To the Graduate Council:

I am submitting herewith a dissertation written by Gerry Lynn Sexton entitled "Cadmium Toxicity in *Glycine max* and Analysis of the Model Legume, *Lotus japonicus*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

L.Evans Roth, Major Professor

We have read this dissertation

and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and

Dean of the Graduate School

Cadmium Toxicity in *Glycine max* and Analysis of the Model Legume, *Lotus japonicus*.

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Gerry Lynn Sexton

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DEDICATION

For Jerry, Harold, Elmer and Fred.

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ABSTRACT

Lotus

Lotus japonicus has been proposed as a model system for the study of plants that form determinate nodules and is now in the early stages of use as a model. Several groups have begun to describe the genetics and gross morphology of the plant.

L. japonicus nodules share many characteristics with other determinate-type nodules. The cells of most nodules are arranged into distinct functional zones. This organization differs markedly between determinate and indeterminate nodules. Most authors rely on a set of, what are believed to be, standard characteristics of a given nodule type when studying their experimentally altered plants, to determine what if any effect their experimental conditions had on the plant. This is especially important in the use of model systems. Over the course of time, initially reported characteristics become accepted as rule, and any variation on them is considered to have been induced by the experiment. I will show in this study that many of these so called "typical" features represent only one of several possibilities. I furthermore think that descriptions of these characteristics should reflect this fact. For example, infection threads of *L. japonicus* are usually broad and resemble those found in alfalfa, which forms indeterminate nodules, while most threads found in soybean and cowpea are thin. However, *L. japonicus* can form single file infection threads, and broad threads have been observed in soybean.

Some characteristics are unique to a given species. There are several such differences observed between *L. japonicus* and soybean. First, *L. japonicus* roots contain numerous cells with large deposits of tannins which are not found in the roots of soybean or cowpea. Second, the infected cells of *L. japonicus* have large centrally located vacuoles

that are notably absent from healthy soybean nodules. Third, the prominent scleroid layer found in soybean nodules is absent in *L. japonicus*. I support *L. japonicus* as a viable model system but feel that natural inter- as well as intra-species variations must be acknowledged and discussed.

Cadmium

Since cadmium is becoming an increasingly important environmental pollutant, I studied the effects of cadmium on soybean nodules by using electron microscopy and X-ray microprobe analysis. I have found that, although plants can accumulate modest amounts of cadmium without significant observable effects, exposures to cadmium at levels in the range of 1.0 to 10mM in laboratory conditions result in a total disarray of infected cells, nodules and plants at 28 days post inoculation. Cellular effects were also seen earlier and at lower concentrations. The earliest and strongest accumulations of cadmium were found in the polyphosphate bodies of bacteroids which, because of their internal location, could only be exposed after the metal had traversed the cell and symbiosome membranes as well as having penetrated the cortical layers of the nodule. Cadmium was also found in nuclei of infected cells in the euchromatin, heterochromatin and nucleoli, results suggesting that cadmium binds to DNA in host cells. Binding to the DNA in bacteroid chromatin could also be expected. The metal was also localized in cell walls of most nodule cells, in amyloplasts, in granules inside tonoplasts, and in the cytosol of almost all cell types in treated plants. Cadmium was also localized in polyphosphate bodies in control cells, though at a lesser frequency and with weak X-ray peaks. Other metals localized were calcium and magnesium, found in both control and treated tissues about equally, and iron which was similarly found, except that it was concentrated more strongly in polyphosphate

bodies of treated than untreated cells. I suggest that the primary pathogenicity of cadmium is due to changes in the properties of DNA, the destruction of membranes and the death of bacteroids, though numerous other processes are undoubtedly also affected. The finding of cadmium in numerous locations and causing such widespread devastation in nodule cells helps explain why yields of soybeans in fields treated with industrial sewage sludge and otherwise cadmium-contaminated are so quickly and often permanently reduced. The finding of cadmium in both control and treated nodules and in plants that may or may not show toxic effects suggests that cadmium can be inserted in the food chain for human beings both by ingestion and inhalation at significant levels beyond those presently recognized.

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NOMENCLATURE

BSO	buthionine sulfoximine
CTEM	conventional transmission electron microscopy
d	day
df	degrees freedom
EELS	electron energy loss spectrometry
GSSGR	glutathione reductase
h	hour(s)
h-PC	homo-phytochelatins
KeV	kiloelectron volts
MT	metallothioneins
PC	phytochelatins
PGA	polygalacturonic acid
p.i.	post inoculation
PM	plasma membrane
PNS	plant nutrient solution
PPB	polyphosphate bodies
(R)ER	(rough) endoplasmic reticulum
s	second
spp.	species
STEM	scanning transmission electron microscopy
TEM	transmission electron microscopy

1. Literature Review and Introduction

1.1 Introduction

The growth and function of all biological organisms is limited by the presence or absence of certain key elements and compounds. Biologically active nitrogen is generally the most limiting nutrient for plants. Atmospheric nitrogen is useless to most higher organisms and must be "fixed" (reduced) to be used. As a result, nitrogen must be transformed into a biologically useful form such as NH_3. This is often accomplished by bacteria living in the soil. These bacteria include free living groups such as *Klebsiella*, *Azotobacter* and cyanobacteria (69). Many plants simply absorb nitrogen compounds from the soil after it is released by these bacteria. However, some plants have evolved a symbiotic relationship with another group of bacteria which provide the plants a more steady source of nitrogen. The plants in return provide an environment free from desiccation, with controlled oxygen levels (nitrogenase is sensitive to oxygen), and a source of carbon for energy. As a result these plants are better able to grow on nitrogen-poor soils.

The most agriculturally important group of plants capable of forming this symbiotic relationship is the legumes. Legumes are important to humans because they allow farmers to grow crops with less nitrate and ammonia fertilizer; thereby, reducing the cost of farming, both economically and environmentally. They are important in developing countries because farmers there may not be able to afford to supplement the soil with processed nitrogen. The study of legumes and their symbionts may one day lead to development of more efficient symbiotic systems as well as new symbioses with plants that cannot form such relationships now.

The topic of dinitrogen fixation will be covered briefly here. For a more in-depth coverage of the topic the reader is advised to investigate the excellent reviews by Dart (21), Gresshoff and Rolfe (95), Halverson and Stacey (41) and Kijne (59).

1.1.1 The Nodule

The plant-microbe symbiosis is established within a specialized organ called a nodule. This structure serves to contain the microsymbionts and regulate the symbiosis. The nodule is typically found below ground on the root, but can also appear on adventitious roots above ground on some plants such as *Sesbania* (83).

Only a small group of soil microbes can successfully induce the formation of nodules on legumes. With a few exceptions, these bacteria generally fall into three genera, the fast growing *Rhizobium* spp. and the slow growing *Bradyrhizobium* spp. and the Azorhizobia (30, 52, 69, 82, 114).

Some non-legume species such as *Alnus* spp. and *Casuarina cunninghamiana* (113) are not infected with either *Bradyrhizobium* spp. or *Rhizobium* spp. and instead form nitrogen fixing nodules with actinomycete-like microbes.

1.1.2 Nodule Types

Three major types of nodules are formed by plants: determinate, indeterminate and collar or ring nodules. Nodule shape is determined by the host plant rather than the infecting bacteria and is not specific to a given genus. Related species can form different types of nodules, such as *Vigna unguiculata* (cowpea) which forms round, determinate nodules while *V. owahuensis* forms elongate, indeterminate nodules (21,86).

Most cells that compose a nodule are not unique to just that organ. Cells such as the vascular elements and parenchyma cells are found throughout the plant. Two cell types, however, are found only in the nodule. These include the infected cells which contain the microsymbiont and the uninfected cells that carry out additional biochemical conversions of nitrogenous compounds exported by infected cells (105, 122). Infected cells are specialized to be capable of phagocytosis, a process that is unusual for plant cells

due to problems with turgor (59). During this process infected cells engulf bacteria, enclosing them in unique organelles called symbiosomes. Not all symbiotic hosts have these specialized cells. For instance, the non-legume *Parasponia* does not bring the symbiotic bacteria into the cytoplasm of its nodule cells, instead maintaining the bacteria within the confines of modified infection threads called "fixation threads" where they actively fix nitrogen (88,95).

1.1.2.1 Determinate nodules

In determinate nodules, such as those of *G. max* and *V. unguiculata,* the meristem is dispersed throughout the nodule during early development but eventually subsides to a few pockets at the edge of the bacterial zone and then disappears, leaving a spherical organ (95). These nodules remain attached to the root through a small connected group of cells and often develop ridges along the outside (21). Such nodules are generally sectioned into roughly concentric zones which include the outer cortical cells including the epidermis, the inner cortical cells which include parenchyma and vascular cells, and the infected zone which includes the infected and uninfected cells.

1.1.2.2 Indeterminate nodules

Indeterminate nodules, a second, major nodule type, contain a persistent meristem which continually produces new cells, resulting in an outward growth that gives the nodule a more cylindrical shape (21, 122).

These nodules can be divided into three zones, each of which represents a different stage of development. The zone most distal to the root stele contains the active meristem in which mitotic activity is limited (79). The meristem persists throughout the development and productive life of the nodule (21).

Just proximal to the meristematic region is the infected zone. Infection threads ramify in this region and enter the phagocytic cells. Infected cells mature in this zone and

nitrogen fixation begins. This region contains the protein leghemoglobin, which helps regulate the amount of oxygen the bacteroids are exposed to and gives this middle zone a pink color, and the infected and uninfected cells which serve similar functions to those of determinate nodules.

The third most proximal zone is the area of senescence where infected cells begin to shut down and finally die, and leghemoglobin is degraded, giving this region a brown or green color (79). In the infected cells of this zone, nitrogen fixation ceases, and the symbiosomes are disrupted.

1.1.2.3 Collar nodules

This type of nodule, found on plants such as *Lupinus* spp., form a flat collar that may encircle the entire root (21). The meristem begins as a hemisphere at the tip of the nodule and eventually localizes at the edges of the nodule; growth from these peripherally located meristems results in growth around the nodule (21).

1.1.3 The Infection Process

Successful recognition, nodule initiation, infection and nodule formation are complicated events, and much research remains to be done. The establishment of a complete functional nodule involves the products of many plant and bacterial genes in nine steps: (1) chemoattraction of the bacteria, (2) host-symbiont selection by activation of transcriptional regulators such as Nod D, (3) bacterial entry into the plant, (4) formation of plant meristems and nodule initiation, (5) infection thread formation and ramification, (6) nodule growth, (7) bacteria release, (8) mature infection and (9) senescence. Many of these steps occur simultaneously so their sequence is approximate.

1.1.3.1 Chemoattraction

This process begins in the rhizosphere outside the root where the plant root exudes several substances, including amino acids, sugars and carboxylic acids, that act as chemoatractants to many of the bacterial species found there (35, 59). The fact that the *Rhizobium* spp. are not the only organisms attracted to these exudates suggests that exudates act only to attract bacteria and do not serve as a method of specific bacterial selection (35, 59).

1.1.3.2 Selection

Other components of the root exudate, flavonoids and isoflavonoids, serve a very different role, a selection mechanism, matching the appropriate host and symbiont (6, 28, 93, 109). These compounds exert their effects through a complex series of events that result in the activation of a specific group of genes in the bacteria that in turn code for proteins that help trigger the nodulation events that follow.

The first step in this process is the activation of certain trascription regulators such as Nod D. These components are activated by flavonoids in *Rhizobium* spp. and isoflavonoids in *Bradyrhizobium* spp. (6, 93, 96). Nod D binds upstream of a group of genes known collectively as the *nod* genes (7). It is this group of interactions, flavonoid or isoflavonoid, with Nod D and other transcriptional regulators that is believed to, at least in part, confer specificity.

More than one microsymbiont is typically capable of forming effective nodules on a given host, and many *Rhizobium* spp. are capable of inducing nodules on a number of host species. For instance, the slow growing *Rhizobium* strain RP501, which was isolated from *Parasponia*, also form effective nodules on *Macroptilium atropurpureum* (siratro) and *V. unguiculata* (cowpea) (67). *B. japonicum* USDA 110 also forms nodules on both *V. unguiculata* and *G. max.*

1.1.3.3 Entry into the plant

Infecting bacteria gain access to plants in several ways. The method used is determined by the plant, e.g., *Arachis hypogaea* (peanut) where both *Rhizobium* spp. strain 32H1 (106) and *Rhizobium* spp. strain 8A10 (18) enter the plant via a crack in the epidermis at sites of lateral root emergence (19, 18, 95). In some cases, bacteria gain access to the root of the appropriate plant by taking advantage of normal root processes. As lateral or adventitious roots erupt from the epidermis, they crack the surface, leaving the interior of the plant open to infection (18, 19, 59). In other systems, e.g., *Parasponia*, the microorganism has evolved a method of inducing the outer cortical cells of the root to divide, which mimics the situation in systems that infect via root hairs. These cells eventually produce a callus-like structure that breaks the epidermis, forming a crack through which the bacteria gain entry to the plant (59).

A second, and perhaps more common, method of infection involves inducing root hair curling and then penetration into the deformed cell via a specialized structure called an infection thread. This system is used by microsymbionts infecting *Glycine* spp. (95).

In root hair infection, the bacteria first attach themselves to the root covering it (95). Infection typically occurs at the zone of root hair emergence just prior to their formation (14, 21, 95). Once the root hair emerges, the bacteria induce it to curl (45, 59, 95, 115), entrapping the bacteria attached to the surface (115).

1.1.3.4 Formation of nodule meristems

At some point after the bacteria attach to the root, they release a group of compounds that induce the plant to form a meristem (77, 103). This growing region produces the cells that will make up the mature nodule where the infection threads eventually carry the bacteria and release them.

1.1.3.5 Infection thread formation

Invasion of the plant root begins when the bacteria weaken or digest the epidermal cell wall and form an invagination that is walled off by the host cell. The bacteria continue to divide at the point of contact, and the processes of bacterial division and host cell wall deposition produce the growing tube-shaped infection thread (94, 95, 107) that crosses the cytoplasm and vacuolated space of the trichoblast and proceeds to the site of nodule meristematic activity (59, 95). Once this point has been reached the initial thread ramifies into smaller threads that enter the phagocytic cells and eventually release the bacteria.

The wall material that composes the thread has been shown to be of plant origin (94, 107, 115), although there is evidence that the wall of the thread differs in several aspects from the root hair cell wall and may represent a slightly modified structure (13, 115). Vesicles with wall material (115), microtubules, endoplasmic reticulum and Golgi bodies (94, 115) can be seen in the vicinity of the thread, all of which suggest that the plant supplies the material that composes the wall. The infection thread wall is composed of cellulose, polygalacturonic acid (PGA), methyl-esterified PGA and xyloglucan (89, 107). Polysaccharides are not the only components of infection threads. It has been shown that infection threads also contain a group of proteins that are similar in structure to the plant nodulins (107). These proteins, called proline-rich proteins, are part of the extensin superfamily of wall proteins (58, 107) and are found in the matrix material of the infection thread (107). The origin of the matrix material is controversial, but the evidence points to the plant as well as the bacteria (13, 59, 107), a reasonable finding since the thread is the location where microsymbiont and host first contact one another.

The number, size and fate of infection threads seems to vary from species to species. In soybean, for example, infection threads are relatively difficult to find both in early and late stages of nodule development (36). These threads tend to be thin, one or two bacteria in width, with little matrix material surrounding the bacteroids. However, larger

threads can occasionally be found (97, 24). It has been suggested that the threads degenerate in soybean after the bacteria are released into the host cell (4, 36), and Bal (4) reports vesicles in soybean that contain wall-like debris. This is quite different from the situation in alfalfa (85) and *Lotus* (reported here) where the threads can be rather large with copious amounts of matrix material and seem to persist until senescence. In some species such as *L. japonicus* and *V. radiata* (mung bean) (80), both types of threads can be readily found, a result suggesting that for all species thread width varies rather than being a specific type. Perhaps thinking of threads as one type or the other is no longer useful. Threads should instead be described as one "type" with much variation.

1.1.3.6 Phagocytosis

Once the thread has entered the dividing cortical cells of the root, the bacteria must "take up residence" in specialized cells for the symbiotic relationship to be established (except systems such as *Parasponia* which retain the bacteria in "fixation threads") (104). Release of bacteria into the host cell cytoplasm requires a signaling mechanism. If the bacteria do not produce the appropriate signal, they can be degraded in the infection thread or destroyed by lysosomal action after entry (81). The process of release is a phagocytotic event, and as such it would be expected that an elaborate signaling process takes place for the event to be successful (36, 80, 99).

Very little thread wall or matrix material is endocytosed along with the bacteria. The bacteria are enveloped in a vesicle that is, at first, composed of the host PM (80, 99). The initial composition of the symbiosome membrane is the source of some controversy. Some believe that the membrane is generated at the point of bacterial release entirely from the plasma membrane (73, 80), while others suggest that the initial symbisome membrane has a percentage of plasma membrane (PM), as well as Golgi bodies and endoplasmic reticulum (ER) generated membrane (99). The composition of this membrane gradually

changes over a short period of time, due to the addition of vesicles from the Golgi apparatus and rough endoplasmic reticulum (RER). This membrane and its contents are known as a symbiosome, the functional unit of the symbiotic system.

1.1.3.6.1 Symbiosome

Over the course of time vesicles fuse with the symbiosome altering the composition of both its membrane and luminal space. Several proteins similar to those found in lysosomes have also been found in symbiosomes. Kinnback, Mellor and Werner (60) reported the presence of α-mannosidase isozyme II, a lysosomal protein, in the symbiosome space, and several proteases (116) and at least one protease inhibitor (34) have also been found in the symbiosome. Symbiosome membranes also contain ATPase H^+ pumps which serve to move H^+ ions into the symbiosome space (9) lowering its pH by 1-1.6 points in isolated symbiosomes (116).

Given their similarities to lysosomal compartments, it would be easy to hypothesize that symbiosomes are simply lysosomes. However, there is evidence that some proteins, such as nodulin 24 (29), are unique to the symbiosomes. Also, the bacteria maintain some measure of control over the pH of symbiosomes by actively removing malate/succinate from the symbiosome space (54) and producing ammonia which has been shown to inhibit lysosomal proteases (62, 72). So, clearly, symbiosomes create an environment controlled in part by the plant and in part by the bacteria. That the symbiosomes environment are not totally under host control, that the bacteria are not degraded, and that there is a group of proteins specific to the symbiosome strongly suggest that this structure is a unique organelle, a view put forth by Mellor and Werner (73).

During senescence, the bacteroids are digested within the symbiosome. In fact, vesicles containing membrane whirls and polyphosphate bodies (PPB) can be seen even within young infected cells, are likely the remnants of symbiosomes and may represent a

structure formed by the fusion of several symbiosomes (98). This characteristic further supports the hypothesis that symbiosomes eventually become lysosome-like in nature.

1.1.3.7 Mature infection

After release from the thread into a functional and complete symbiosome, the establishment of a mature infection proceeds. The bacteria begin the transformation into bacteroids. At some point between release and maturity, the bacteria begin to fix nitrogen. The symbiosome membrane receives vesicles from the host which gradually change its composition (72 and references, 73 and references, 99, 122 and references). Once the bacteria have fully transformed and are fixing nitrogen, they are called bacteroids.

1.1.3.8 Senesence

In the last stage, the nodule begins to senesce. During this stage of development, the bacteroids degenerate and are destroyed. The symbiosomes in soybean coalesce into ever larger vesicles which seem to function as lysosomes, degrading all contents within. The infected cells themselves are eventually destroyed leaving only the cell walls.

1.1.3.9 Nodulins

Nodulins are plant proteins that are specific to the nodule. They are directly tied to the establishment of a successful nodule. The nodulin genes are typically expressed around the onset of nitrogen fixation (76). The proteins can be classified into two groups dependent on when they are produced. The early nodulins form early in nodule development, and help establish the initial symbiotic relationship. Late nodulins are produced latter in development and maintain mature nodule function. Typical late nodulins include, leghemoglobin, an O_2 regulating protein, uricase, responsible for part of the nitrogen fixation pathway, glutamine synthetase, which is also part of the nitrogen fixation pathway and some symbiosome specific proteins.

