identification of calcium oxalate crystals in both plant and animal tissues is frequently difficult. Usually such crystals are assumed to be calcium oxalate rather than proved to be so. The actual determination of the chemical composition of such crystals is often avoided since the individual crystals or aggregates are usually small, not present in large numbers and/or embedded in other cellular material which makes their collection, purification and analysis difficult. The present study used several different methods which have been successful to varying extents in crystal identification in plant and animal tissues. Although other methods exist for the determination of calcium oxalate (see Hodgkinson, 1978; Franceschi and Horner, 1980) the

methods used were believed to be the most useful for determining the chemical composition of such crystals in protein bodies.

The most accurate methods used in this study to determine the chemical composition of the calcium-rich crystal inclusions were x-ray diffraction and infra-red spectrometry. With either method the form of calcium oxalate of which the crystals are composed of can also be determined. Since both these methods are highly sensitive, they should be used if possible for determining the chemical composition of such crystals. However, disadvantages with both methods when applied to the crystals found in protein bodies do occur. One disadvantage is that these crystals must be extracted, collected and purified prior to analysis. Fortunately, in carrot an excellent system for obtaining relatively pure collections of crystals was found after numerous attempts at purification failed. During germination and early seedling growth most if not all of these crystals are not degraded unlike other endosperm components. Therefore these crystals, relatively free of cellular material, could be collected from endosperm remains. Another disadvantage occurs because individual crystals and aggregates are very small (1.0 to 10.0 µm) and a large number are required for analysis. There-

fore the chemical composition of individual crystals or aggregates can not be investigated.

EDX analysis is helpful in ruling out most other chemical compounds even though it does not indicate the exact chemical compound composing such crystals. Through its use such crystals were shown to be rich in Ca and contain no other elements higher than atomic number 10. The major advantage of EDX analysis over other methods is that small individual crystals of aggregates or singly occurring crystals could be analyzed separately for differences which might be present. The EDX analysis results obtained in the present study are consistent with the crystals being calcium oxalate. In conjunction with other methods, EDX analysis is a valuable method in determining the chemical composition of such crystals.

Microincineration is a useful method for the *in situ* chemical identification of such crystals. A drawback, as detailed below with regard to the crystal solubility studies, is the assumption that the crystals are oxalate when they are insoluble in acetic acid.

The insolubility of a crystal in water, acetic acid and dilute sodium hydroxide and its solubility in mineral acids such as hydrochloric acid indicates that a crystal is most likely calcium oxalate. While crystal solubility studies are useful as a check of the chemical

composition of crystals the major drawback to this method is that the identification of crystals is based on the elimination of various other compounds which might be present rather than providing a positive identification. With this method one must always be concerned with the possibility that some unexpected chemical compound which has similar solubility properties to calcium oxalate may be present. For example it is assumed that barium and strontium oxalate do not occur since barium and strontium are rare in plant tissues. However, Lott and Buttrose (1978b) found globoid crystals in Brazil nut that contained measurable amounts of barium. Thus caution must be used in such generalizations. Solubility studies in conjunction with EDX analysis should permit an almost certain identification of the type of oxalate.

Results obtained from specific staining for calcium oxalate using the methods of Silver and Price (1969) and Yasue (1969) were not conclusive. Although most crystals stained, some did not. This could indicate that these few crystals were not composed of calcium oxalate however the tissues were treated with acetic acid and dilute sodium hydroxide prior to staining thus removing any dicarboxylic salts other than oxalate. Also EDX analysis indicated only Ca was present.

Chaplin (1974) in a study on the demonstration of calcium oxalate in animal tissue indicated that the two best methods for staining calcium oxalate were those of Pizzolato (1964) which is the method used by Silver and Price (1969) and Yasue (1969). Chaplin (1974) indicated that while both methods were inconsistent, the method of Pizzolato (1964) was especially so. Thus specific staining of such inclusions should be done in conjunction with other tests before stating the chemical composition of such crystal inclusions.

Light microscopy, although of limited use in determining the identity of such crystals, was valuable. With both brightfield and polarizing modes the larger globoid crystals and calcium oxalate inclusions were observable within the protein bodies. Use of polarized light quickly established whether calcium-rich crystals were present and their location in a given section since such crystals are birefringent. Crystal solubility, microincineration and staining all required light microscopy as a quick and accurate means of determining the outcome of various treatments.