1.1.3.10 Nod factors

In the *Rhizobium*, the genes involved in nodule formation are called the *nod* genes. The common *nod* genes (*nod ABCIJ*) are involved in nodule formation and are functionally interchangeable within all *Rhizobium* species (30). When the *nod* genes are activated, the *Rhizobium* secrete a special group of carbohydrate/lipid molecules called the *nod* factors. These factors are capable of inducing root hair deformation, cortical cell division and some other steps in the infection process on their own. In fact their addition is sometimes sufficient to induce the formation of full, empty nodules (30).

1.2 Cadmium

1.2.1 Introduction

1.2.1.1 Cadmium and Human Health

1.2.1.1.1 Acute exposure

Although somewhat rare, cases of acute Cd poisoning do occur. Workers in metal production facilities can become exposed if the ore containing the metal is heated to sufficient temperature (11, 12, 25). The first sign of acute poisoning from inhalation is respiratory distress due to chemical pneumanitis and edema. Estimates vary, but a fatal dose is about 5 mg/m^3 in air (32).

Ingestion can also be a source of acute toxicity. In the 1940's and 50's, many cases of acute poisoning were reported after Cd was used as a substitute for scarce Cr in the manufacture of cooking utensils (32). Cadmium contamination has also been reported after acidic foods and drinks were stored in contact with Cd-plated surfaces (32). The rapid onset of severe vomiting that often accompanies acute exposure through ingestion helps prevent many non-lethal cases from developing long-term health problems (32).

1.2.1.1.2 Chronic exposure

Most people are exposed to some level of Cd daily, intake generally coming from the food we eat (70%) and can be greatly increased by smoking (32, 118), the latter allowing more time for absorption and deposition. The half-life of Cd in the body is varied but can be extremely long. Some estimates are as short as six months (90) to as long as over 30 years (61); most were greater than seven years and less than 20 (32). The first symptom generally recognized is proteinuria, and the kidney appears to be the major target of Cd toxicity, typically suffering renal tubular dysfunction. Unlike acute exposure, where

renal function may eventually return to normal, in chronic exposure, the effects usually are permanent (32).

There is also ample evidence that chronic exposure to Cd results in alterations in Ca chemistry within the body. In many people exposed to the metal in the workplace, kidney stones were found (2, 26, 31, 68), composed of calcium phosphate (3). Many of these people also had elevated levels of Ca in their urine (57), continued long after exposure ceased (56). The blood levels of Ca remained near normal in people with elevated Ca in their urine (56) and, when radiological examinations were conduced, pseudofractures suggesting osteomalacia could be seen. Osteoporosis was also reported in some workers (32).

Most of the studies showing the affects of chronic and acute poisoning by Cd have been reported for industrial workers. Farmers may also be at elevated risk when working with fertilizers that have been used to recycle industrial wastes. Much more work needs to be done to better determine the effects on the general public, who are exposed daily through the food they eat.

1.2.2 Properties

Cadmium exists in all soils at a low background level, reaching amounts in uncontaminated soil of 0.01 to 0.7 μg/g soil (101). It remains in the soil for a relatively long period of time and is readily taken up by plants, a phenomena that makes Cd hazardous to humans.

Cadmium was discovered in 1817 by a German chemist named F. Stromyer. It is not found in a pure state in nature (23). It is a soft, ductile, silver-white metal with a specific gravity of 8.642, a melting point of 320.9 °C and an atomic weight of 112.4 (23). It is concentrated in sulfide minerals and usually found in zinc, zinc-lead and zinc-lead-copper ores (10).

More than 65% of the world's cumulative production of Cd has occurred in the past 30 years. Approximately 90% of Cd is used for the following: electroplating (29%), stabilizers (25%), pigments (23%) and batteries (13%) (23). In electroplating Cd is used in combination with iron, zinc and other metals to coat many items such as nuts, bolts and screws (23). When used as a pigment in paints, plastics or rubber, Cd compounds produce a range of colors, e.g., CdS-BaSO4 yellow, CdS-BaSO4-ZnS orange and CdS-BaSO4-CdSe dark red (23). Due to rising health concerns, Cd use and production has been reduced somewhat in the past few decades (10).

Cadmium enters the environment in three ways. The first is atmospheric deposition on soil. Approximately 90% of air-borne Cd pollution comes from industrial sources including 73% from non-ferrous metal production, 20% from waste incineration, and the remaining 7% from a combination of iron and steel production, industrial applications, combustion of coal, oil and wood and rubber-tire wear (23). The other 10% of all Cd deposited in soils via the atmosphere comes from natural sources including 65% from volcanoes, 25% from decaying vegetation, 12% from airborne soil and 2% from forest fires. (23)

A second important source is industrial sewage. Waste that is normally separated into liquid and solid components for disposal (22). Approximately 90% of the Cd present in sewage will come out with the sludge portion in this process (22), and in many cases, the cheapest way to dispose of sludge is through its application to agricultural fields which results in accumulation of Cd in the soil. Most of this metal will remain in the top 20 cm of the soil (118), where most crop plants receive their nutrients. Other methods of disposing of the sludge include burning which allows the Cd to become airborne, eventually settling in the fallout of the incinerator.

Another important agricultural source of Cd is phosphate fertilizers which in the United States, add up to 200 mg Cd/Kg fertilizer per year (10). Fertilizers can become further contaminated with Cd through the practice of adding industrial waste to them.

This practice, termed recycling, has recently attracted the attention of the national press. Through a series of regulatory loop holes, some companies have been able to take hazardous waste ash from incinerators, scrubbers and mining operations and re-label it as fertilizer supplement. Once its designation has been changed, it can be added to fertilizer without modification. To further exacerbate the problem, fertilizer manufactures do not have to test or label their product with the amount of heavy metal present and metal levels can become quite high. Companies producing fertilizers are only required to test and indicate levels of the beneficial components, all others being labeled as inert ingredients without being tested. In theory, this "recycling" provides industries with a cost effective and safe way to dispose of materials that would otherwise wind up in hazardous waste landfills.

Much of this material, especially ash from stack scrubbers, contains significant levels of Cd and Pb. Since they are not indicated as ingredients in the fertilizer farmers unknowingly apply them to their crops. Most plants accumulate Cd in their roots, while some, such as spinach and tobacco, translocate high levels to their leaves (117, 118). Many plants continue to function after exposure to the metal without noticeable outward signs that the plant is under stress, the result being that contaminated plants are harvested and sold, thereby contributing to our daily intake of Cd. It is believed that this chronic exposure to Cd is a far more serious health concern than acute exposure (32, 33).

For example a story first reported in the Seattle Times (123, 124, 125, 126) was about a small town in Washington state and a fertilizer company named Cenex. In 1986, Cenex built a settling pond in this small town called Quincy. The pond was designed to capture runoff produced when fertilizer was cleaned from farm equipment. In 1990 the

pond was closed but still contained over 38,000 gallons of settled material that was found to contain high levels of Cd, Be and Cr and was labeled as hazardous waste. The material needed to be removed, but it was determined that removal would cost in excess of $170,000. In an effort to save money, the company decided to pay a local farmer $10,000 to spread the material over his 100-acre leased farm, the thought being the land would act as a filter and remove all the toxic materials from the sludge. Instead the corn crop that year was destroyed, and, for years after, production on the 100-acre farm was greatly reduced. The company attempted to reduce the toxic affects by repeatedly flooding the fields but to no avail. After a couple of years, the toxic effects were still dramatic, and a complaint by the land owner was filed with the EPA after he lost the farm to foreclosure brought on by the lands inability to produce a healthy crop. The EPA investigated and found that there was enough Cd, Be and Cr in the sludge that the pond qualified as a superfund site!

After this event, local farmers began to realize that they too were experiencing yield problems and that they had used fertilizer supplied by Cenex. They began to wonder if the fertilizer they were using could be contaminated like the sludge from Cenex's settling pond. Crops were not the only things being affected; some of the farmers had lost cattle to high rates of cancer. They had hair samples from themselves and their children analyzed and discovered high levels of Cd, Al, Sb, Pb and As.

It was eventually determined that Cenex was indeed the source of local contamination. Now the problem became figuring out where the heavy metal originated. It was finally determined that Aluminum Company of America (Alcoa) was the likely source of the toxic material used by Cenex. They had sold more than 200,000 tons of hazardous waste from their smelter factory in Addy, Washington to a company called L-Bar products which produced fertilizer and road de-icer from the material. One of the company's products, a fertilizer called CalMag®, was sold to the Cenex company, which then resold it to farmers in the Quincy area.

The worst case of heavy metal toxicity to plants through the application of fertilizer occurred in Tifton, Georgia. A farmer there applied a soil liming product called "Lime Plus" produced by the Sogreen company to over 1,000 acres of peanuts. The entire crop died from metal toxicity because the liming agent contained, among other things, 10% Zn and 3.6 % Pb, Cd and Cr (123-126).

The problem is being exported as well. In Charleston, South Carolina, the Stoller Chemical Company was fined $1 million for exporting 3,000 tons of toxic materials containing high levels of Cd and Pb to Bangladesh and Australia in 1992. Unfortunately some of the material had already made it to crop fields in Bangladesh and gardens and pasture fields in Australia before it was recalled (123-126).

Clearly, studies must be conducted to find out how heavy metals such as Cd are used and stored by plants, especially if hazardous material recycling practices as described continue in this country.

1.2.3 Cadmium Effects On The Cell

1.2.3.1 Lipid Peroxidation

Transition elements such as Cd may act as catalysts in reactions which cause the oxidative deterioration of macromolecules (111). Often, free radicals of oxygen are generated which are then responsible for oxidative destruction and also for lipid peroxidation, DNA damage, depletion of sulfhydryls, and altered calcium chemistry (111).

Exactly how Cd is involved in the generation of free radicals is not well understood (1, 111, 112), since Cd is not believed to be directly involved in the generation of these destructive compounds. There are several possible indirect routes for Cd to have the same effect however.

One possibility is that Cd may after the free concentrations of some other metal such as Fe which would then be capable of producing free radicals. Another possibility is the

interaction of Cd with antioxidants, e.g., the free radical scavenger glutathione disulfide, a protein maintained in the reduced form by glutathione reductase (GSSGR EC 1.6.4.2). Cadmium is known to inhibit GSSGR (1) and,therefore, could contribute to the increased levels of free radicals by preventing them from being scavenged (1, 111, 112).

Regardless of the mechanism, the addition of Cd to animal cells results in an increase of lipid peroxidation (1, 111, 112).

1.2.3.2 Cadmium Effects on Other Metal Chemistry

Cadmium is often found associated with other metals in tissue. Rauser and Ackerley (92) reported that Cd can be found in electron-dense granules that also contain other metals such as Ca, Fe, Pb, Zn and sometimes Ni and P. My data show Cd associated with Fe, Ca and P.

1.2.3.2.1 Calcium

I hypothesized at the beginning of this study that one of the ways that Cd produces a toxic affect in a given cell is by interfering with the normal cytological biochemistry of other metal ions, particularly Ca. There is much evidence in the literature to suggest this including reports of Cd movement across plasma membrane (PM) and tonoplast membranes being facilitated by Ca transporters (44, 101) and by reports of Cd competing directly with Ca for binding to such proteins as calmodulin (20).

In a series of experiments conducted by Hinkle et al. (44), Cd was transported into cultured pituitary cells via a voltage-gated Ca channel, and the addition of the Ca channel blockers, dihydropyridines, verapamil and diltiazem, afforded the cells some protection against the toxic effects of Cd. Cadmium toxicity was increased on the other hand through the addition of the Ca channel agonists, Bay K8644 and K^+. The addition of high levels of Ca decreased Cd toxicity, a result suggesting Ca and Cd compete for transport into the cell via this voltage-gated Ca channel.

Karez et al. (55) demonstrated Ca/Cd interactions in the algae, *Cricosphaera elongata* , and found that increasing levels of Cd competitively inhibited Ca uptake. Uptake of Cd by the cells, unlike Hinkle's pituitary cells, appeared to be inhibited by the Ca channel blocker verapamil.

Cadmium may also enter mitochondria by utilizing a Ca transporter. Jarvisalo (50) showed that Ca transport into mitochondria via a Ca transporter was blocked by the presence of Cd (43).

Chao et al. (20) demonstrated that Cd, at 5 μM, could activate the phophodiesterase activity of calmodulin to 60% of maximal, compared with 70% activation by the same amount of Ca. More importantly, in the presence of low levels of Ca, Cd could fully activate calmodulin and thus could have dramatic effects on the cell's ability to control Ca/calmodulin interaction.

Zinc appears to be another target of Cd toxicity. One possible mechanism is the interference with Zn fingers in the control of DNA (118). Cadmium-substituted Gal-4, a Zn-requiring factor involved in the regulation of galactose utilization in yeast, was found to have a secondary structure that was similar to the naturally occurring Zn-substituted proteins (5).

1.2.3.3 Cadmium Affects on Proteins

Cadmium has an affinity for sulfhydryl (-SH) groups and is believed to exert its toxic effects on proteins by interfering with the normal functions of -SH groups (111). In fact it is believed that Cd's interaction with -SH groups is one of the major methods of Cd toxicity. Cells have taken advantage of Cd's affinity for -SH groups to reduce its toxicity by evolving Cd-binding proteins that chelate Cd, by having large numbers of -SH groups. Perhaps the most important group of proteins of this type in plants are the phytochelatins.

1.2.3.4 Disruption of Organellar Function

Cadmium has profound affects on particular subcellular organelles such as the nucleus, mitochondria, chloroplasts, PM and symbiosomes.

Cadmium is capable of changing the conformation of DNA, destabilizing the molecule by interfering with AGCT sequences (53), binding to the phosphate group (53, 65), and interacting extensively with bases, especially guanine (65).

The functions of the mitochondria have been reported to be interrupted by Cd. Hawkes et al. (43) showed that in crickets and locusts, the mitochondria showed signs of membrane disruption and swelling in response to Cd. Silverberg (108) reports that mitochondria in the fresh water algae, *Ankistrodesmus falcatus*, *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* granules which contain Cd and as seen in crickets and locusts, the mitochondria were swollen and had disruptions of the inner membrane.

Chloroplasts of *Nostoc* showed adverse effects when exposed to 6.36 μM Cd. The thylakoid membranes became disrupted, and the chloroplasts developed large vacuous spaces (27).

I show that the PM as well as the symbiosome membrane became disrupted and eventually disintegrated after exposure to 10mM $CdCl_2$. Destruction of the PM obviously results in cell death, this may occur by Cd's action on lipid peroxidation or more indirectly by affecting the nucleus which in turn prevents the cell from actively maintaining the PM. My evidence is that symbiosome membrane damage becomes apparent before that of the PM.

1.2.4 The Cell's Ways Of Dealing With Cadmium

Plants have developed many mechanisms for coping with exposure to toxins such as heavy metals. Unlike animals or many bacteria, plants cannot move away from a toxic material and had to develop both systemic and cellular mechanisms for tolerating heavy

metals. Different species may have evolved unique strategies of protection from damage, but more likely different species employ similar strategies, just in varying proportions.

1.2.4.1 Whole plant

The general consensus of most authors seems to be that metals associate with roots to a much larger degree than stems, leaves or fruit (47 and refs). One exception to this may be tobacco which accumulates high levels of Cd in its leaves (117). Noblin (66) demonstrated that Bush Bean and Pea roots contained most of the plant's total Cd. Cereal plants mobilized two to three times more Cd to the shoots than dicots but still had more Cd in their root system than in shoots (47). One possible explanation for the observation that dicots mobilized less of the metal to the shoot than monocots is the presence of a stele in dicots, which limits movement of ion-carrying water through the roots and into the vascular tissue (47).

Cataldo (17) found that after a 48-hour pulse of Cd, *Glycine max* roots contained 92% of the plants total Cd. After 21 days the level had dropped only slightly to 84%, with much of the mobilized metal being relocated in the seed (7.8%).

1.2.4.2 Binding in the cell wall

Cells are believed to possess several mechanisms for dealing with toxic levels of Cd. One of the first parts of a cell to encounter Cd is the cell wall, and Cd binds readily to pectic and hemicellulosic wall polymers (118). Many fractionation studies have shown that when cells are broken apart and the individual components are examined for Cd content, the cell wall often contains large amounts (17, 47, 118). My studies have shown, through X-ray microanalysis, that Cd is deposited in the cell wall which may act as an affinity column and remove the charged Cd^{2+} ion (118). Inouhe (47) found that almost 50% of Cd in dicots can be localized in the cell wall fraction. As much as 35% of total tissue Cd in soybean leaves and 36% of Cd in soybean root may be located in the cell wall (17).

Not everyone is in agreement on Cd binding to cell wall components. Rauser and Ackerley using X-ray microprobe analysis could not localize Cd in the cell wall, instead finding most in granules in the nuclei, cytoplasm and vacuoles (92).

1.2.4.3 Membrane as a barrier to Cd movement

Avoidance is a plausible method of Cd tolerance. If the plants could exclude Cd, the toxicity problem would be solved with little or no effort. Cell membranes are known to exclude many charged components and can often regulate the movement of ions into and out of the cell, but Cd is not easily controlled by the membrane of higher plants. There is strong evidence that Cd can utilize Ca channels to cross the PM (44), a result suggesting that the PM of plants is probably not an effective barrier to Cd uptake. Furthermore, exclusion of Cd could also mean the prevention of Ca movement, something clearly detrimental to the cell.

1.2.4.4 Chelation in the cytoplasm

There is strong evidence that a group of specialized proteins called phytochelatins (PC) act in higher plants to sequester Cd. The presence of Cd induces the production of this family of -SH-rich proteins, and pre-exposure to low levels of Cd results in the production of PC's and provides a higher level of protection against subsequently higher levels of Cd (38, 39). PC will be discussed further in section 1.2.5.7.

Other chelators include organic acids such as malate and perhaps more importantly citrate (118, 120, 121). As much as 68-74% of Cd in monocots was located in the cytoplasm of root cells, while dicot root cells could have levels as high as 50% of total Cd located in their cytoplasm (47).

1.2.4.5 Movement to the vacuole

Cadmium can utilize Ca transporters to gain access to some organelles inside the cell, particularly in the tonoplast. In plants, this large vacuole serves many specialized functions including storage for ions and has been shown to concentrate up to 100% of the Cd present in tobacco protoplasts (117).

Cadmium is a charged ion, which typically has difficulty moving freely across membranes. It is not known if Cd is translocated into the vacuole as a free ion or is first bound to some protein in the cytoplasm and then the complex is transported across the tonoplast membrane (117). Cadmium-binding proteins (BP) have been localized in the cytoplasm and the tonoplast of tobacco leaves, but glutathione, the precursor for PC production, was located in the cytoplasm and not the vacuole of tobacco leaves (117). These data suggest that the Cd is first bound to PC's in the cytoplasm and then the complex is transported into the vacuole. The possibility of the Cd-BP and Cd being transferred to the vacuole independently cannot be excluded with these data however. It is unlikely that Cd movement into the cell is accomplished by a Cd-specific transporter since the metal has no known metabolic function, which suggests that instead the use of a transporter specific for another metal, possibly Ca.

Salt and Wagner (101) have demonstrated the existence of a H^+/Cd antiporter on the tonoplast membrane, allowing movement of Cd into the tonoplast coupled with an increase in tonoplast pH, a finding which suggests that Cd is moved into the tonoplast in exchange for the movement of H^+ into the cytoplasm. The addition of the proton ionophores, FCCP and gramicidin, which destroy the pH gradient, lowered the Cd accumulation in the vacuole by as much as 80%. These studies do not exclude the possibility that Cd is also transported into the tonoplast as a Cd-BP complex, but do suggest that the transport of Cd into the vacuole may be via a Ca channel. These data suggest more than one mechanism for moving Cd into the tonoplast.

Ortiz et al. (84) demonstrated the existence of another type of transporter capable of moving Cd across the tonoplast membrane of yeast. Cadmium-sensitive mutants were deficient in the production of an ABC (ATP-binding cassette) type membrane transport protein. Mutants that over-expressed this protein were tolerant of higher levels of Cd, suggesting that more Cd had been relocated to the tonoplast. This protein, named HMT1, is related to a transport protein associated with cystic fibrosis. These authors suggest that there are two types of Cd-BP complexes, differing in -SH content and molecular weight, and hypothesize that the low-weight protein may bind Cd in the cytoplasm and be transferred to the vacuole, where the high-weight protein then binds Cd and prevents it from being transported back out of the vacuole.

1.2.4.6 Formation of Cd precipitates

Cells may also form dense crystals of Cd. There is evidence that mitochondria form Cd crystals (108), although I was unable to detect granules in the mitochondria of soybean nodule cells. Cadmium has also been located in dense granules within the nucleus, cytoplasm and vacuoles of cortical cells by Rauser et al. (78). I did detect some granulation along the cell wall and/or the plasma membrane and Cd-containing granules within the cytoplasm of soybean nodule cells.

1.2.4.7 Phytochelatins

In 1957 two researchers named Margoshes and Vallee discovered a protein in horse kidney that helped regulate metals and called them metallothioneins (MT) (91). A survey of different eukaryotic organisms indicated that MT existed in higher plants (87), and Cd binding to members of this class of proteins was reported in 1977 (15). As time passed and evidence grew, it became apparent that the metal-binding proteins of plants, algae and some fungi differed markedly from those found in animals.

In 1985 at the Second International Meeting on Metallothionein and Other Low Molecular Weight Metal-binding Proteins (91), a revised nomenclature for MT was devised to deal with the new discoveries being made. The system defined MT as any polypeptide that shared a list of characteristics with horse kidney MT. The list included: low molecular weight, high metal content, high cysteine content, absence of aromatic amino acids and histidine, abundance of the sequence Cys - X - Cys where X is any amino acid except Cys, and spectroscopic characteristics of metal thiolates. The superfamily of MT were then divided into 3 subclasses:

Class I : polypeptides with locations of cysteine closely related to those of horse renal MT.

Class II: polypeptides with locations of cysteine distantly related to those of horse renal MT.

Class III: atypical, non-translationally synthesized metal thiolate polypeptides (91).

In 1985 Grill et al. (39) described a protein in *Rauvolfia serpentina* that contained several γ-carboxyaminde linkages, thereby allowing the classification of these metal binding proteins as type III MT. This class of proteins was given a series of names including cadystin (63, 64), γ-glutamyl peptides (70), poly (γ-glutamylcysteinyl) glycine (48), and the now more widely accepted term phytochelatins (38, 39) by various researchers. Phytochelatins are not limited to higher plants however, being found in algae and fungi as well. Some plants were found to contain an alternative form of PC called homo-phytochelatins (h-PC).

PC are small metal-binding proteins that have a molecular weight of 10,000 to 13,800 (8, 16, 119) and appear to be a heterogeneous group of proteins that have the general structure of (γ-Glu-Cys)n-Gly, where glutamic acid forms the N terminus and glycine the C terminus. The generalized structure of h-PC is (γ-Glu-Cys)n-β Ala. In

legumes, of the 43 species examined so far, 7 were found to contain strictly PC, 13 strictly h-PC and the other 23 a mixture of the two. There appeared to be a strong correlation between the type of PC or h-PC found in the cell and the presence of glutathione or homo-glutathione in the cell. If glutathione was present exclusively in the cell, only PC was formed, but, if homo-glutathione was present, only h-PC was formed. If a mixture was present, then the cell formed a mixture of PC and h-PC. This finding led to the hypothesis that PC and h-PC are produced by post-translational modifications of glutathione and homo-glutathione respectively.

Glutathione is produced in a two-step process. The first combines Glu and Cys to form γ-Glu-Cys by the enzyme γ-glutamylcysteine synthetase (EC 6.3.2.2); the second involves the addition of Gly to the end of this molecule by the enzyme glutathione synthetase (EC 6.3.2.3) to form glutathione (71). Low activity of either enzyme in yeast mutants prevented them from forming Cd binding complexes (91). The addition of buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, strongly reduced the amount of PC produced in response to Cd in a series of plant species (40, 110). When normally sub-toxic levels of Cd were added to the cells, their growth stopped or slowed (46, 110).

Evidence suggests that there is not a gene coding for the synthesis of PC but that production of PC is a post-translational modification of glutathione (or homo-glutathione) (91). The evidence suggesting the lack of a PC-specific gene includes the fact that ribosomes are not known to form the γ-peptide bond involved in PC construction (91). Also, for h-PC there exists no known codon for ß-Ala (91). Further evidence includes the rate of response; PC production occurs too rapidly to directly involve protein synthesis (91).

In summary, Cd has been shown to be the strongest inducer of PC production, followed by Cu and to a much lesser extent Zn (91). It is believed that Cd binds to the -SH

1.2.5 General Model of How the Cell Handles Cadmium

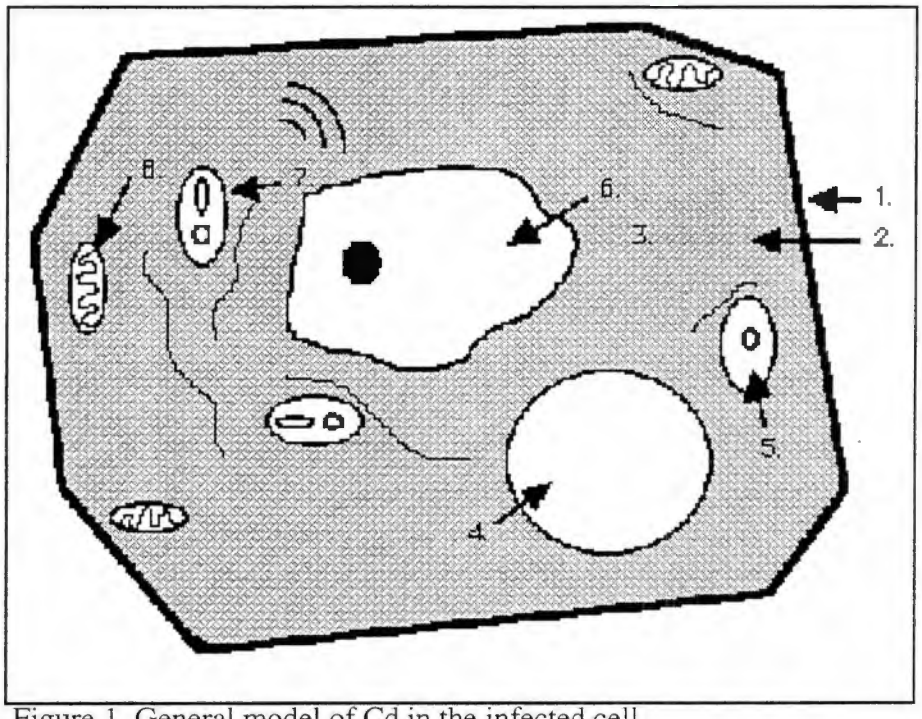


Figure 1. General model of Cd in the infected cell.

What happens to Cd in an infected cell? It should be noted that these events are not sequential.

1) A substantial amount of Cd becomes bound to the cell wall, perhaps as much as 50% (118).

2) Cadmium that is not initially bound to the cell wall is readily transported across the plasma membrane, perhaps by Ca channels or transporters.

3) Once in the cytoplasm, Cd binds to many of the cytosolic components. Cadmium has a particular affinity for -SH groups on proteins. In addition, phytochelatins may bind Cd in the cytosol (91).

4) Either bound to phytochelatins or as free ions, Cd is transported into the tonoplast. Here it is prevented from interfering with most cytological biochemistry. It may be maintained in the tonoplast by binding to larger forms of phytochelatins.

5) Cadmium binds to some component of the amyloplast, perhaps ferritin, although no crystals were observed.

6) Cadmium present in the cytosol crosses the nuclear membrane (likely to simply diffuse through nuclear pores) and once there binds to euchromatin, heterchromatin and the nucleolus. It is capable of disrupting the structure of DNA by binding to the phosphate groups as well as the bases, especially guanine (65, 53).

7) Cadmium crosses the symbiosome membrane and the bacteroid membranes, entering the bacteroid where it is sequestered in the PPB. It is likely that Cd is capable of interfering with bacteroid DNA in a fashion similar to its interference with eukaryotic DNA. It is unclear whether Cd is transported across the symbiosome membrane as a free ion or bound to the small organic acids that serve as carbon sources for the bacteroids. Eventually, Cd may destroy the symbiosome membrane, disrupting symbiosome structure.

8) Cadmium has been reported to disrupt the cristae of mitochondria in fresh water algae (108) and certain insects (43).

group of the cysteine residue. Indeed, binding ratios for Cd:Cys were 1:2 to 1:3 and for Cu were 1:2 to 1:6 (91).

1.2.6 Conclusions

Cadmium has become a significant health threat to humans in the past century. Most Cd that humans and other organisms are exposed to is generated through the actions of industry. The problem is exacerbated by the lack of adequate disposal and the recycling of Cd-contaminated wastes into fertilizer without notification or testing. Cadmium affects most organisms adversely and has been shown to destroy food crops. It is generally accepted that Cd exerts its toxic effects by interfering with several different components of the cell.

There is little doubt that Cd interferes with Ca chemistry. Many studies show competition for binding to several important proteins by the two metals, perhaps the most significant being their competition for sites on the Ca-regulating protein calmodulin. Cadmium has also been shown to cause damage to proteins directly by interfering with -SH groups.

Soybean is a crop plant that is particularly sensitive to Cd, and, because of its importance in the nutrition of humans and feed animals, provides an interesting subject for the study of Cd tolerance and toxicity in plants. The symbiotic relationship established within nodules is a delicate one, and to my knowledge no one has as yet focused on Cd toxicity in this structure.

1.3 Lotus

1.3.1 Introduction

The use of *Arabadopsis thaliana* as a model for the study of plant biology has proven immensely useful. However, since this plant cannot provide important information concerning nitrogen fixation or the symbiosis established between plant and microbe, it became apparent that one or two model systems should be selected for legumes. *Lotus japonicus* has been proposed for the study of determinate nodules (42).

Several species were discussed for which a large amount of data existed, but because of biological concerns they were not chosen. These concerns included the large tetraploid genome of most agriculturally important species as well as the economic problems involved in the sharing of data about cash crops.

While there are few data concerning *L. japonicus*, it possesses several desirable characteristics. The plant is small, allowing it to be grown rather easily under laboratory conditions. The genome is small, having only 6 haploid chromosomes, 1 large, 3 intermediate and 2 small in size (42) and only 400 Mb of DNA (37). *L. japonicus* can produce up to 6000 seeds, 20 per non-shattering pod, and each seed is tiny, weighing 1.2 mg (42). Flowers are found in pairs on peduncles that radiate from axials along the branches (42), and the mature plant is bushy, having limbs that can reach 30 cm in length (42, 51). Harvest can continue for up to 6 months (42). Both fast growing *R. loti* and slow growing *Bradyrhizobium* spp. (*Lotus*) successfully induce nodule formation on the plant (49). The symbiotic (*sym*) genes of the commonly used bacterium *R. loti* are located on the chromosome similar to the situation in *Bradyrhizobium* species, and not on a sym plasmid, more typical of *Rhizobium* species (42). Both *Agrobacterium tumefacens* and *A. rhizogenes* successfully transform *Lotus* (37). The plant is nodulated via root hair curling

and forms determinate nodules similar to those formed on soybean and cowpea (42, 51); nodule internal structure will be described in detail in the second section.

While work on *Lotus* began slowly, the model is gradually gaining support. Most work involves the genetic aspects of *L. japonicus* (37, 51, 125). There are currently over 20 isolated mutants, including those for non-nodulating, super nodulating, and non-fixing phenotypes (37).

1.4 X-ray Microanalysis

One of the most important techniques utilized by this study involves the use of X-rays generated by a sample exposed to an electron beam to localize the heavy metal cadmium. This technique deserves a short description here for those readers that are not familiar with the principles of the technique.

To understand the principles of X-ray microanalysis, we must first discuss how the electron beam interacts with the specimen. As the beam strikes a given sample, energy is given off in a variety of forms which lie along the electromagnetic spectrum.

Those electrons that pass through the specimen are of two types. Electrons that lose some amount of energy are said to be inelastically scattered and are most typically used for both conventional imaging and can also be separated into their respective energy levels in an effort to determine the chemical composition of the sample. This process is termed electron energy loss spectrometry or EELS.

Another group of electrons lose little or no energy as they pass through the specimen but are deflected to high angles and are therefore said to be elastically scattered. If these electrons are deflected to a sufficiently high angle, they will exit the specimen opposite to the direction of the incident or original electron beam and are now said to be backscattered. Backscattered electrons can be used in both imaging and chemical analysis of the specimen. This analysis is possible because heavier atoms result in more backscatter than smaller ones and so produce a stronger signal on the detector.

Secondary electrons are inelastically scattered, but, rather than being a direct product of the incident beam, they originate from the specimen itself. These electrons travel toward the incident beam and are used in imaging, providing information about the

surface of the specimen. These electrons are most typically used in scanning electron microscopy.

Visible light may also generated when the incident beam strikes the sample. Most biological molecules produce very little of this energy, and the resolution is the same as that for light microscopy, so this energy source provides little valuable information for electron microscopy.

Perhaps most significant to analytical analysis, X-rays are produced also. Two types of X-rays are generated, the analytically useful 'characteristic X-rays' and 'Bremsstrahlung X-rays'.

Characteristic X-rays are produced as a result of a high energy electron from the incident beam striking an electron in the inner shell of a specimen atom. This collision results in the inner shell electron being ejected from the atom, which leaves a vacant space and ionizes the atom. An electron from one of the outer shells of the atom then falls into this vacant space and produces a photon of X-ray energy in the process. Since the energies of these shells is discrete, the energy of the X-ray produced as the outer shell electron falls into the lower orbital is also discrete. According to Mosley's law, any particular atom has a unique shell configuration, and therefore fills the vacant lower orbital in a characteristic fashion. A careful analysis of the X-ray energy signature produced by the atoms of a sample will therefore provide information on the chemical composition of the specimen.

A practical problem in X-ray studies is that some peaks overlap and are not seen because another peak overpowers it. Similarly a peak could be misinterpreted. In the study presented here, the atoms of most interest are Cd and Ca. The X-ray energies of interest for these are the Lα and Lβ for Cd at KeVs of 3.133 and 3.528 respectively and the Kα and Kβ for Ca at KeVs of 3.690 and 4.012 respectively. For the samples examined in this study, the Cd peak energies overlap with the Mα and Mβ of U at energy levels of 3.171 and 3.37 KeVs respectively. For this reason, the samples were not post-stained

with uranyl acetate. There is also an overlap with the energy peaks for K. Cadmium is distinguished from K by paying close attention to the energy values of the given peaks and by the fact that the Lα and Lβ for Cd produce peak heights that are similar, resulting in a characteristic double peak. The Kα and Kβ for K are more significantly different and, therefore, typically only the Kα peak is observed for K.

A problem arises in some biological studies when sections are not stained with U/Pb to avoid peak overlap. The lack of stain can make structure recognition difficult. Another problem in biological studies is that living systems are variable in, for example, their exposure and up-take of Cd. The only way to deal with this problem is to perform many scans and to analyze the data statistically to establish significance.

In addition, processing of samples for study in EM may result in some leaching of the desired material. This can only be offset by comparing data collected through X-ray microanalysis with other techniques.

The Hall method can be used to quantitate this type of data (100).

Despite the drawbacks, X-ray microanalysis remains one of the few methods where the exact location of a desired metal can be determined directly.

1.5 Research envisioned from reviewing the literature

As with most studies, the work described here provides a springboard of ideas for many additional experiments. I would like to take this opportunity to discuss some of the more interesting ones.

1.5.1 Lotus

This study utilizes both TEM and LM to compare mature *L. japonicus* nodules to *G.max* nodules. A follow-up study, concentrating on early infection events would prove very useful. In addition, to further *L. japonicus* as a model system, a complete microscopic study of the plant is needed. Perhaps eventually leading to a complete "histological" atlas of wild-type *L. japonicus*.

1.5.2 Cadmium

There are two sets of experiments that could enhance our understanding of Cd toxicity in soybean, as well as plants in general. The first set are directly related to the work presented here and seems to be a natural progression. Of all the literature surveyed this thesis is to be the only report on the effects of Cd on the nodule of any legume. It would be of great interest to determine if the toxic effects presented here eventually lead to the demise of the plant as a whole. To accomplish this goal, an experiment could be designed that would determine if the addition of nitrate to the nutrient media would reverse the adverse affects of Cd on the plant.

In addition, a careful time course study could help identify the stage of development that proves to be the most susceptible to Cd toxicity. In so doing, this might further pinpoint the exact mechanisms responsible for cellular damage by Cd.

Wider ranging research could result in the utilization of plants to remove toxic levels of Cd from contaminated soils. This "biological mining" is already being done for Ni. Plants could be engineered to translocate most of the Cd they are exposed to into the harvestable areas of the plant and after harvest the Cd could be removed and recycled. Another possibility would be the development of agricultural plants that translocate little or no Cd to edible areas, resulting in a safer food supply.

Bibliography

1. Acan, N.L. and Tezcan, E.F. 1995. Inhibition Kinetics of Sheep Brain Glutathione Reductase by Cadmium Ion. Biochem. Mol. Med. 54:33-37.

2. Ahlmark, S., Axelsson, B., Friberg, L. and Piscator, M. 1961. Further Investigations into Kidney Function and Proteinuria in Chronic Cadmium Poisoning. Int. Congr. Occup. Health 13:201-203.

3. Axelsson, B. 1963. Urinary Calculus in Long-Term Exposure to Cadmium. Pages 939-942 in: Proceedings of the 14th International Congress on Occupational Health, Sangro, P., Akoun, G. and Beate, H.L., eds. Excerpta Medica Foundation, Madrid, Amsterdam.

4. Bal, A.K. 1985. Vacuolation and Infection Thread in Root Nodules of Soybean. Cytobios 42:41-47.

5. Baleja, J.D., Marmorstein, R., Harrison, S.C. and Wagner, G.J. 1992. Solution Structure of the DNA-Binding Domain of Cd2-GAL 4 from *S. cerevisiae*. Nature 356:450-453.

6. Banfalvi, Z., Nieuwkoop, A., Schell, M. and Stacey, G. 1988. Regulation of *nod* gen expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214:420-424.

7. Barbour, W.M., Wang, S.P. and Stacey, G. 1992. Molecular Genetics of *Bradyrhizobium* Symbioses. Pages 648-684 in: Biological Nitrogen Fixation, Stacey, G., Burris, R.H. and Evans, H.J., eds. Chapman and Hall, New York.

8. Bartolf, M., Brennan, E. and Price, C.A. 1980. Partial Characterization of a Cadmium-Binding Protein from the Roots of Cadmium-Treated Tomato. Plant Physiol. 66:438-441.

9. Bassarab, S., Mellor, R.B. and Werner, D. 1986. Evidence for Two Types of Mg++ ATPase in the Peribacteroid Membrane from *Glycine max* Nodules. Endocytobiosis and Cell Res. 3:189-196.

10. Bernard, A., Chang, A.C., El-Amamy, M.M., et al. 1986. *Cadmium*, pp. 1-396. Springer-Verlag, Berlin.

11. Beton, D.C., Andrews, G.S., Davies, H.J., Howells, L. and Smith, G.F. 1966. Acute Cadmium Fume Poisoning: Five Cases with One Death from Renal Necrosis. Br. J. Ind. Med. 23:292-301.

12. Blejer, H.P. 1966. Death Due to Cadmium Oxide Fumes. Ind. Med. Surg. 35:363-364.

13. Callaham, D.A. and Torrey, J.G. 1981. The Structural Basis for Infection of Root Hairs of *Trifolium repens* by *Rhizobium*. Can. J. Bot. 59:1647-1664.

14. Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A. and Bauer, W.D. 1984. Anatomical Analysis of the Development and Distribution of *Rhizobium* Infections in Soybean Roots. Can. J. Bot. 62:2375-2384.

15. Casterline, J.L. and Barnett, N.M. 1977. Isolation and Characterization of Cadmium-Binding Components in Soybean Plants. Plant Physiol. 59 suppl.:124.