IV Summary of Findings

Until this thesis, the study of protein bodies of the Umbelliferae had been neglected. A number of important findings were made in this thesis with regard

to the structure and composition of the protein bodies in several species of the Umbelliferae. The endosperm consisted of two cell types based on protein body structure. One cell type contained protein bodies composed of structurally homogeneous proteinaceous matrix and a number of variously sized globoid crystals. Using EDX analysis such globoid crystals were found to contain Mg, P and K or less frequently Mg, P, K and Ca. The other cell type contained protein bodies composed of structurally homogeneous proteinaceous matrix and commonly multiple crystal inclusions either termed druses or aggregates, or less frequently individual crystals. In this thesis, for the first time, such crystal inclusions in protein bodies were indentified, conclusively, as calcium oxalate. In the embryo only one type of protein body was found. These protein bodies commonly consisted of proteinaceous matrix which often appeared flocculent and only one globoid crystal in a given section. Using EDX analysis to study the various tissues of carrot and caraway embryos the globoid crystals were found to contain various combinations of Mg, P, K and Ca with P always present and no other trace element distribution pattern distinguishable. No membrane was found surrounding either globoid crystals in the endosperm or embryo,

or the crystals of calcium oxalate. Not surprising Ca determinations indicated that species with an abundance of such crystals in their protein bodies have much higher Ca contents in their seeds than seeds without such crystals. It was also found that during germination and early seedling growth the calcium oxalate crystal inclusions were not used directly even when the seedlings had no other Ca supply.

APPENDIX A

Other Plants which may contain Calcium Oxalate Crystal Inclusions in Their Protein Bodies

Family and plant	Reference
Balsaminaceae	
Cardiospermum halicacabum	Pfeffer, (1872); Kohl, (1889)
Berberidaceae	
Berberis vulgaris	Pfeffer, (1872); Kohl, (1889)
Buxaceae	
Simmondsia chinensis	Buttrose and Lott, (1978b)
Compositae	
Silybum marianum	Pfeffer, (1872); Kohl, (1889)
Centaurea jacea	Pfeffer, (1872); Kohl, (1889)
Centaurea maculosa	Lüdtke, (1890)
Tragopogon major	Pfeffer, (1872); Kohl, (1889)
Corylaceae	
Corylus avellana	Pfeffer, (1872); Kohl, (1889) Vaughan, (1970); Lott and Buttrose, (1978c)
Dipsacaceae	
Cephalaria procera	Pfeffer, (1872); Kohl, (1889)
Lecythidaceae	
Bertholletia excelsa	Kohl, (1889)
Leguminosae	
Lupinus luteus	Pfeffer, (1872); Kohl, (1889)
Phaseolus	Kohl, (1889)
Myrtaceae	
Eucalyptus erythrocorys	Buttrose and Lott, (1978b)
Eucalyptus citriodora	Beltrati, (1978)
Eucalyptus maculata	Beltrati, (1978)

APPENDIX A cont'd

Ranunculaceae	
Helleborus foetidus	Pfeffer, (1872); Kohl, (1889)
Paeonia peregrina	Pfeffer, (1872); Kohl, (1889)
Paeonia procera	Pfeffer, (1872); Kohl, (1889)
Staphyeaceae	
Staphylea pinnata	Pfeffer, (1872); Kohl, (1889)
Umbelliferae	
Aethusa cynapium	Pfeffer, (1872); Kohl, (1889)
Coriandrum sativum	Pfeffer, (1872); Kohl, (1889)
Cuminum cyminum	Winton, (1916)
Vitaceae	
Vitis vinifera	Pfeffer, (1872); Kohl, (1889)
Cissus antartica	Pfeffer, (1872); Kohl, (1889)

BIBLIOGRAPHY

Altschul, A.M., Yatsu, L.Y., Ory, R.L. and Engleman, E.M., (1966), Seed proteins. Ann. Rev. Plant Physiol. 17, 113-136.

Arnott, H.J., (1973), Plant Calcification, 609-627 IN: BIOLOGICAL MINERALIZATION, I. Zipkin (ed.), John Wiley and Sons, Toronto.

Asada, K. and Kasai, Z., (1962), Formation of myo-inositol and phytin in ripening rice grains. Plant Cell Physiol. 3, 397-406.

Ashton, F.M., (1976), Mobilization of storage protein of seeds. Ann. Rev. Plant Physiol. 27, 95-117.

Ashton, W.M., and Williams, P.C., (1958), The phosphorus compounds of oats. I The content of phytate phosphorus. J. Sci. Food Agric. 9 505-511.