16. Casterline, J.L. and Barnett, N.M. 1982. Cadmium-binding Components in Soybean Plants. Plant Physiol. 69:1004-1007.

17. Cataldo, D.A., Garland, T.R. and Wildung, R.E. 1983. Cadmium Uptake Kinetics in Intact Soybean Plants. Plant Physiol. 73:844-848.

18. Chandler, M.R. 1978. Some Observations on Infection of *Arachis hypogaea* L. by *Rhizobium*. J. Exp. Bot. 29:749-755.

19. Chandler, M.R., Date, R.A. and Roughley, R.J. 1982. Infection and Root-Nodule Development in *Stylosanthes* Species by *Rhizobium* . J. Exp. Bot. 33:47-57.

20. Chao, S.H., Suzuki, Y., Zysk, J.R. and Cheung, W.Y. 1984. Activation of Calmodulin by Various Metal Cations as a Function of Ionic Radius. Mol. Pharm. 26:75-82.

21. Dart, P. 1977. Infection and Development of Leguminous Nodules. Pages367-472 in: A Treatise on Dinitrogen Fixation, Hardy, R.W.F. and Silver, W.S., eds. John Wiley & Sons, New York.

22. Davis, R.D. 1984. Cadmium - a Complex Environmental Problem : Part II Cadmium in Sludges Used as Fertilizer. Experientia 40:117-234.

23. Dobson, S. 1992. *Cadmium-Environmental Aspects*, pp. 1-156.World Health Organization, Geneva.

24. Dunlap, J.R. 1998. Personal communication.

25. Dunphy, B. 1967. Acute Occupational Cadmium Poisoning: a Critical Review of the Literature. J. Occup. Med. 9:22-26.

26. Elinder, C.G., Edling, C., Lindberg, E., Kagedal, B. and Vesterberg, O. 1985. Assessment of Renal Function in Workers Previously Exposed to Cadmium. Br. J. Ind. Med. 42:754-760.

27. Fernandez-Pinas, F., Mateo, P. and Bonilla, I. 1995. Ultrastructural Changes Induced by Selected Cadmium Concentrations in the Cyanobacterium *Nostoc* UAM208. J. Plant Physiol. 147:452-456.

28. Firmin, J.L., Wilson, K.E., Rossen, L. and Johnston, A.W.B. 1986. Flavonoid Activation of Nodulation Genes in *Rhizobium* Reversed by other Compounds Present in Plants. Nature 324:90-92.

29. Fortin, M.G., Zelechowska, M. and Verma, D.P.S. 1985. Specific Targeting of Membrane Nodulins to the Bacteroid-enclosing Compartment in Soybean Nodules. EMBO J. 4:3041-3046.

30. Franssen, H.J., Vijn, I., Yang, W.C., Bisseling, T. 1992. Developmental Aspects of the Rhizobium-legume Symbiosis. Plant Mol. Biol. 19:89-107.

31. Friberg, L. 1950. Health Hazards in the Manufacture of Alkaline Accumulators with Special Reference to Chronic Cadmium Poisoning. Acta Med. Scand. 138:1-124.

32. Friberg, L., Elinder, C.G. and Kjellstrom, T. 1992. *Cadmium*, pp. 1-280.World Health Organization, Geneva.

33. Friberg, L., Piscator, M., Nordberg, G. and Kjellstrom, T. 1974. *Cadmium in the environment*, pp. 1-248.CRC Press, Cleveland.

34. Garbers, C., Menkbach, R., Mellor, R.B. and Werner, D. 1988. Protease (Thermolysin) Inhibition Activity in the Peribacteroid Space of *Glycine max* Root Nodules. J. Plant Physiol. 132:442-445.

35. Gaworzewska, E. and Carlile, M. 1982. Positive Chemotaxis of *Rhizobium leguminosarum* and Other Bacteria Towards Root Exudates from Legumes and Other Plants . J. Gen. Microbiol. 128:1179-1188.

36. Goodchild, D.J. and Bergersen, F.J. 1966. Electron Microscopy of the Infection and Subsequent Development of Soybean Nodule Cells. J. Bact. 92:204-213.

37. Gresshoff, P.M. and Jiang, Q. 1997. Classical and Molecular Genetics of the Model Legume *Lotus japonicus*. MPMI 10:59-68.

38. Grill, E., Gekeler, W., Winnacker, E.L. and Zenk, H.H. 1986. Homo-Phytochelatins are Heavy Metal-Binding Peptides of Homo-Glutathione Containing Fabales. FEBS Letters 205:47-50.

39. Grill, E., Winnacker, E.L. and Zenk, M.H. 1985. Phytochelatins: The Principal Heavy-Metal Complexing Peptides of Higher Plants. Science 230:674-676.

40. Grill, E., Winnacker, E.L. and Zenk, M.H. 1987. Phytochelatins, a Class of Heavy-Metal-Binding Peptides from Plants, are Functionally Analogous to Metallothioneins. Proc. Natl. Acad. Sci. 84:439-443.

41. Halverson, L.J. and Stacey, G. 1986. Signal Exchange in Plant-Microbe Interactions. Microbiol. Rev. 50:193-225.

42. Handberg, K. and Stougaard, J. 1992. *Lotus japonicus,* an autogamous, diploid legume species for classical and molecular genetics. Plant J. 2:487- 496.

43. Hawkes, F. and Kihn, Y. 1989. Action of cadmium on mitochondria: CTEM and EELS studies in two orthopteran insects during meiosis and spermiogenesis. Cytobios 57:43-54.

44. Hinkle, P.M., Kinsella, P.A. and Osterhoudt, K.C. 1987. Cadmium Uptake and Toxicity via Voltage-Sensitive Calcium Channels. J. Biol. Chem. 262:16333-16337.

45. Hollis, A., Kloos, W. and Elkan, G. 1981. DNA:DNA Hybridization Studies of *Rhizobium japonicum* and Related *Rhizobiaceae*. J. Gen. Microbiol. 123:215-222.

46. Huang, B., Hatch, E. and Goldsbrough, P.B. 1987. Selection and characterization of Cadmium Tolerant Cells in Tomato. Plant Sci. 52:211-221.

47. Inouhe, M., Ninomiya, S., Tohoyama, H., Joho, M. and Murayama, T. 1994. Different Characteristics of Roots in the Cadmium-Tolerance and Cd-binding Complex Formation Between Mono- and Dicotyledonous Plants. J. Plant Res. 107:201-207.

48. Jackson, P.J., Unkefer, C.J., Doolen, J.A., Watt, K. and Robinson, N.J. 1987. Poly (gamma-glutamylcysteinyl) Glycine: Its Role in Cadmium Resistance in Plant Cells. Proc. Natl. Acad. Sci. 84:6619-6623.

49. Jarvis, B.D.W., Pankhurst, C.E. and Patel, J.J. 1982. *Rhizobium loti,* a New Species of Legume Root Nodule Bacteria. Int. J. Syst. Bacteriol. 32:378-380.

50. Jarvisalo, J.O., Kilpio, J. and Saris, N.E.L. 1980. Toxicity of Cadmium to Renal Mitochondria when Administered in Vivo and in Vitro. Environ. Res. 22:217-223.

51. Jiang, Q. and Gresshoff, P.M. 1993. *Lotus japonicus* - a model plant for structure-function analysis in nodulation and nitrogen fixation. Pages 97-110 in: Plant Biotechnology and Development, Gresshoff, P.M., ed. CRC Press, Boca Raton.

52. Jordan, D.C. 1982. Transfer of Rhizobium japonicum Buchanan 1980 to Bradyrhizobium gen. nov., a Genus of Slow-Growing, Root Nodule Bacteria from Leguminous Plants. Int.J.Syst.Bacteriol. 32:136-139.

53. Kang, S. and Wells, R.D. 1994. Zinc Destabilizes DNA Watson-Crick Pairs at AGCT. J. Biol. Chem. 269:9528-9532.

54. Kannenberg, E.L. and Brewin, N.J. 1989. Expression of a Cell Surface Antigen from *Rhizobium leguminosarum* 3481 is Regulated by Oxygen and pH. J. Bact. 171:4543-4548.

55. Karez, C., Allemand, D., Renzis, G., Gnassia-Barelli, M., Romeo, M. and Puiseux-Dao, S. 1990. Ca-Cd Interaction in the Prymnesiophyte *Cricosphaera elongata*. Plant Cell Environ. 13:483-487.

56. Kazantzis, G. 1979. Renal Tubular Dysfunction and Abnormalities of Calcium Metabolism in Cadmium Workers. Environ. Health Perspect. 28:155-160.

57. Kazantzis, G., Flynn, F.V., Spowage, J.S. and Trott, D.G. 1963. Renal Tubular Malfunction and Pulmonary Emphysema in Cadmium Pigment Workers. Q. J. Med. 32:165-192.

58. Kieliszewski, M. and Lamport, D.T.A. 1994. Extensin: Repetitive Motifs, Functional Sites, Post-Translational Codes, and Phylogeny. Plant J. 5:157- 172.

59. Kijne, J.W. 1992. The *Rhizobium* Infection Process. Pages349-398 in: Biological Nitrogen Fixation, Stacey, G., Burris, R.H. and Evans, H.J., eds. Chapman & Hall, New York.

60. Kinnback, A., Mellor, R.B. and Werner, D. 1987. Alpha-mannosidase Isoenzyme II in the Peribacteroid Space of *Glycine max* Root Nodules. J. Exp. Bot. 38:1373-1377.

61. Kjellstrom, T., Lind, B., Linnman, L. and Elinder, C.G. 1975. Variation of Cadmium Concentration in Swedish Wheat and Barley. Arch. Environ. Health 30:321-328.

62. Knowles, S.E. and Ballard, F.J. 1975. Selective Control of the Degradation of Normal and Aberrant Proteins in Reuber H35 Hepatoma Cells. Biochem. J. 156:609-619.

63. Kondo, N., Imai, k., Isobe, M. and Goto, T. 1984. Cadystin A and B, Major Unit Peptides Comprising Cadmium Binding Peptides Induced in a Fission Yeast . Tetrahedron Lett. 25:3869-3872.

64. Kondo, N., Isobe, M., Imai, k., Goto, T. and Murasugi, A. 1983. Structure of Cadystin, the Unit-Peptide of Cadmium-Binding Peptides Induced in a Fission Yeast, *Schizosaccharomyces pombe*. Tetrahedron Lett. 24:925-928.

65. Langlais, M., Tajmir-Riahi, H.A. and Savoie, R. 1990. Raman Spectroscopic Study of the Effects of Ca, Mg, Zn, and Cd Ions on Calf Thymus DNA: Bindings Sites and Conformational Changes. Biopolymers 30:743-752.

66. Leita, L., De-Nobili, M., Mondini, C. and Baca-Garcia, M.T. 1993. Response of Leguminosae to Cadmium Exposure. J. Plant Nutr. 16:2001-2012.

67. Marvel, D., Kuldau, G., Hirsch, A., Richards, E., Torrey, J. and Ausubel, F. 1985. Conservation of Nodulation Genes Between *Rhizobium meliloti* and a Slow-Growing *Rhizobium* Strain that Nodulates a Nonlegume Host. Proc. Natl. Acad. Sci. 82:5841-5845.

68. Mason, H.J., Davison, A.G. and Wright, A.L. 1988. Relations Between Liver Cadmium, Cumulative Exposure, and Renal Function in Cadmium Alloy Workers. Br. J. Ind. Med. 45:793-802.

69. Mathews, C.K. and Van Holde, K.E. 1990. *Biochemistry*, pp. 1-1129.Benjamin/Cummings, Redwood City:

70. Mehra, R.K. and Winge, D.R. 1988. Cu (I) Binding to the Schizosaccharomyces pombe Gamma-Glutamyl Peptides Varying in Chain Lengths. Arch. Biochem. Biophys. 265:381-389.

71. Meister, A. and Anderson, M.E. 1983. Glutathione. Annu. Rev. Biochem. 52:711-60.

72. Mellor, R.B. 1989. Bacteroids in the Rhizobium-legume Symbiosis Inhabit a Plant Internal Lytic Compartment: Implications for Other Microbial Endosymbioses. J. Exp. Bot. 40:831-839.

73. Mellor, R.B. and Werner, D. 1987. Peribacteroid Membrane Biogenesis in Mature Legume Root Nodules. Symbiosis 3:75-100.

74. Mitra, R.S., Gray, R.H., Chin, B. and Bernstein, I.A. 1975. Molecular Mechanism of Accomodation in *E. coli* to Toxic Levels of Cd^{2+} . J. Bacteriol. 121:1180-1188.

75. Mutoh, N. and Hayashi, Y. 1988. Isolation of Mutants of Schizosaccharomyces pombe Unable to Synthesize Cadystin, Small Cadmium-Binding Peptides. Biochem. Biophys. Res. Commun. 151:32-39.

76. Nap, J.P. and Bisseling, T. 1990. Nodulin Function and Nodulin Gene Regulation in Root Nodule Development. Pages 181-229 in: Molecular Biology of Symbiotic Nitrogen Fixation, Gresshoff, P.M., ed. CRC press, Boca Raton.

77. Ndoye, I., DeBilly, F., Vasse, J., Dreyfus, B. and Truchet, G. 1994. Root Nodulation of *Sesbania rostrata*. J. Bact. 176:1060-1068.

78. Newcomb, E.H. and Tandon, S.R. 1981. Uninfected Cells of Soybean Root Nodules: Ultrastructure Suggests Key Role in Ureide Production. Science 212:1394-1396.

79. Newcomb, W. 1976. A Correlated Light and Electron Microscopic Study of Symbiotic Growth and Differentiation in *Pisum sativum* Root Nodules . Can.J.Bot. 54:2163-2186.

80. Newcomb, W. and McIntyre, L. 1981. Development of Root Nodules of Mung Bean (*Vigna radiata*): a Reinvestigation of Endocytosis. Can. J. Bot. 59:2478-2499.

81. Noel, K.D., Stacey, G., Tandon, S.R., Silver, L.E. and Brill, W.J. 1982. Rhizobium japonicum Mutants Defective in Symbiotic nitrogen Fixation. J. Bacteriol. 152:485-494.

82. Nutman, P.S. 1955. The Influence of the Legume in Root-Nodule Symbiosis. Biol. Rev. 109-151.

83. Olsson, J.E. and Rolfe, B.G. 1985. Stem and Root Nodulation of the Tropical Legume *Sesbania rostrata* by *Rhizobium* Strains ORS-571 and WE7. J. Plant Physiol. 121:199-210.

84. Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G. and Ow, D.W. 1992. Heavy Metal Tolerance in the Fission Yeast Requires an ATP-binding Cassette-type Vacuolar Membrane Transporter. EMBO J. 11:3491- 3499.

85. Patel, J.J. and Yang, A.F. 1981. Light and Electron Microscopic Studies of Nodule Structure of Alfalfa. Can. J. Microbiol. 27:36-43.

86. Paterson, D.M. and Wright, S.J.L. 1988. Diffusion gradient plates for herbicide toxicity tests on micro-algae and cyanobacteria. Letters Appl. Microbiol. 7:87-90.

87. Premakumar, R., Winge, D.R., Wiley, R.D. and Rajagopalan, K.V. 1975. Copper-Chelatin: Isolation from Various Eucaryotic Sources. Arch. Biochem. Biophys. 170:278-288.

88. Price, G.D., Mohapatra, S.S. and Gresshoff, P.M. 1984. Structure of Nodules Formed by *Rhizobium* strain ANU289 in the nonlegume *Parasponia* and the Legume Siratro (*Macroptilium atropurpureum*). Bot. Gaz. 145:444-451.

89. Rae, A.L., Bonfante-Fasolo, P. and Brewin, N.J. 1992. Structure and Growth of Infection Threads in the Legume Symbiosis with *Rhizobium leguminosarum*. Plant J. 2:385-395.

90. Rahola, T., Aaran, R.K. and Miettinen, J.K. 1972. Half-Time Studies of Mercury and Cadmium by Whole-Body Counting. Pages553-562 in: Assessment of Radioactive Contamination in Man, International Atomic Energy Agency Unipublishers, New York.

91. Rauser, W.E. 1990. Phytochelatins. Annu. Rev. Biochem. 59:61-86.

92. Rauser, W.E. and Ackerley, C.A. 1987. Localization of Cadmium in Granules Within Differentiating and Mature Root Cells. Can. J. Bot. 65:643-646.

93. Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. 1986. Flavones Induce Expression of Nodulation Genes in *Rhizobium*. Nature 323:632-635.

94. Robertson, J.G. and Lyttleton, P. 1982. Coated and Smooth Vesicles in the Biogenesis of Cell Walls, Plasma Membranes, Infection Threads and Peribacteroid Membranes in Root Hairs and Nodules of White Clover. J. Cell Sci. 58:63-78.

95. Rolfe, B.G. and Gresshoff, P.M. 1988. Genetic Analysis of Legume Nodule Initiation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:297-319.

96. Rossen, L., Shearman, C.A., Johnston, A.W.B. and Downie, J.A. 1985. The *nodD* Gene of *Rhizobium leguminosarum* is Autoregulatory and in the Presence of Plant Exudate Induces the *nodA,B,C* Genes. EMBO J. 4:3369-3373.

97 Roth, L.E. 1998. Personal communication.

98. Roth, L.E., Dunlap, J.R. and Stacey, G. 1987. Localizations of Aluminum in Soybean Bacteroids and Seeds. Appl. Environ. Microbiol. 53:2548-2553.

99. Roth, L.E. and Stacey, G. 1989. Bacterium Release into Host Cells of Nitrogen-fixing Soybean Nodules: the Symbiosome Membrane comes from Three Sources. Euro.J.Cell Bio. 49:13-23.

100. Russ, J.C. 1984. Thin Sections in STEM and TEM. Pages164-173 in: Fundamentals of Energy Dispersive X-ray Analysis, Ashby, M., Charles, J., Evans, A.G., et al, eds. Butterworths, London.

101. Salt, D. and Wagner, G.J. 1993. Cadmium Transport Across Tonoplast of Vesicles from Oat Roots: Evidence for a $Cd^{2(+)}$ /$H^{(+)}$ Antiport Activity. J. Biol. Chem. 268:12297-12302.

102. Schirmer, R.H. and Krauth-Siegel, R.L. 1989. Glutathione Reductase. Pages 553-596 in: Glutathione: Chemical, Biochemical and Medical Aspects Part A, Dolphin, D., Poulson, R. and Avramovic, O., eds. Wiley, New York.

103. Schmidt, J., Wingender, R., John, M., Wieneke, U. and Schell, J. 1988. *Rhizobium meliloti* nod A and nod B genes are Involved in Generating Componds that Stimulate Mitosis of Plant Cells. Proc. Natl. Acad. Sci. 85:8578-8582.

104. Scott, K.F. and Bender, G.L. 1990. The *Parasponia-Bradyrhizobium* Symbiosis. Pages 231-250 in: Molecular Biology of Symbiotic Nitrogen Fixation, Gresshoff, P.M., ed. CRC Press, Boca Raton .

105. Selker, J.M.L. and Newcomb, E.H. 1985. Spatial Relationships Between Uninfected and Infected Cells in Root Nodules of Soybean. Planta 165:446-454.

106. Sen, D. and Weaver, R. 1984. A Basis for Different Rates of N2 [nitrogen]-Fixation by the same Strains of *Rhizobium* in Peanut and Cowpea Root Nodules. Plant Sci. Lett. 34:239-246.

107. Sherrier, D.J. and Vandenbosch, K.A. 1995. Localization of Repetitive Proline-rich Proteins in the Extracellular Matrix of Pea Root Nodules. Protoplasma 183:148-161.

108. Silverberg, B.A. 1976. Cadmium-Induced Ultrastructural Changes in Mitochondria of Freshwater Green Algae. Phycologia 15:155-159.

109. Spaink, H.P., Bakkers, J., Bladergroen, M., et al. 1996. The Molecular Basis of Host Specificity of *Rhizobia*. Pages 299-306 in: Biology of Plant-Microbe Interactions, Stacey, G., Mullin, B. and Gresshoff, P.M., eds. International Society for Molecular Plant-Microbe Interactions, St. Paul .

110. Steffens, J.C., Hunt, D.F. and Williams, B.G. 1986. Accumulation of non-protein metal-binding polypeptides (gamma-glutamyl-cysteinyl)n-glycine in selected cadmium-resistant tomato cells. J. Biol. Chem. 261:13879-13882.

111. Stohs, S.J. and Bagchi, D. 1995. Oxidative Mechanisms in the Toxicity of Metal Ions. Free Radic. Biol Med. 18:321-336.

112. Szymanska, E. and Laskowska Klita, T. 1993. Effect of Cadmium (CdCl2) on Lipid Peroxidation and Activities of Antioxidant Enzymes. Acta Biochim. Poland 40:144-146.

113. Torrey, J.G. 1976. Initiation and Development of Root Nodules [caused by an unidentified actinomycete-like soil microorganism] of *Casuarina* [*cunninghamiana*] (*Casuarinaceae*). Amer. J. Bot. 63:335-344.

114. Trinick, M.J., Miller, C. and Hadobas, P.A. 1991. Formation and Structure of Root Nodules Induced on *Macroptilium atropurpureum* Inoculated with Various Species of *Rhizobium*. Can. J. Bot. 69:1520-1532.

115. Turgeon, B.G. and Bauer, W.D. 1985. Ultrastructure of Infection-thread Development During the Infection of Soybean by *Rhizobium japonicum*. Planta 163:328-349.

116. Udvardi, M.K., Lister, D.L. and Day, D.A. 1991. ATPase Activity and Anion Transport Across the Peribacteroid Membrane of Isolated Soybean Symbiosomes. Arch. Micro. 156:362-366.

117. Vogeli-Lange, R. and Wagner, G.J. 1990. Subcellular Location of Cadmium and Cadmium-Binding Peptides in Tobacco Leaves. Implication of a Transport Function for Cadmium-Binding Peptides. Plant Physiol. 92:1086-1093.

118. Wagner, G.J. 1993. Accumulation of Cadmium in Crop Plants and its Consequences to Human Health. Adv. Agron. 51:173-212.

119. Wagner, G.J. and Trotter, M.M. 1982. Inducible Cadmium Binding Complexes of Cabbage and Tobacco. Plant Physiol. 69:804-809.

120. Wang, J., Evangelou, B.P., Nielsen, M.T. and Wagner, G.J. 1991. Computer Simulated Evaluation of Possible Mechanisms for Quenching Heavy Metal Ion Activity in Plant Vacuoles. Plant Physiol. 97:1154-1160.

121. Wang, J., Evangelou, B.P., Nielsen, M.T. and Wagner, G.J. 1992. Computer Simulated Evaluation of Possible Mechanisms for Sequestering Metal Ion Activity in Plant Vacuoles. Plant Physiol. 99:621-626.

122. Werner, D. 1992. Physiology of Nitrogen-Fixing Legume Nodules: Compartments and Functions. Pages 399-431 in: Biological Nitrogen Fixation, Stacey, G., Burris, R.H. and Evans, H.J., eds. Chapman and Hall, New York.

123. Wilson, D. 1997. Throughout the Country: Example after Example of hazardous Wastes Being Turned into Fertilizer. Seattle Times July 4

124. Wilson, D. 1997. Here's What's Known, and not Known, about Toxics, Plants and Soil. Seattle Times July 3

125. Wilson, D. 1997. Fear in the Fields - How Hazardous Wastes Become Fertilizer. Seattle Times July 3

126. Wilson, D. 1997. Fear in the Fields - McDermott Drafting Bill to Regulate Fertilizers. Seattle Times July 13

2. *Lotus*

2.1 Abstract

Lotus japonicus has been proposed as a model system for the study of plants that form determinate nodules and is now in the early stages of use as a model. Several groups have begun to describe the genetics and gross morphology of the plant.

L. japonicus nodules share many characteristics with other determinate-type nodules. The cells of most nodules are arranged into distinct functional zones, though this organization differs markedly between determinate and indeterminate nodules. Most authors rely on a set of what are believed to be standard characteristics of a given nodule type when studying their experimentally altered plants to determine what if any effect their experimental conditions had on the plant. This is especially important in the use of model systems. Over the course of time, initially reported characteristics become accepted as rule, and any variation on them is considered to have been induced by the experiment. I show in this study that many of these so called "typical" features represent only one of a range of possibilities and furthermore believe that descriptions of these characteristics should reflect this fact. For example, infection threads of *L. japonicus* are usually broad and resemble those found in alfalfa, which forms indeterminate nodules, while most threads found in soybean and cowpea are thin. However, *L. japonicus* can form single file infection threads, and broad threads have been observed in soybean.

However, some characteristics are unique and invariant in a given species and several were observed in which *L. japonicus* differs from soybean. First, *L. japonicus* roots contain numerous cells with large tannin deposits not found in the roots of soybean or cowpea. Second, the infected cells of *L. japonicus* have large central vacuoles notably absent from healthy soybean nodules. Third, the prominent scleroid layer found in soybean nodules is absent in *L. japonicus*. I support *L. japonicus* as a viable model

system but feel that natural inter- as well as intra-species variations must be acknowledged and discussed.

Additional keywords: nitrogen fixation, symbiosis, symbiosome, tannins.

2.2 Introduction

The study of cellular biology has been greatly enhanced through the use of model systems. As examples related to plant-microbe interactions, studies of *Escherichia coli* and *Arabidopsis thaliana* (13, 14) have contributed immensely to our understanding of basic biology and have served as benchmarks to which novel discoveries can be compared.

However, model systems have limitations. For example, though *A. thaliana* has many very good general-model characteristics, it cannot form nodules(12). Therefore, recent interest has been mounting to develop a useful plant models to study nodulation.

Much research into the cell biology of nodule-forming plants has been done on such varied species as soybean, pea, and cowpea (cf. 25, 16, 18, 5, 2), but work on such a wide collection of plant species divides scarce resources and may slow the progress of basic studies into the nodulation process. A model system capable of nitrogen fixation that also embodies many of the characteristics of *A. thaliana* is needed and would accelerate research progress (12). *L. japonicus* has been proposed as such a model system for the study of plants that form determinate type nodules (10).

Many of the characteristics of this plant such as small genome size and genomic transformation via *Agrobacterium tumefaciens* lend themselves to genetic manipulation. Other features such as a short generation time (from seed to flowering plant in seven weeks) and prolific seed production (as many as 6000 seeds/ plant) are further advantages (10).

In the present study, I examined nodules produced on *L. japonicus* roots by infection with two *Bradyrhizobium* spp. strains of bacteria and two *Rhizobium* spp. strains most often used for molecular-biology studies of *L. japonicus* nodulation. My data show that these *Rhizobium* spp. strains are effective in inducing the formation of nodules, but

some observed characteristics, such as a high degree of infected-cell vesiculation throughout nodule development and the presence of phenolic compounds in the nodule and root near the nodule, raise questions about the compatibility of these strains with *L. japonicus*.

My studies have yielded three major results. First, I characterized the cell-structure features of this system more fully than has been done previously. Second, I compared nodule differences induced by the several microbial species typically used for nodulation studies. Third, my analysis of these findings has focused on four characteristics that will require special care in interpreting certain types of studies and that will limit the validity of comparisons with other similar nodulation systems.

2.3 RESULTS

2.3.1 Nodules formed by inoculation with strains NZP 2037 or NZP 2235

L. *japonicus* nodules are slightly elliptical to spherical in shape and have a diameter of one to two millimeters when mature (Fig. 1). Nodules are found on both primary and lateral roots, typically in clusters of two to four. Nodules can be divided into five zones based on cell location and morphology (Fig. 2).

Zone one: This outer most band of cells composing the epidermis of the nodule is typically two to four cells deep (Figs. 1 & 2). The cytoplasm in these cells is thin and pressed against their cell walls by a large central vacuole, so that the nucleus and few organelles present protrude into the vacuole space. In some epidermal cells, the wall appears broken and the contents gone, perhaps in the process of being sloughed off the nodule. These cells are comparable to the periderm and outer cortex described for soybean by Parsons and Day (2, 22).

Zone two: This zone forms a thin band around the nodule and is often only one cell deep. Where one cell abuts another, the walls are thickened and contain a dark-staining material similar to a suberized lamella (Figs. 3 & 4). The cytoplasm is thin and pressed against the wall by the tonoplast, that contains amorphous fibrillar material. The larger organelles, such as mitochondria, nucleus and starch-free plastids, extend away from the wall, making cytoplasmic invaginations into the tonoplast. The rough endoplasmic reticulum (RER) forms long sheets often parallel to the cell wall. The cells are elongated and in close contact with the surrounding cells, so that intercellular spaces are notably absent; and in a band intermittently interrupted by single, large, thin-walled cells. This zone occupies roughly the same location as the scleroid cells of soybean (23, 22), but typical scleroid cells are absent in *L. japonicus*.

Plate 1. Wild type *L. japonicus* nodules.

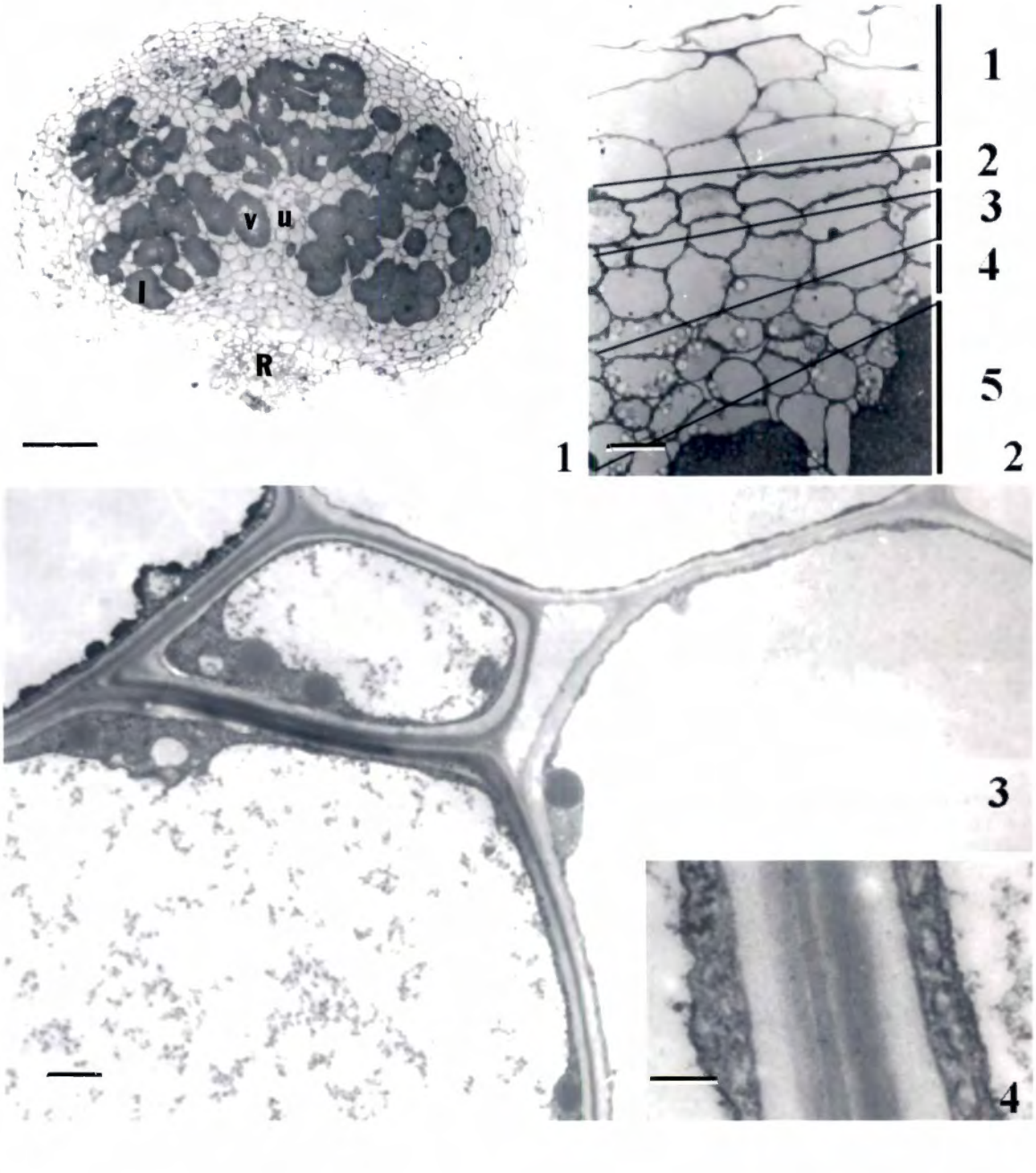
(Figures 1 through 4)

Figure 1. Light micrograph of an *L. japonicus* (NZP-2037) nodule in cross section. Large infected cells (I) containing numerous large central vacuoles (V) can be seen surrounded by smaller uninfected cells (U). A portion of the root (R) is at the bottom to provide orientation. bar = 80 μm

Figure 2. Magnified image of previous nodule with zones one to five marked. Note the darkened walls of the cells in zone two and numerous starch granules in zone four. bar = 40 μm

Figure 3. Electron micrograph showing cells of zones one (upper), two and three (lower). Note the dark material deposited along the wall of the zone-two cell.
bar = 1 μm

Figure 4. Enlarged image of zone two cell wall showing dark osmophilic material embedded in the walls. bar = 0.2 μm


v u
l
R
1
2
3
4
5
1
2
3
4

Zones three & four: Cells are similar to one another in size and shape, occurring three to seven cells deep (Figs. 1 & 2). They are more polyhedral in shape, and their walls lack any noticeable secondary thickening. The cytoplasm of these cells is also pressed against the cell wall by a large tonoplast. Intercellular spaces and starch are noticeably absent in zone three but present in zone four. The nodule often contains few vascular bundles, which are located between zones three and four. Zones two, three, and four are comparable to the inner cortex of soybean described by Parsons and Day (23, 22).

Zone five: The largest and innermost portion of the nodule is composed of both infected and uninfected cells, the latter being distributed throughout zone five, and as with cells of the inner and outer cortex, the majority of their cellular volume is filled by the tonoplast that presses the cytoplasm against thin walls. These cells are typically three to five times smaller than the surrounding infected cells and often contain several starch granules in well developed amyloplasts (Figs. 1, 5 & 6).

Unlike all other cells in the nodule, most of the volume of infected cells contain cytoplasm and symbiosomes. Vacuoles do appear, however, the larger often located perinuclearly (Figs. 5, 6 & 11). The function of these vacuoles is unknown, but they are notably absent from some healthy wild-type determinate-nodulating plants such as soybean, while present in others like cowpea. The nucleus is centrally located, enlarged, and typically contains one or more prominent nucleoli. Moderate amounts of RER and Golgi bodies are distributed throughout the cytoplasm, while the mitochondria and plastids are located at the periphery of the cell, a pattern typical of infected cells in other species.

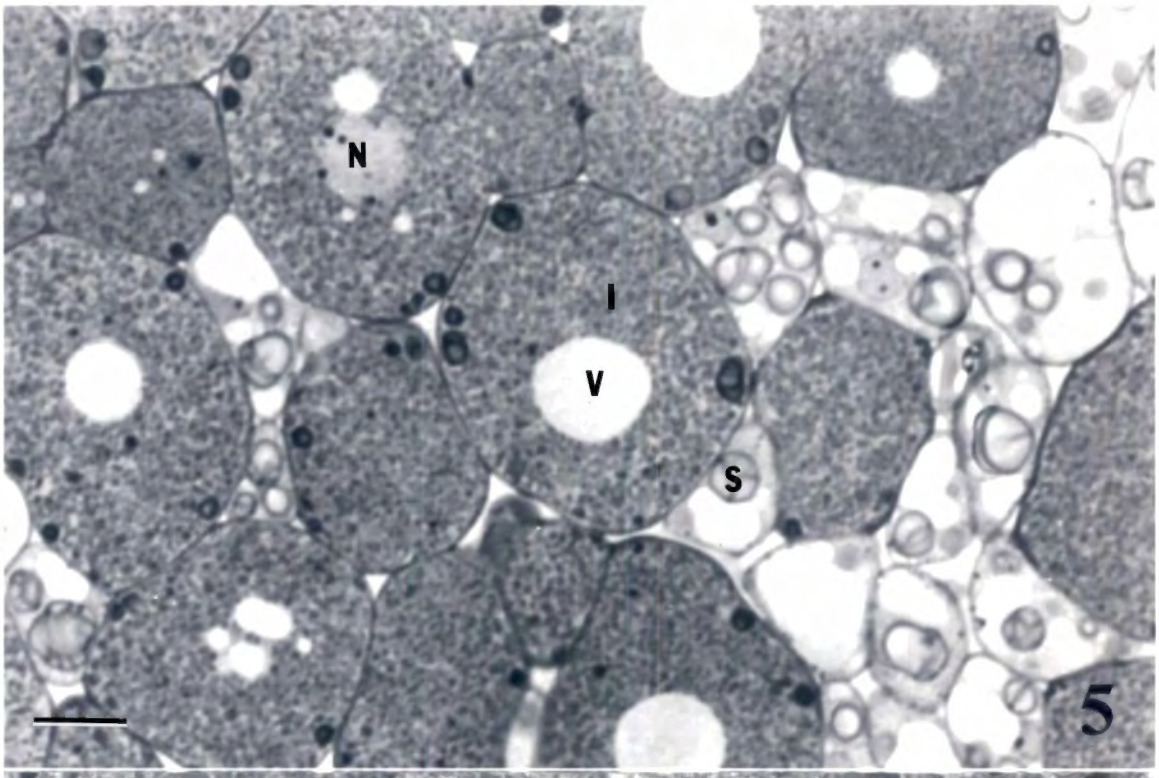
The symbiosomes are evenly distributed throughout the cytoplasm, and most contain one to three bacteroids, though as many as six have been observed. The unbranched *R. loti* bacteroids were devoid of polyphosphate bodies (PPB) but often contain poly β-hydroxybutyrate (PHBA) at the ends of their long axis (Fig. 9).

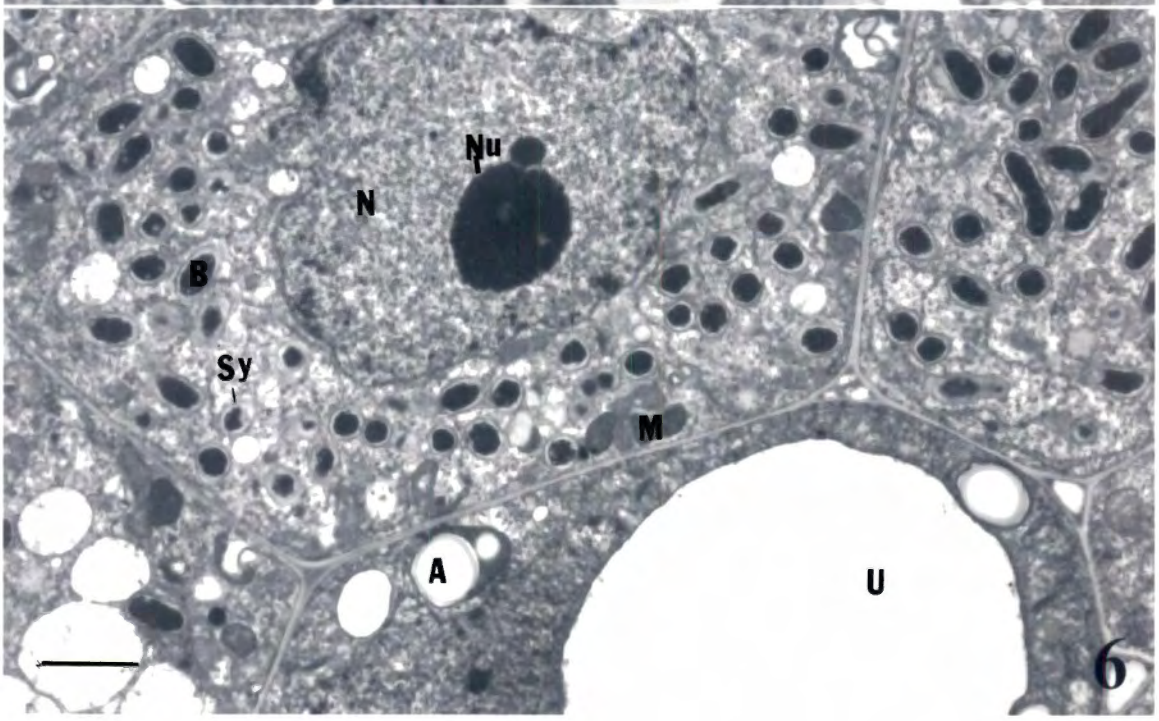
Plate 2. Light and electron micrographs of infected cells.