Beltrati, C.M., (1978), Morphological and anatomical studies of the seeds and seedlings of *Eucalyptus citriodora* and *E. maculata*. Rev. Biol. Trop. 26, 213-215.

Black, O.F., (1918), Calcium oxalate in the Daskeen Amer. J. of Bot. 5, 447-451.

Buttrose, M.S., (1971), Ultrastructure of barley aleurone cell as shown by freeze-etching. Planta 96, 13-26.

Buttrose, M.S., (1978), Manganese and iron in globoid crystals of protein bodies from *Avena* and *Casuarina*. Aust. J. Plant Physiol. 5, 631-639.

Buttrose, M.S. and Lott, J.N.A., (1978a), Inclusions in seed protein bodies in members of the Compositae and Anacardiaceae: comparison with other dicotyledonous families. Can. J. Bot. 56, 2062-2071.

Buttrose, M.S. and Lott, J.N.A., (1978b), Calcium oxalate druse crystals and other inclusions in seed protein bodies: Eucalyptus and jojoba. Can. J. Bot. 56, 2083-2091.

Chang, C.C. and Beevers, H., (1968). Biogenesis of oxalate in plant tissues. Plant Physiol. 43 1821-1828.

Chaplin, A.J., (1974), Some observations on the demonstration of calcium oxalate in tissue sections. Stain Tech. 49, 165-173.

Clark, H.E., (1936). Effect of ammonium and nitrate nitrogen on the composition of the tomato. Plant Physiol. 11, 5-24.

Craig, S., Goodchild, D.J. and Hardham, A.R., (1979), Structural aspects of protein accumulation in developing pea cotyledons. I. Qualitative and quantitative changes in parenchyma cell vacuoles. Aust. J. Plant Physiol. 6, 81-98.

Crombie, W.M.L., (1954), Oxalic acid metabolism in Begonia semperfloreus. J. Expt. Bot. 5, 173-183.

Cutter, E.G., (1978), PLANT ANATOMY, PART I: CELLS AND TISSUES (2nd Edition), 44-47, Edward Arnold, London.

De Kock, P.C., Ohta, Y., Inkson, R.H.E. and Knight, A.H., (1973). The effect of oxalate and ethylenediaminetetraacetic acid on absorption of calcium into Lemna. Physiol. Plant. 28, 379-382.

Dwarte, D. and Ashford, A.E., Personal communication from D. Dwarte and A.E. Ashford. N.S.W., Australia.

Ergle, D.R. and Guinn, G., (1959), Phosphorus compounds of cotton embryos and their changes during germination. Plant Physiol 34, 476-481.

Fernald, M.L., (1970), GRAY'S MANUAL OF BOTANY (8th edition), 1078-1105, D. Van Nostrand Co., New York.

Franceschi, V.R. and Horner, H.T., Jr., (1980), Calcium oxalate crystals in plants. Bot. Review 46, 361-428.

Frank, E., (1972). The formation of crystal idioblasts in Canavalia ensiformis D.C. at different levels of calcium supply. Z. Pflanzenphysiol. 67, 350-358.

Gentile, A.C.,(1954). Carbohydrate metabolism and oxalic acid synthesis by Botrytis cinerea. Plant Physiol. 29, 257-261.

Guilliermond, A., (1941), THE CYTOPLASM OF THE PLANT CELL. Chronica Botanica Co., Waltham, MA.

Hartig, T., (1855), Über das Klebermehl. Bot. Ztg. 13. 881-882.

Hayaishi, O., Simazono, H., Katagiri, M. and Saito, Y., (1956). Enzymatic formation of oxalate and acetate from oxaloacetate. J. Am. Chem. Soc. 78, 5126-5127.

Hodgkinson, A., (1978), OXALIC ACID IN BIOLOGY AND MEDICINE, Academic Press, New York and London.

Hofsten, A. von, (1973), X-ray analysis of micro-elements in seeds of Crambe abyssinica. Physiol. Plant 29, 76-81.

Jacobsen, J.V., Knox, R.B. and Pyliotis, N.A., (1971), The structure and composition of aleurone grains in the barley aleurone layer. Planta 101, 189-209.

Jacobsen, J.V. and Pressman, E., (1979), A structural study of germination in celery (Apium gravolens L.) seed with emphasis on endosperm breakdown. Planta 144, 241-248.

Jacobsen, J.V., Pressman, E. and Pyliotis, N.A., (1976), Gibberellin induced separation of cells in isolated endosperm of celery seed. Planta 129, 113-122.