Figures 5 and 6

Figure 5. Light micrograph of *L. japonicus* (NZP-2235) infected (I) and uninfected cells (U). Note the large central vacuoles (V) in the infected cells as well as numerous starch grains (S) in both infected as well as uninfected cells. Several nuclei (N) can be seen in the infected as well as uninfected cells with prominent nucleoli (Nu). bar = 15 μm

Figure 6. Electron micrograph of infected cells containing numerous released bacteria (B) in symbiosomes. The cytoplasm contains several small vacuoles (V), and mitochondria (M) and plastids (A) at the periphery of the cell in both cell types. A large, central nucleus (N) with prominent nucleolus (Nu) can be seen in the infected cell at the top of the image. The uninfected cell at the bottom of the image contains several starch-filled amyloplasts (A) and a large tonoplast. bar = 2 μm


N
I
V
S
5


Nu
N
B
Sy
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U
6

Both broad and thin infection threads were found in *L. japonicus* (Figs. 7 & 8), and both released bacteria into the host cytoplasm. While thin infection threads were occasionally found, most were broad, round or oval in cross section with as many as nine bacteria in cross section. The total volume of matrix is greater in those nodules infected with NZP 2235 (*L. j.*-2235) than those infected with NZP 2037 (*L. j.*-2037). The broad threads found in *L. japonicus* were similar in structure to those seen in some plants such as alfalfa, which form indeterminate nodules (24). The bacteria inside these threads were tightly packed and filled with PHBA. In addition, persistent threads with PHBA-loaded bacteria could be seen in mature infected cells.

Roots of these plants were much like those of other legumes with one exception. In osmicated tissues, numerous individual cells were seen, each with a very large osmiophilic vacuole (Figs. 13 & 14) that tested positive for tannins when stained with a ferrous chloride stain specific for these compounds (11). Cells containing tannins were distributed throughout the cortex of the root, one or more layers deep beneath the epidermis, but always remained outside the pericycle. No two tannin-filled cells were observed in proximity to one another but were regularly spaced along the root.

2.3.2 Nodules infected by infection with USDA 110 or USDA 3469

I inoculated *L. japonicus* with the *B. japonicum* strain USDA 110 and *Bradyrhizobium* strain USDA 3469. Although USDA 110 failed to stimulate nodule formation, USDA 3469 induced the formation of nodules that exhibited the same zoning pattern as those infected with both *R. loti* strains (Fig. 11). Two types of infected cells were observed. In one type, the bacteria were enclosed only in what appeared to be very large infection threads without evidence of release into host cells (Fig. 12). In the other type, bacteria had been released into the cytoplasm, where some appeared to be in large

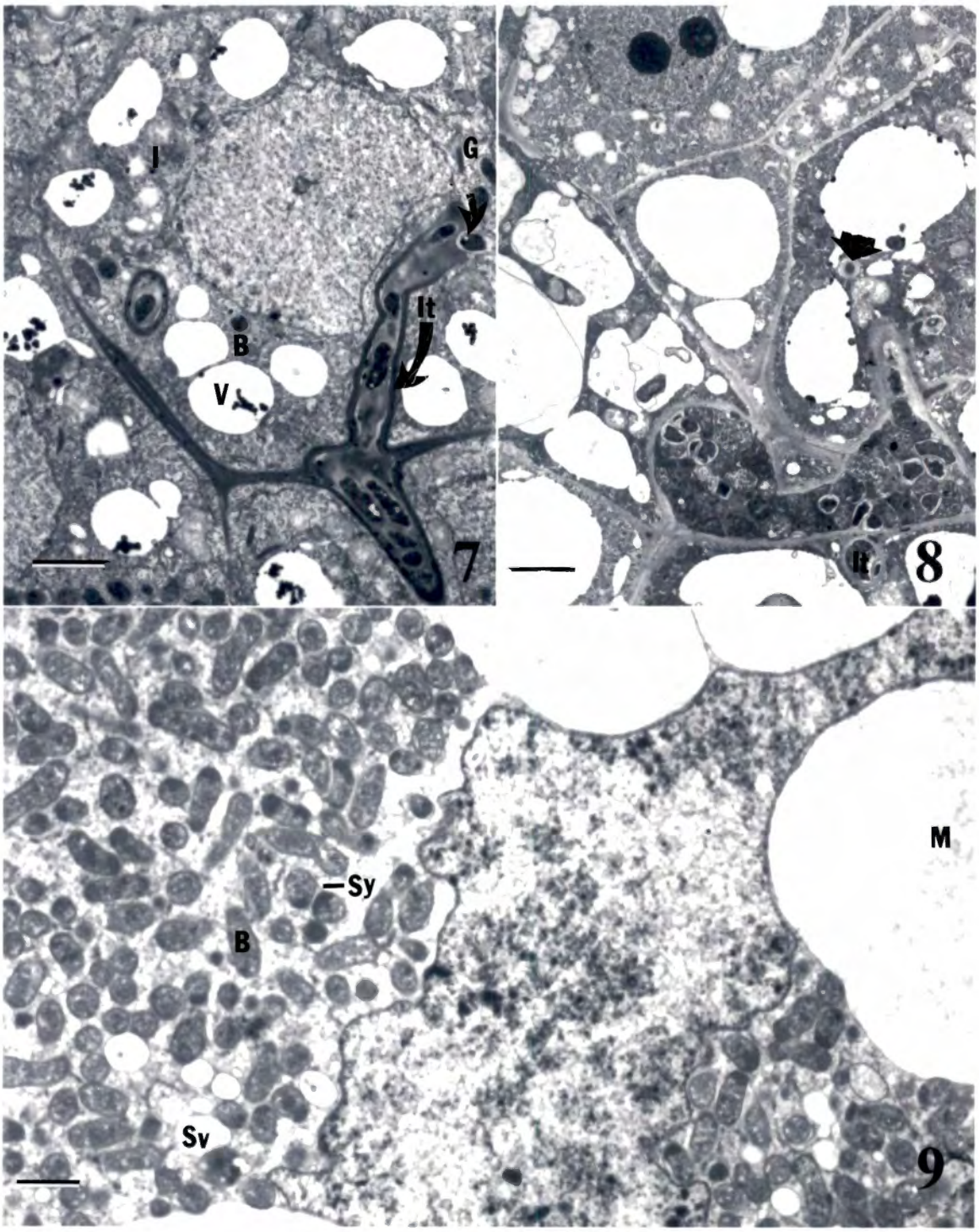
Plate 3. Electron micrographs of *L. japonicus* infected with NZP-2037 and NZP-2235.

Figures 7 through 9

Figure 7. Electron micrograph of *L. japonicus* (NZP-2235) infected cells with two infection threads (It), one of which is showing release (R). The thread is near the nucleus, a condition often associated with release in other legumes such as soybean. Several Golgi bodies (G) can be seen near the release point. In addition, previously released bacteria (B) can be seen elsewhere in the cytoplasm, perhaps endocytosed from another thread. The young infected cell contains numerous large vacuoles (V). bar = 2 μm

Figure 8. Electron micrograph of *L. japonicus* (NZP-2037) early infection. A large thread (It) can be seen in the lower left quadrant of the image. In addition a cross section of a thread where the bacteria are arranged in single file can be seen in the cytoplasm of one cell (arrow). A third thread with three bacteria in cross section can be seen in the cell at the bottom right. bar = 3 μm

Figure 9. Electron micrograph of mature infected cell of *L. japonicus* (NZP-2037). The cytoplasm is filled with numerous bacteroids (B), each contained within its own tight symbiosome membrane (Sy). Numerous small vesicles (Sv) can be seen throughout, and several large vacuoles are visible, each containing amorphous material (M) and located near the nucleus. bar = 2 μm


I
G
It
B
V
7
It
8
M
Sy
B
Sv
9

groups numbering over 50 and surrounded by a symbiosome membrane, while others were free of any membrane and lying loose in a disorganized cytoplasm (Fig. 11). The bacteria were short, contained PPB, and accumulated PHBA. These nodules often showed signs of senescence.

2.3.3 Characteristics of the formed nodule

The volumetric fractions (area occupied by infected cells vs. the total area within the infected zone) were calculated for *L. j.*-2037, *L. j.*-2235 and *Glycine max* infected with USDA-110 (*G. m.*-110) (Table 1). The infected zone of *G. max* nodules contained a greater percentage of space occupied by infected cells (73%) than either *L. j.*-2037 (54%) or *L. j.*-2235 (67%).

In addition, the numbers of infected and uninfected cells occupying zone five were tabulated (Figs. 15, 16 & 17). *L. j.*-2235 and *G. m.*-110 both contained approximately 50% infected cells, while *L. j.*-2037 only contained about 25% infected cells (Table 1).

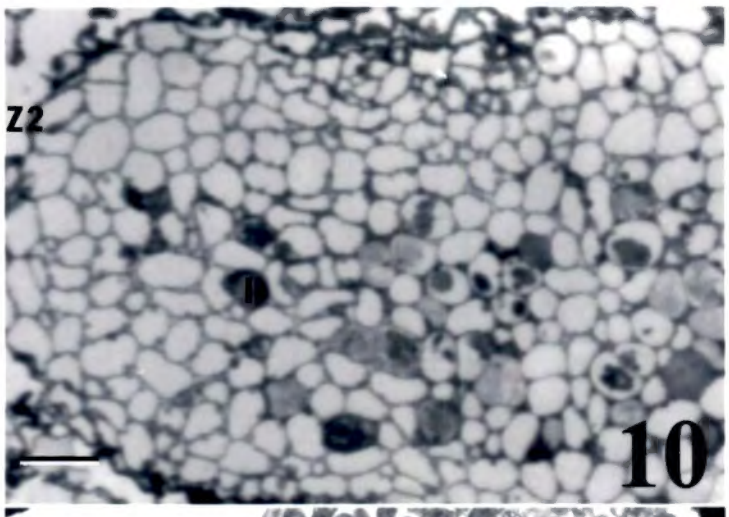
Two statistical procedures were used to analyze the significance of the data. These Kruskal-Wallis test showed that the volumetric levels differed significantly yielding a X^2 (df=2) = 8.91 and giving a p value of 0.012. To determine which of the three levels (*L. j.*-2037, *L. j.*-2235, *G. m.*-110) differed, a Mann-Whitney test was used. Values for *L. j.*-2037 and *G. m.*-110 differed significantly providing a p value of 0.004. All analysis was done with the assistance of the University of Tennessee statistical analysis team in room 200 of the Stokely Management building.

Plate 4. Light and electron micropraphs of *L. japonicus* infected with USDA-3469.

Figures 10 and 11

Figure 10. Light micrograph of an *L. japonicus* (USDA-3469) infected nodule. The general organization matches that reported for NZP-2037 and NZP-2235 infected nodules, but it is obvious that infection is incomplete, since only a few cells contain released bacteria. The darkened walls of zone two cells (Z2) can be seen along the periphery of the nodule. It is unlikely that these nodules were capable of fixing nitrogen. bar = 56 μm

Figure 11. Electron micrograph of an infected cell of *L. japonicus* (USDA-3469) with many bacteria (B) free in the remnants of cytoplasm. Some groups are still contained within a membrane-bound vesicle. bar = 2 μm



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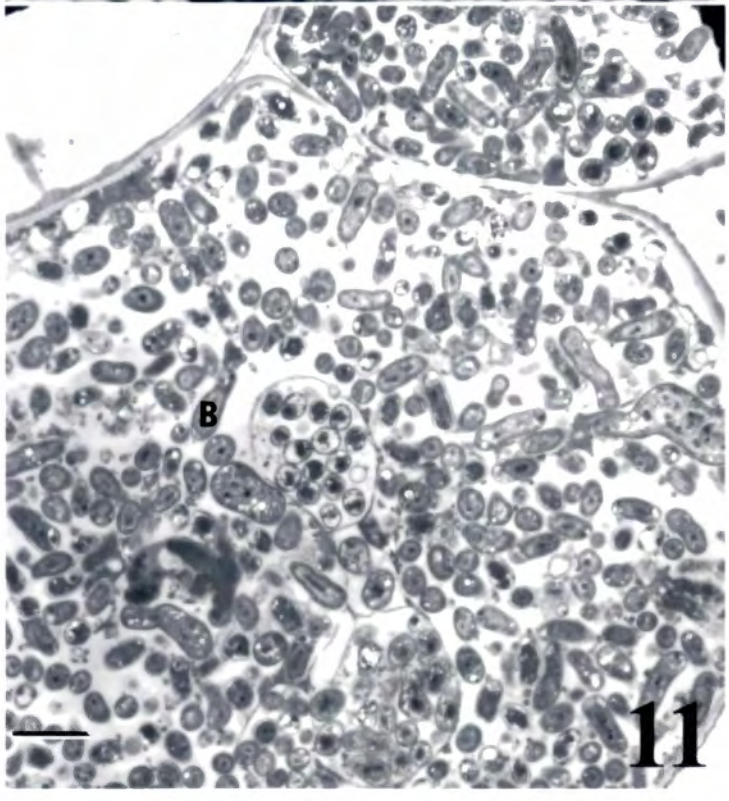


11

Figure 12. Electron micrograph showing remains of an infected cell of *L. japonicus* (USDA-3469) in which the bacteria are enclosed within what appear to be very large infection threads (I), or vacuoles from which no release is occurring.

bar = 0.8 μm

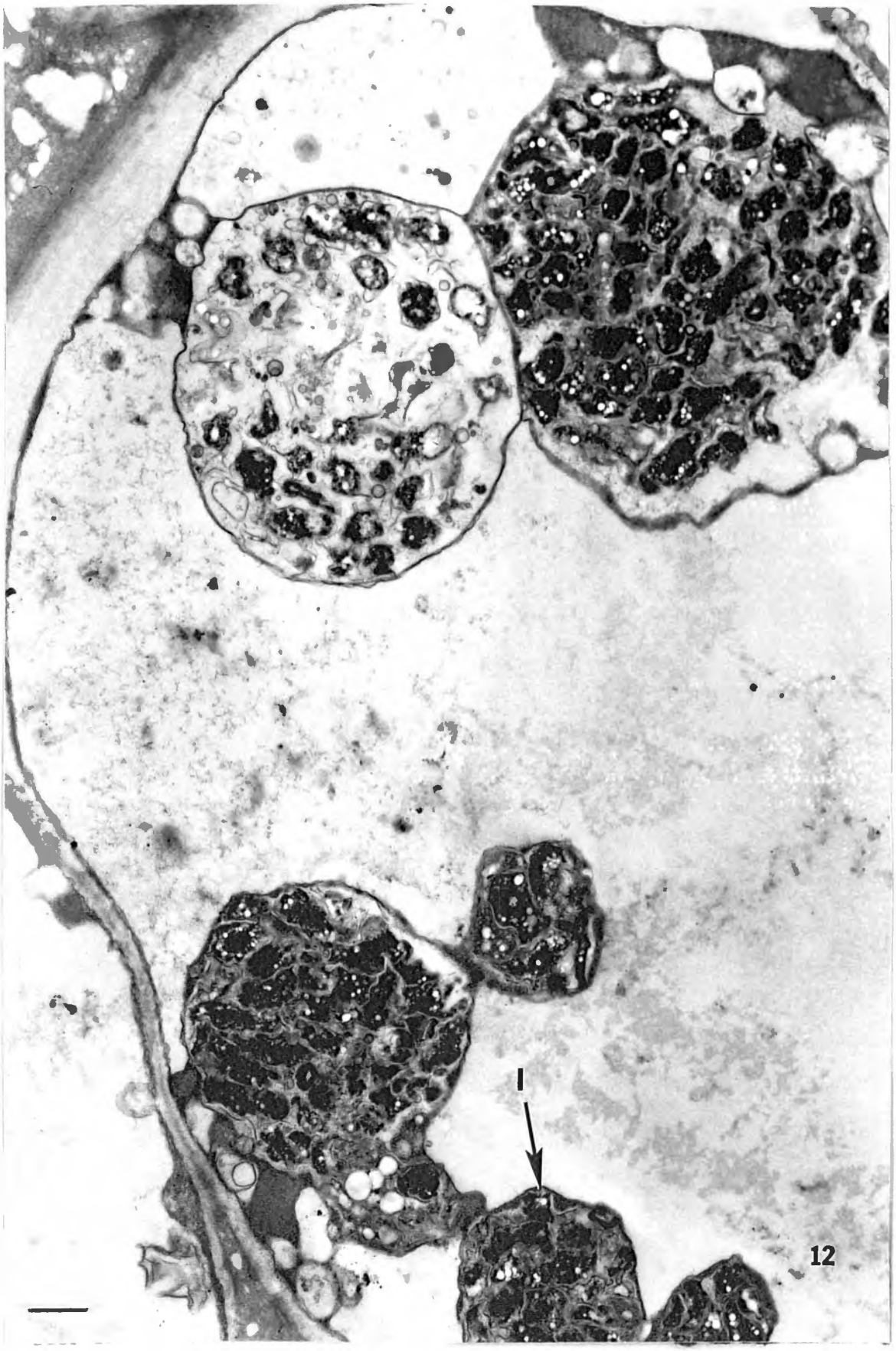

12

Figure 13 Light micrograph of tannin containing cell in the root cortex of *L. japonicus*. bar = 5.5 μm

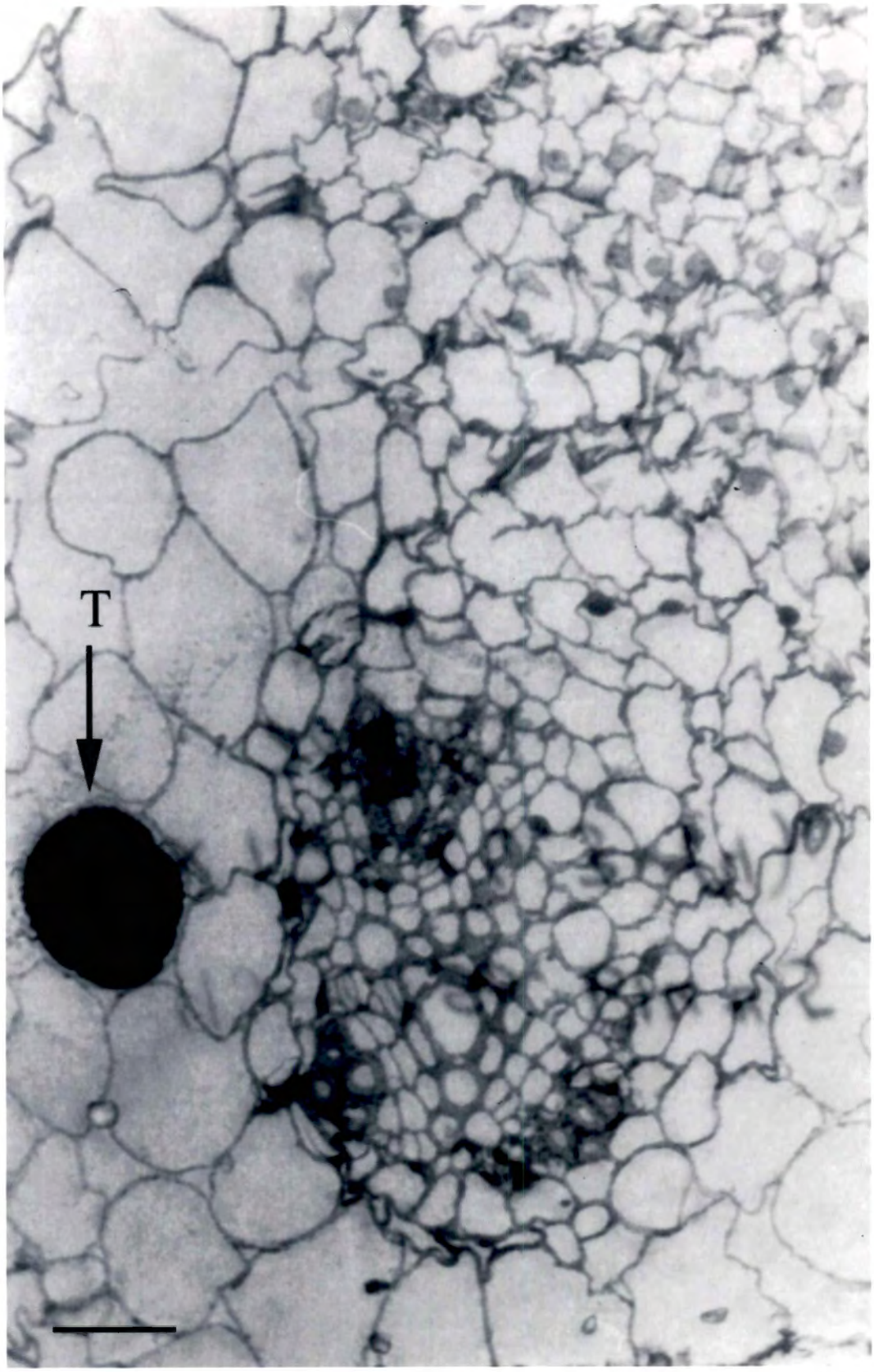

T

Figure 14. Electron micrograph of a similar cell showing that the tannin is contained entirely within the tonoplast. Other cellular organelles as well as cytoplasm can be seen. bar = 0.7 μm


N

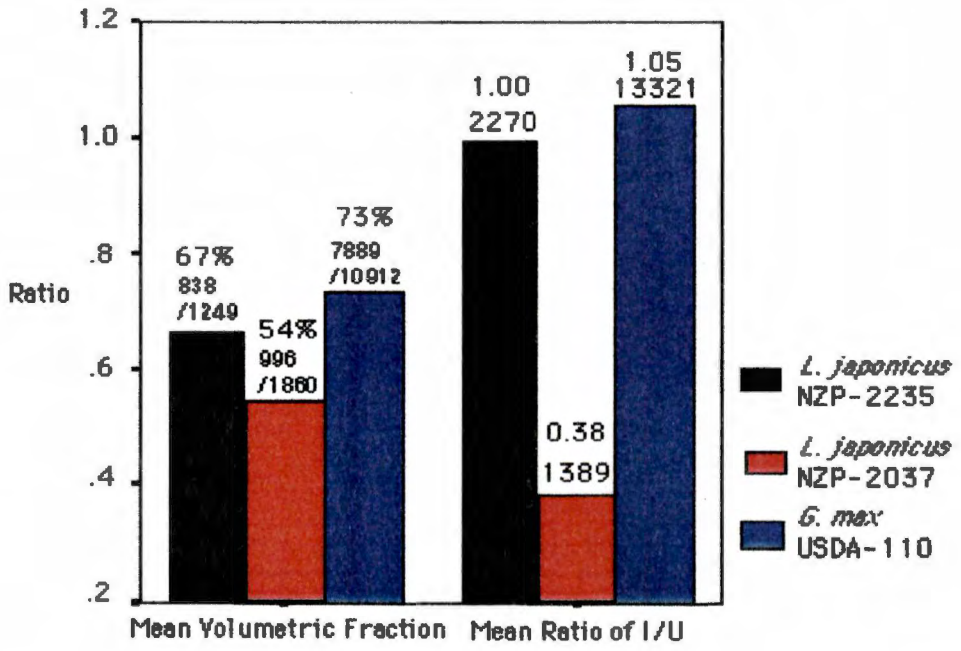


Figure 15. Mean volumetric fraction (left) and mean ratio infected cells (right) (I) to uninfected cells (U). The percentage composition of the infected zone for *G. m.*-110 and *L. j.*-2235 is almost evenly distributed between the infected and uninfected cells. *L. j.*-2037, however, contains a much higher percentage (75%) uninfected cells than infected cells (25%).

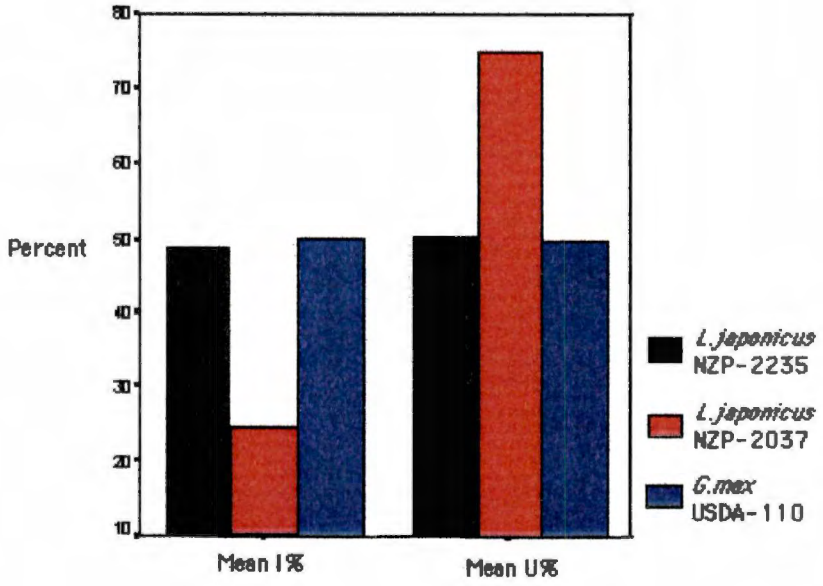


Figure 16. Mean percentage of total infected zone area filled with infected (left) (I) and uninfected cells (right) (U) in *L.japonicus*-NZP-2037, *L. japonicus*-NZP-2235 and *G.max*-USDA-110. The infected zones of *L. j.*-2235 and *G. m.*-110 both contain roughly 50% infected and 50% uninfected cells. *L. j.*-2037, however contains 25% infected cells and 75% uninfected, suggesting that the infected cells of *L. j.*-2037 are larger in size than *G. m.*-110.

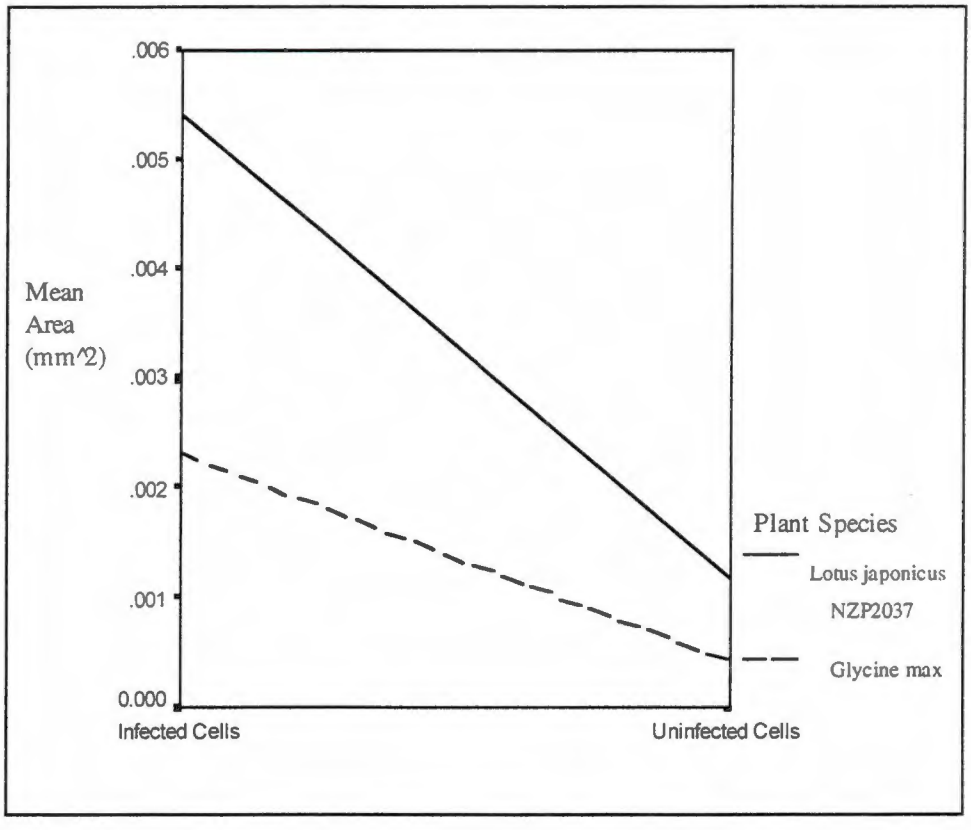


Figure 17. Mean area of infected and uninfected cells in *L. japonicus* - NZP-2037 and *G. max* - USDA-110. Infected and uninfected cells of NZP-2037 infected *L. japonicus* on average occupy a greater area. The size variation is greater between infected cells.

Table 1. Summary statistics from which Figures 13 and 14 were derived.

Plant species	measure	N	Min.	Max.	Mean	Std. Dev.
L. japonicus NZP-2235	Volumetric fraction	6	0.52	0.83	0.67	0.12
	Ratio I/U	6	0.67	1.54	1.00	0.29
	I %	6	40.00	60.69	49.24	6.85
	U %	6	39.31	60.00	50.76	6.85
L. japonicus NZP-2037	Volumetric fraction	10	0.24	0.76	0.54	0.13
	Ratio I/U	10	0.13	1.43	0.39	0.38
	I %	10	11.28	58.82	24.74	13.75
	U %	10	41.18	88.72	75.26	13.75
L. japonicus combined	Volumetric fraction	16	0.24	0.83	0.59	0.14
	Ratio I/U	16	0.13	1.54	0.62	0.46
	I %	16	11.28	60.69	33.93	16.71
	U %	16	39.31	88.72	66.07	16.71
G. max USDA-110	Volumetric fraction	13	0.51	0.84	0.73	0.09
	Ratio I/U	13	0.52	1.71	1.06	0.34
	I %	13	34.39	63.07	50.20	7.97
	U %	13	36.93	65.61	49.80	7.97

Table 2. Summary comparisons of the differences between nodules in *L. japonicus* and *G. max*.

Characteristic	*L. japonicus*	*G. max*
Nodule type	determinate	determinate
Infection thread size	most contain three to nine bacteria in cross section, occasionally single file	most single file, wider threads occasionally reported (7, 27)
Infection thread persistence	persists in mature cells, easy to find	degrades rapidly (1), difficult to find
Matrix material	sparse to copious depending on bacterial strain	sparse
Vesicles in infected cells	Large, centrally located and common	Small, rare in mature infected cells
Invading bacteria	*Rhizobium* spp. typical, not pleomorphic	*B. japonicum* typically not pleomorphic
Stele structure	triarch (9)	tetrarch (9)
N2 translocation compounds	amides (9)	ureides (9)
Scleroid layer	absent	present just under epidermis

2.4 DISCUSSION

L. japonicus nodule morphology resulting from *R. loti* inoculations resembles that of other determinate nodules such as those formed by soybean (8) and siratro (Price et al. 1984). There are, however, three significant morphological differences between *L. japonicus* and agriculturally important determinate nodulating plants.

2.4.1 The presence of large vesicles in infected cells

In *L. japonicus*, these cells contain large, centrally located vesicles notably absent from wild-type healthy infected soybean cells. While their exact function is unknown, there are a number of possibilities. Several studies can be cited to suggest that they indicate suboptimal growth conditions or growth in the presence of toxic substances. Unpublished observations (27) showed many such vesicles in all infected cells in drought-stressed and probably also Al stressed soybean plants grown in acid soil at the University of Tennessee Plateau Agricultural Experiment Station near Crossville, Tennessee. These perinuclear vesicles usually contained membranous whorls, and in a few cases also contained intact bacteroids devoid of their symbiosome membranes. Findings in these cases of stressed growth suggest that membrane fusion had occurred between vesicles and symbiosomes and that bacteroids were taken into the vesicles where they were degraded in what were apparently primary lysosomes. Another observation of perinuclear vesicles was reported in a study of laboratory-grown soybeans (28). Here, a high aluminum content in cotyledon amyloplasts in the seeds used was established by using X-ray microprobe analysis, Numerous perinuclear vesicles were found in infected cells and shown to contain PPB with X-ray spectra identical to the PPB of USDA 110 bacteroids in infected cells. Therefore, such vesicles were demonstrated to be involved in bacteroid destruction.

Vesicles found in *L. japonicus*, however, were largely devoid of membrane whorls or other material that would suggest a definitive role in bacteroid degradation. Therefore, these vesicles may serve a functional role in healthy, active nodules in *L. japonicus*. Large vesicles such as these have been reported in alfalfa (24) and are occasionally seen in cowpea (personal observation). Bieberdorf (3) suggests that these vesicles appear and enlarge, as a result of coalescence of smaller vesicles as nodules age but provides little evidence in support of these claims. The converse is also possible; that is, the vesicles may be remnants of the central vacuole and occupy space in the infected cell to maintain turgor until the space can be filled with cytoplasm and symbiosomes.

The role of perinuclear vesicles is unexplained and merit more study in *L. japonicus* and other legumes. If the presence of these large vesicles represents suboptimal growing conditions, standard growing conditions used in most laboratories will require improvements. If they represent a normal growth pattern in *L. japonicus* nodules, they represent a significant departure from the growth and differentiation of infected cells in soybean.

2.4.2 Location and function of specialized tannin cells

I have shown that *L. japonicus* produces large amounts of root-associated tannins. Distributed throughout the cortex of *L. japonicus* roots, specialized cells contain tannins in large central vacuoles that occupy nearly the whole cell volume. These specialized cells are absent from the roots of soybean and cowpea, but are found in other *Lotus* species (19-21).

Tannins serve several roles in plant roots. They are polymeric flavolans (6) and are produced via the same pathway as the non-polymeric flavolans, some of which have been found to function as signal molecules inducing nodulation genes of potential infecting bacteria (26).

Tannins are also known to be produced by the root systems of certain plant species as a protective mechanism against pathogenic infection (6). This process is most often associated with protection against fungal pathogenesis but has also been shown to adversely affect the growth of soil microbes including potential symbionts. Some bacterial strains have been able to develop resistance to tannin compounds and therefore may have a selective advantage over non-resistant strains (15), e.g., strain NZP-2037 (19-21). However, the correlation between tannin resistance and symbiont effectiveness may not be direct. Cooper and Rao showed that some strains sensitive to tannins still formed effective nodules (6).

The unique ability of *L. japonicus* to produce large quantities of root-associated tannins may limit the range of infection capability and nodulation-inducing effectiveness of bacteria on *Lotus* roots (19-21). While the importance of the presence of tannin-filled cells is unknown, they represent a significant departure from other determinate legume species.

2.4.3 Variant nodule characteristics

Like many biological processes, nodulation characteristics of *L. japonicus* is subject to variation. These altering characteristics are sometimes typical of a given inoculum, but often variation can even be seen within the same plant, which may represent differences in micro-environment (e.g., inoculum dose, root ontogeny, physiological fitness ect.).

Infection thread widths range from single file to in excess of nine bacteria in cross section; *L. j.*-2235 contained fewer bacteria in cross section on average than did infection threads in *L. j.*-2037. In addition, *L. j.*-2235 threads contained more matrix material than their *L. j.*-2037 counterparts.

When volumetric fraction data and percent infected/uninfected cell data are compared, the infected zone of *L. j.*-2037 was occupied with approximately 54% infected cell area but contained only 25% infected cells. *L. j.*-2235 contained approximately 67%

infected cell area and 50% infected cells. *G. max* contained 73% infected-cell area in the infected zone, composed of 50% infected cells. For all three, these data suggest that infected cells are larger on average than uninfected cells. In addition, these data demonstrate that infected cells of *L. j.*-2037 are proportionally larger with respect to their uninfected cells than either *L. j.*-2235 or *G. max*.

Varying characteristics are not unique to *L. japoncius*. *Vigna radiata* (mung bean) as well as *G. max* form threads which vary in size from single file to many bacteria in width (7, 17, 27). *L. japonicus* and *V. radiata* seem more likely to show a wide range of thread width than *G. max*.

On the other hand, certain characteristics are not generally subject to variation. For instance, *L. japonicus* forms determinate nodules, its stele is arranged as a triarch and it transports assimilated nitrogen in the form of amides. Thus far, no known datum demonstrates that these traits vary in wild-type *L. japonicus* (Table 2).

Some variations, however, have been induced to change. Bond (4) was able to induce *G. max* (cv. Peking) nodules to take on an indeterminate (at least meristematic) shape in about 15% of transformed nodules. In addition to change in outward appearance, these nodules formed a persistent meristem and took on the same zoning pattern of indeterminate nodules.

2.4.4 Discussion summary

In summary, several differences between *L. japonicus* and other determinate-nodulating plants were found. Notably, certain characteristics such as infection-thread type and *Rhizobium* spp. as infecting bacteria may be more closely related to indeterminate nodulating plants. In addition, unique features such as root-tannin cells and cortical cell-layer differences are unique to *L. japonicus*. However, no model system will reflect all the characteristics that are unique to an individual system. Thus I feel that *L. japonicus* will

prove to be a useful model system for the study of determinate nodulation but its differences will need to be factored into comparisons with other legumes and accounted for in experimental designs.

2.5 MATERIALS AND METHODS

L. japonicus - Gifu seeds were obtained from Dr. Peter Gresshoff, Center for Legume Research at The University of Tennessee. To prepare for germination, seeds were scarified lightly with fine sandpaper, surface sterilized in a 2% hypochlorite / 0.01% Tween 20 solution for 10 min., then rinsed three times with sterile water (12). Seeds were germinated on filter paper saturated with distilled water in a petri dish in the dark until root tips could be seen, typically 5-6 d. Seedlings were then transferred to either clear plastic pouches that contained 25 ml of plant nutrient solution (PNS) (see appendix for composition) or flats containing vermiculite and watered with PNS adjusted to pH 7.0. After 5 d, the plants were inoculated with one of four strains of bacteria; *R. Loti* NZP 2235 or NZP 2037 or *B. japonicum* USDA 3469 or USDA 110. Strain NZP 2037 was obtained from Dr. Gresshoff; strains USDA 110, USDA 3469 and NZP 2235 were obtained from Dr. Gary Stacey, Center for Legume Research at The University of Tennessee. Plants were grown in either a growth chamber with a light intensity of 100 μmol m^{-1} s^{-1} and an 18h/6h day/night cycle at a temperature of ca. 21°C. or in a greenhouse under combined natural and supplemental lighting intensity of 100 to 300 μmol m^{-1} s^{-1}.

Nodules were harvested 4 weeks p.i.. They were halved in 1% glutaraldehyde in phosphate buffer at pH 6.8, then transferred to fresh fixative for 1 h at room temperature. Samples were post-fixed for 1 h in 1% osmium tetroxide in phosphate buffer (27). Samples were then dehydrated in an acetone step gradient and embedded in Spurr's resin. The embedded nodules were semi-thin sectioned (1 mm) and stained with methylene blue for light microscopy. For electron microscopy, sections were cut (100 nm), post-stained with uranyl acetate and lead citrate and viewed with an Hitachi H-600 transmission electron microscope.

Cytochemical tests for tannins were carried out using a ferrous sulfate histochemical stain (11)(see appendix for formulation and procedure)

Data on volumetric fractions and numbers of infected vs. uninfected cells were tested with the Kruskal-Wallis method, and then compared using a Mann-Whitney test..

Bibliography

1. Bal, A.K. 1985. Vacuolation and Infection Thread in Root Nodules of Soybean. Cytobios 42:41-47.

2. Bhuvaneswari, T.V., Turgeon, B.G. and Bauer, W.D. 1980. Early Events in the Infection of Soybean (*Glycine max* L. Merr). Plant Physiol. 66:1027-1031.