Johnson, F.B., (1956), A method for demonstrating calcium oxalate in tissue sections. J. Histochem. Cytochem. 4, 404-405.

Johnson, F.B., (1958), Further observations on the identification of calcium oxalate in tissue sections. J. Histochem. Cytochem. 6, 405.

Johnson, F.B. and Pani, K., (1962), Histochemical identification of calcium oxalate. Arch. Path. 74, 347-351.

Joy, K.W., (1964). Accumulation of oxalate in tissues of sugar-beet, and the effect of nitrogen supply. Ann. Bot. 28, 689-701.

Kingsbury, J.M., (1964), POISONOUS PLANTS OF THE UNITED STATES AND CANADA, Prentice-Hall, Englewood Cliffs, N.J.

Kohl, F.G., (1889), Anatomisch-physiologische untersuchung der Kalksalze und Kieselsäure in der Pflanze. 1-196, N.G. Elwert'sche, Marburg.

Kornberg, H.L. and Krebs, H.A., (1957). Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. Nature 179, 988-991.

Kossa, J. von, (1901), Über die im Organismus künstlich Verkalkungen. Beitr. Path. Anat. 29, 163-202.

Lolas, G.M. and Markakis, P., (1975), Phytic acid and other phosphorus compounds of beans (Phaseolus vulgaris L.) J. Agric. Food Chem. 23, 13-15.

Lott, J.N.A., (1980), Protein bodies, 589-623, IN: THE BIOCHEMISTRY OF PLANTS, A COMPREHENXIVE TREATISE. Vol. 1., P.K. Stumpf, and E.E. Conns (eds.), Academic Press, New York.

Lott, J.N.A. and Buttrose, M.S., (1978a), Globoids in protein bodies of legume seed cotyledons. Aust. J. Plant Physiol. 5, 89-111.

Lott, J.N.A. and Buttrose, M.S., (1978b), Thin-sectioning, freeze-fracturing, energy dispersive x-ray analysis, and chemical analysis in the study of inclusions in seed protein bodies: almond, Brazil nut, and quandong. Can. J. Bot. 56, 2050-2061.

Lott, J.N.A. and Buttrose, M.S., (1978c), Location of reserves of mineral elements in seed protein bodies: macadamia nut, walnut, and hazel nut. Can. J. Bot. 56, 2072-2082.

Lott, J.N.A., Greenwood, J.S. and Vollmer, C.M., (1978), An energy dispersive x-ray analysis study of elemental loss from globoid crystals in protein bodies as a result of osmium tetroxide fixation. Can. J. Bot. 56, 2408-2414.

Lott, J.N.A., Larsen, P.L. and Darley, J.J., (1971), Protein bodies from the cotyledons of Cucurbita maxima. Can. J. Bot. 49, 1777-1782.

Lott, J.N.A. and Spitzer, E., (1980), X-ray analysis studies of elements stored in protein body globoid crystals of Triticum grains. Plant Physiol. 66, 494-499.

Lott, J.N.A., Spitzer, E. and Vollmer, C.M., (1979), Calcium distribution in globoid crystals of Cucurbita cotyledon protein bodies. Plant Physiol. 63, 847-851.

Lott, J.N.A. and Vollmer, C.M., (1973), The structure of protein bodies in Cucurbita maxima cotyledons. Can. J. Bot. 51, 687-688.

Lott, J.N.A. and Vollmer, C.M., (1979), Composition of Globoid Crystals from Embryo Protein Bodies in Five Species of Cucurbita. Plant Physiol. 63, 307-311.

Lüdtke, F., (1890), Beitrage zur Kenutniss der Aleuronkörner. Jahrb. Wissen. Bot. 21, 62-127.

Lui, W.S.T. and Altschul, A.M., (1967), Isolation of globoids from cotton seed aleurone grain. Arch. Biochem. Biophys. 121, 678-684.

Martin, A.C., (1946), The comparative internal morphology of seeds. Amer. Mid. Natur. 36, 513-660.

Matheson, N.K. and Strothers, S., (1969), The ultiization of phytate by germinating wheat. Phytochem. 8, 1349-1356.

Mollenhauer, H.H. and Totten, C., (1971), Studies on seeds and fixation of seeds. J. Cell Biol. 48, 387-394.

Nahapetian, A. and Bassiri, A., (1976), Variations in concentrations and interrelationships of phytate, phosphorus, magnesium, calcium, zinc, and iron in wheat varieties during two years. Agric. Food Chem. 24, 947-950.

Ogawa, M., Tanaka, K. and Kasai Z., (1975), Isolation of high phytin containing particles from rice grains using an aqueous polymer two phase system. Agric. Biol. Chem. 39, 695-700.

Osmond, C.B., (1967). Acid metabolism in Atriplex I. Regulation of oxalate synthesis by the apparent excess cation absorption in leaf tissue. Austral. J. Biol. Sci. 20, 575-587.

Pernollet, J., (1978), Protein bodies of seeds: ultrastructure, biochemistry, biosynthesis and degradation. Phytochem. 17, 1473-1480.

Pfeffer, W., (1872), Untersuchuggen über die Proteinkörner und die Bedeutung des Asparagins beim Keimen der Samen. Jahr. Wissen. Bot. 8, 429-574.

Pizzolato, P., (1964), Histochemical recognition of calcium oxalate. J. Histochem. Cytochem. 12, 333-336.

Prinz, M., Harlow, G. and Peter, J.(eds), (1978). SIMON AND SHUSTER'S GUIDE TO ROCKS AND MINERALS, Simon and Schuster, New York.

Rasmussen, G.K. and Smith, P.F., (1961). Effects of calcium; potassium and magnesium on oxalic, malic and citric acid content of Valencia orange leaf tissue. Plant Physiol. 36, 99-101.

Raven, J.A. and Smith, F.A., (1974), Significance of hydrogen ion transport in plant cells. Can. J. Bot. 52, 1035-1048.

Raven, J.A. and Smith, F.A., (1976), Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol. 76, 415-431.

Richardson, K.E. and Tolbert, N.E., (1961). Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J. Biol. Chem. 236, 1280-1284.

Rompel, J., (1905), Krystalle von Calcium oxalate in der Frucht wandt der Umbelliferen und ihre Verwerthung für die Systematik. Sitzungsten. Akad. Wiss. Wien 104, 417-476.

Russ, J.C., (1972), Energy dispersive analysis of x-rays and the scanning electron microscope, 115-132 IN: THIN-SECTION MICROANALYSIS, PROCEEDINGS OF A SYMPOSIUM. EDAX Laboratories, Raleigh, N.C.

Seal, S.N. and Sen, S.P., (1970). The photosynthetic production of oxalic acid in Oxalis corniculata. Plant Cell Physiol. 11, 119-128.

Sharma, C.B. and Dieckert, J.W., (1975), Isolation and partial characterization of globoids from aleurone grains of Arachis hypogaea seed. Physiol. Plant. 33, 1-7.

Silver, V.A. and Price, J.L., (1969), Demonstration of calcium oxalate crystals in plant tissues by the Pizzolato ($AgNO_3$-H_2O_2) method. Stain Tech. 44, 257-259.

Spitzer, E. and Lott, J.N.A., (1980), Thin-section, freeze-fracture, and energy dispersive x-ray analysis studies of the protein bodies of tomato seeds. Can. J. Bot. 58, 699-711.

Staden, J. van and Comins, N.R., (1976), Energy dispersive x-ray analysis of protein bodies in *Protea compacta* cotyledons. Planta 130, 219-221.

Swift, J.G., and Buttrose M.S., (1972), Freeze-etch studies of protein bodies in wheat scutellum. J. Ultrastruct. Res. 40, 378-390.

THE SADTLER STANDARD SPECTRA FILES, Sadtler Research Laboratories, Philadelphia, PA.

Tully, R.E. and Beevers, H., (1976), Protein bodies of castor bean endosperm: Isolation, fractionation and the characterization of protein components. Plant Physiol. 58, 710-716.

Vaughan, J.C., (1970), THE STRUCTURE AND ULTILIZATION OF OIL SEEDS. Chapman and Hall, London.

Wagner, G. and Loewus, F.A., (1973), The biosynthesis of (+) tartaric acid in *Pelargonium crispum*. Plant Physiol. 52, 651-654.

Webber, E. and Neumann, D., (1980), Protein bodies, storage organelles in plant seeds. Biochem. Physiol. Pflanzen. 175, 279-306.

Winton, A.L., (1916), MICROSCOPY OF VEGETABLE FOODS, 550-567, John Wiley and Sons, New York.

Yang, J.C. and Loewus, F.A., (1975), Metabolic conversion of L-ascorbic acid to oxalic acid in oxalate-accumulating plants. Plant Physiol. 56, 283-285.

Yasue, T., (1969), Histochemical identification of calcium oxalate. Acta Histochem. Cytochem. 2, 83-95.