3. Bieberdorf, F.W. 1938. The Cytology and Histology of the Root Nodules of Some Leguminosae. J. Am. Soc. Agron. 30:375-389.

4. Bond, J.E. 1993. Transformation as a tool to study the genetics of nodulation in *Glycine max*. Ph.D. Thesis, The University of Tennessee.

5. Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A. and Bauer, W.D. 1984. Anatomical Analysis of the Development and Distribution of *Rhizobium* Infections in Soybean Roots. Can. J. Bot. 62:2375-2384.

6. Cooper, J.E. and Raghavendra Rao, J. 1992. Localized Changes in Flavonoid Biosynthesis in Roots of *Lotus pedunculatus* After Infection by *Rhizobium loti*. Plant Physiol. 100:444-450.

7. Dunlap, J.R. 1998 personal communication

8. Goodchild, D.J. and Bergersen, F.J. 1966. Electron Microscopy of the Infection and Subsequent Development of Soybean Nodule Cells. J.Bact. 92:204-213.

9. Gresshoff, P.M. personal communication

10. Handberg, K. and Stougaard, J. 1992. *Lotus japonicus,* an autogamous, diploid legume species for classical and molecular genetics. Plant J. 2:487-496.

11. Jensen, W.A. 1962. *Botanical Histochemistry*, pp. 1-408.W.H. FreemanSan Francisco:

12. Jiang, Q. and Gresshoff, P.M. 1993. *Lotus japonicus* - a model plant for structure-function analysis in nodulation and nitrogen fixation. Pages 97-110 in: Plant Biotechnology and Development, Gresshoff, P.M., ed. CRC Press, Boca Raton.

13. Meyerowitz, E.M. 1987. *Arabidopsis thaliana*. Annu. Rev. Genet. 21:93-111.

14. Meyerowitz, E.M. 1990. *Arabidopsis* advances. TIG 6:1-2.

15. Miller, K.J., Hadley, J.A. and Gustine, D.L. 1994. Cyclic b-1,6-1,3-Glucans of *Bradyrhizobium japonicum* USDA 110 Elicit Isoflavonoid Production in the Soybean (*Glycine max*) Host. Plant Physiol. 104:917-923.

16. Newcomb, W. 1976. A Correlated Light and Electron Microscopic Study of Symbiotic Growth and Differentiation in *Pisum sativum* Root Nodules . Can. J. Bot. 54:2163-2186.

17. Newcomb, W. and McIntyre, L. 1981. Development of Root Nodules of Mung Bean (*Vigna radiata*): a Reinvestigation of Endocytosis. Can. J. Bot. 59:2478-2499.

18. Newcomb, W., Sippell, D. and Peterson, R.L. 1979. The Early Morphogenesis of *Glycine max* soybean and *Pisum sativum* Root Nodules after Inoculation with *Rhizobium*. Can. J. Bot. 57:2603-2616.

19. Pankhurst, C.E., Craig, A.S. and Jones, W.T. 1979. Effectiveness of *Lotus* Root Nodules: I. Morphology and Flavolan Content of Nodules Formed on *Lotus Pedunculatus* by Fast-Growing *Lotus* Rhizobia. J. Exp. Bot. 30:1085-1093.

20. Pankhurst, C.E. and Jones, W.T. 1979. Effectiveness of *Lotus* Root Nodules: II. Relationship Between Root Nodule Effectiveness and *'In Vitro'* Sensitivity of Fast-Growing *Lotus* Rhizobia to Flavolans. J. Exp. Bot. 30:1095-1107.

21. Pankhurst, C.E. and Jones, W.T. 1979. Effectiveness of *Lotus* Root Nodules: III. Effect of Combined Nitrogen on Nodule Effectiveness and Flavolan Synthesis in Plant Roots. J. Exp. Bot. 30:1109-1118.

22. Parsons, R. and Day, D.A. 1990. Mechanism of Soybean Nodule Adaptation to Different Oxygen Pressures. Plant Cell Environ. 13:501-512.

23. Parsons, R. and Day, D.A. 1990. Structure of the Soybean Nodule Cortex: In Relation to Oxygen Diffusion and Regulation of Nodule Activity. In: Proc. 8th Internatl. Cong. Nit. Fix. 360.

24. Patel, J.J. and Yang, A.F. 1981. Light and Electron Microscopic Studies of Nodule Structure of Alfalfa. Can. J. Microbiol. 27:36-43.

25. Pueppke, S.G. 1982. *Rhizobium* Infection Threads in Root Hairs of *Glycine max* (L.) Merr., *Glycine soja* Sieb. & Zucc., and *Vigna unguiculata* (L.) Walp. Can. J. Microbiol. 29:69-76.

26. Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. 1986. Flavones Induce Expression of Nodulation Genes in *Rhizobium*. Nature 323:632-635.

27. Roth, L.E. 1998. personal communication

28. Roth, L.E., Dunlap, J.R. and Stacey, G. 1987. Localizations of Aluminum in Soybean Bacteroids and Seeds. Applied. Environ. Microbiol. 53:2548-2553.

3. Cadmium

3.1 ABSTRACT

Since cadmium is becoming an increasingly important environmental pollutant, I studied the effects of cadmium on soybean nodules by using electron microscopy and X-ray microprobe analysis. I have found that, though plants can accumulate modest amounts of cadmium without significant observable effects, exposures to cadmium at levels in the range of 1.0 to 10 mM in laboratory conditions results in a total disarray of infected cells, nodules and plants at 28 days post inoculation. Cellular effects were also seen earlier and at lower concentrations.

The earliest and strongest accumulations of cadmium were found in the polyphosphate bodies of bacteroids which, because of their internal location, could only be exposed after the metal had traversed the cell and symbiosome membranes, as well as having penetrated the cortical layers of the nodule.

Cadmium was also found in nuclei in infected cells in the euchromatin, heterochromatin and nucleoli, results suggesting that cadmium binds to DNA in host cells. Binding to the DNA in bacteroid chromatin could also be expected. The metal was also localized in cell walls of most nodule cells, in amyloplasts, in granules inside tonoplasts, and in the cytosol of almost all cell types in treated plants. Cadmium was also localized in polyphosphate bodies in control cells, though at a lesser frequency and with weak X-ray peaks. Other metals localized were calcium and magnesium, found in both control and treated tissues about equally, and iron which was similarly found, except that it was concentrated more strongly in polyphosphate bodies of treated than untreated cells.

I suggest that the primary pathogenicity of cadmium is due to changes in the properties of DNA, the destruction of membranes and the death of bacteroids, though numerous other processes are undoubtedly also affected.

The finding of cadmium in numerous locations and causing such widespread devastation in nodule cells helps explain why yields of soybeans in fields treated with industrial sewage sludge and otherwise cadmium-contaminated are so quickly and often permanently reduced. The finding of cadmium in both control and treated nodules and in plants that may or may not show toxic effects suggests that cadmium can be inserted in the food chain for human beings both by ingestion and inhalation at significant levels beyond those presently recognized.

3.2 INTRODUCTION

Cadmium is a trace element that is generally found in association with Zn, Cu and Pb in the natural environment (20). Cadmium serves no known biological function (16, 20) and is toxic to most cells (7, 16, 19). The level of tolerance an organism has for Cd varies between species as well as tissues within an individual organism, concentrates within particular organs of both plants and animals.

The majority of Cd that enters the environment does so as a result of human activity. Industries that are responsible for this release include mining of non-ferrous metals, manufacturing of pigments, burning of industrial wastes and fossil fuels, and applications of sewage sludge and contaminated fertilizers to agricultural crops (15, 18, 20). Fertilizers are of particular interest because they can become contaminated both naturally and through the activity of man. Most phosphotatic fertilizers contain some level of cadmium. Fertilizers in the United States are then further contaminated through the addition of ash from industrial incineration, a practice largely unregulated; manufacturers are not required to analyze the final product to determine and indicate the amounts of heavy metals present and therefore augmented fertilizers can contain very toxic levels of Cd as well as Pb and As (23).

Once inside the plant, the metal is translocated to particular sites, the distributional fate of Cd largely depending on the organism involved. Wagner et al. (19, 20) report that the leaves of tobacco plants accumulate the majority of the Cd load, while Cataldo et al. (1) state that as much as 92% of total Cd can be found in the roots of soybean plants, 8% of which is relocated to the seed during their development. Similar findings were reported by Collins et al. (3).

Even within cells, localization seems to vary with species. Rauser and Ackerley (13) observed the presence of Cd in granules in the cytoplasm, nucleus and vacuole of *Agrostis gigantea* and *Zea mays* cortical cells, while several groups (1, 8, 22) report an almost even 50% distribution in the cell wall and cytoplasm of plant cells. Vogeli-Lange and Wagner (19) show that virtually all Cd and Cd-binding proteins (BP) are located within the tonoplast of tobacco seedlings.

Cells use several mechanisms to remove free Cd ions from circulation and thereby detoxify them. Wagner (20) proposed a general model of cellular detoxification of Cd which included binding to the cell wall, formation of cytoplasmic precipitates and transport of Cd and phytochelatins to the tonoplast.

Toxicity of Cd results from a complex set of chemical interactions. Cadmium has a high affinity for -SH groups, and it is likely that interference with the normal functioning of these groups is one of Cd's principal toxic effects (20). Hinkle et al. (7) showed that the addition of Cd could reduce the incorporation of H^3-labeled leucine into proteins and labeled H^3-thymidine into DNA, results suggesting an adverse effect on translation as well as replication. Cadmium might also interfere with Zn metalloenzymes including RNA and DNA polymerases (24).

To make matters worse, plants bioaccumulate Cd, as low as 10-fold (2, 4, 21) and as high as 1800-fold for roots of soybean compared to the levels in soil in which they were grown (3). Plants can accumulate moderate amounts of Cd without showing adverse effects. Soybean required 4 to 5 μg Cd/g soil to produce a 25% reduction in yield, 10 times greater than the Cd level found in mildly contaminated soil and 100 times the level of uncontaminated soil (20). Since Cd exists at levels below amounts that would be phytotoxic, plants are capable of accumulating moderate levels of Cd without being damaged and can therefore contribute significantly to human intake of Cd.

Approximately 70% of human intake of Cd is from vegetable matter (15). Contamination of agricultural soils and Cd tolerance of plants are therefore of great significance to human health. Many adverse effects have been reported in humans, but the most consistent are renal tubular dysfunction and pulmonary emphysema (5, 6, 15); recent evidence also suggests a role in osteoporosis, especially in post-menopausal women. Effects on human health are worsened by the fact that the half-life of Cd in humans is over 10 years (20).

I report here four findings: First, Cd is preferentially localized in cell walls, nuclei, amyloplasts and cytosolic granules of most nodule cells. Second, infected cells had Cd in the polyphosphate bodies (PPB) of the resident symbiotic bacteroids, and this site is where Cd is found earliest, despite the fact that it had to cross two cell and two bacteroid membranes. Third, low levels of Cd were found in the PPB in control cells. Fourth, by 28 days p.i., nodules show generalized destruction.

Sites of Cd localization can be divided into those that are key to cellular metabolism and those that are secondarily toxic. A summary of pathogenicity in soybean nodules is presented and indicates the numerous sites of metabolic damage.

3.3 RESULTS

The morphology of treated plants did not seem to differ significantly from the controls at 14 days p.i. (Figs. 3 & 4). By 19 day p.i. however, the difference was obvious; large vacuoles were typically found in infected cells (Figs. 5 & 6), and more cells were in the early stages of degradation. By 28 days p.i., the cells of the entire nodule were completely disrupted, bacteroids were arranged in arrays and only a few recognizable cytoplasmic remnants remained (Figs. 7 & 8).

X-ray microanalyses were carried out on the cell walls of numerous cell types; the cytosol of infected, uninfected and inner cortical cells; the nuclear euchromatin, heterochromatin and nucleoli of infected, uninfected and inner cortical cells; amyloplasts of infected, uninfected and inner cortical cells; the vacuole contents of infected, uninfected and outer cortical cells; and the PPB of infected-cell bacteroids. A summary of metals detected is given in Table 1, in which the data from 14, 19 and 28-day-p.i. nodules have been combined. Most spectra contained more than one element of interest along with others (Cu and Os) that came from the grids and fixative used, respectively. Phosphorous, expected to be a component of PPB, was not found since its peak is obscured by the Os peaks.

The highest frequency of Cd detection (73%) occurred in the PPB of the treated plants (Table 1, Figs. 1i-1k & 3). Occurrences of the chemical signature of these PPB included Ca (91% control, 100 % treated), Fe (36% control, 42% treated) and Mg (64% control, 50% treated) (Fig 1h-1k, Table 1). It is of interest that Fe was occasionally found in the PPB of both the control and treated plants.

The walls of all cells composing the nodule, with the exception of the sclerenchyma cells, were found to contain Cd approximately 26% of the time (Table 1, Figs. 1a & 1b)

with a relatively even distribution in all cell types. Only one scan out of 26 (17 of which were Cd-treated) of the scleroid walls showed Cd.

The cytosol of all treated nodule cells, including the scleroid cells, were found to contain Cd, most of which was in free granules (Figs. 1c, 1e, 4 & 9). Similar granules found in the controls contained Ca, Fe, Mg and K, a composition similar to the Cd-containing granules in the treated cells. One granule in the cytoplasm of a control plant showed a small Cd peak (Fig. 1d).

Cadmium was not detected in ribosomes, peroxisomes, mitochondria or endoplasmic reticulum (ER) but was found in nuclei, amyloplasts and tonoplasts of all cells examined, with the exception that these structures were not examined in outer cortical and scleroid cells due to the difficulty of locating them in the sparse cytoplasm in unstained sections. Cadmium detected in these organelles was not generally associated with any granules, although an occasional Cd-loaded granule did appear in the tonoplast. Cadmium seemed to be evenly distributed within all three areas of the nuclei examined: euchromatin, heterochromatin and nucleoli.

The frequency with which both Ca and Fe were detected increased in older treated plants in most areas examined (Table 1). Cadmium was detected more easily in the 19-day p.i. samples. The frequency of detection increased as well as the relative signal strength, which suggests, but does not quantitatively establish, that more Cd may be present in older samples (Table 2, Figs 5 &6).

Cadmium was rarely detected alone, but was often associated with one or more of the following: Ca, Fe and Mg. By far the element most commonly associated with the presence of Cd was Ca. Cadmium was almost always found in the presence of Ca. Treatment of plants with Cd seemed to result in a modest increase in Ca frequency. However, Cd did not seem to distribute itself in the same pattern as Ca (Table 1). Calcium levels were high in both the PPB and the cell walls and relatively evenly distributed

elsewhere. Cadmium distribution was also high in PPB, but not the cell walls, being about evenly distributed in cell walls and other components.

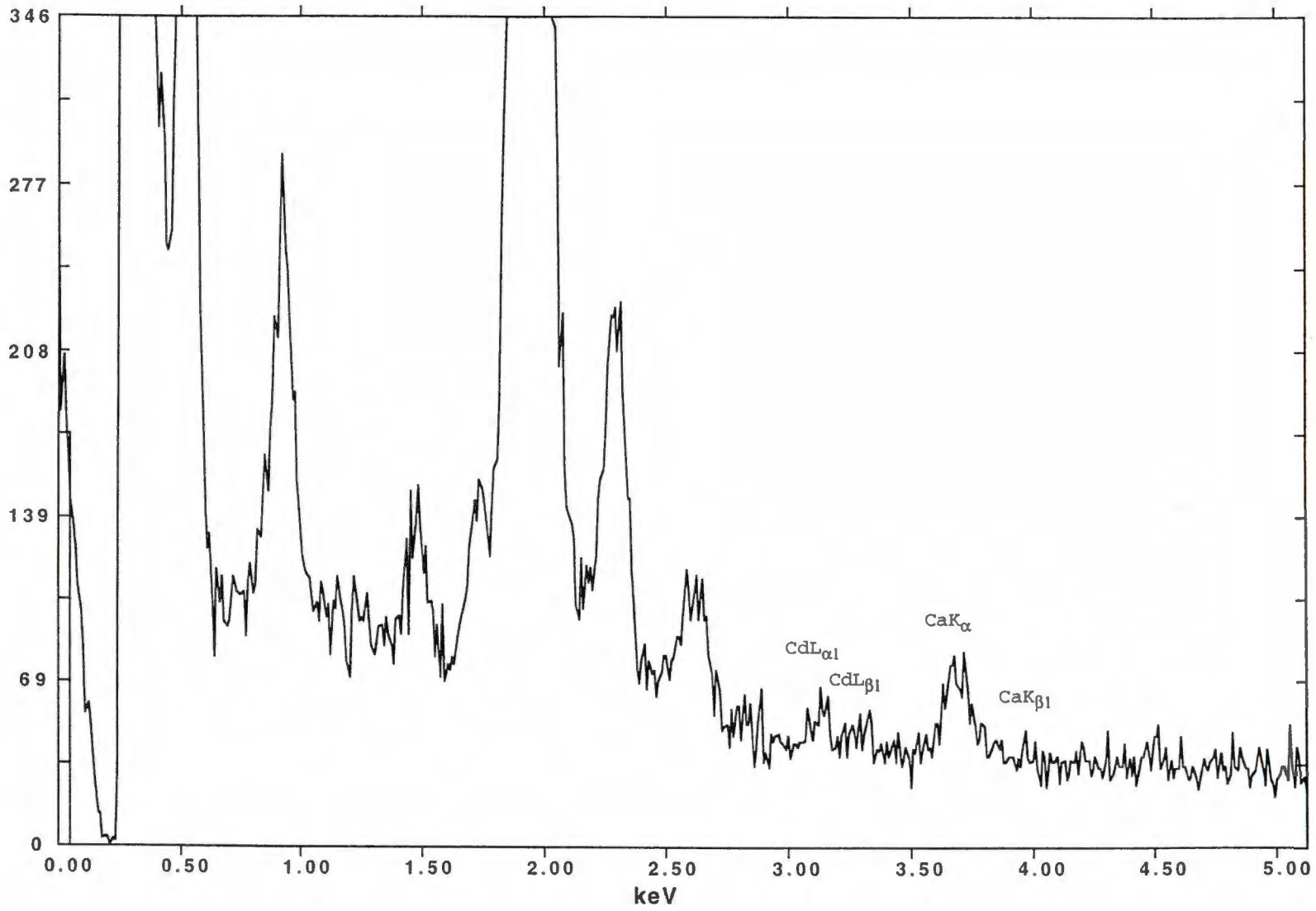
Cadmium was detected in four spectra of control nodules, three in the PPB at both 14 and 19-day p.i., and one in a granule in the cytoplasm of a 14-day p.i. uninfected cell. The signals were weak in comparison to the treated samples but were present none the less (Fig.1a, 1d & 1h).

Table 1. Summary table of the general distribution of ions detected within various subcellular structures covering data from the 412 spectra used. The percent figure is derived from the fraction below it, in which the numerator represents the total number of spectra that demonstrated the presence of Cd and the denominator shows the total number of spectra examined. See the text for more information and for an indication of which cells were sampled in each category.

	Cd		Ca		Fe		Mg	
	control	treated	control	treated	control	treated	control	treated
Cell Wall	0% (0/34)	26% (17/65)	50% (17/34)	55% (36/65)	15% (5/34)	22% (14/65)	44% (15/34)	34% (22/65)
Cytoplasm	5% (1/20)	30% (20/67)	25% (5/20)	30% (20/67)	15% (3/20)	28% (19/67)	40% (8/20)	33% (22/67)
Nucleus	0% (0/22)	29% (17/58)	27% (6/22)	33% (19/58)	23% (5/22)	19% (11/58)	27% (6/22)	21% (12/58)
Amyloplast	0% (0/16)	25% (7/28)	38% (6/16)	21% (6/28)	38% (6/16)	64% (18/28)	19% (3/16)	29% (8/28)
Vacuole	0% (0/21)	18% (4/22)	14% (3/21)	23% (5/22)	5% (1/21)	9% (2/22)	14% (3/21)	18% (4/22)
PPB	27% (3/11)	73% (19/26)	91% (10/11)	100% (26/26)	36% (4/11)	42% (11/26)	64% (7/11)	50% (13/26)

Figure 1a. Spectra of cell wall in control showing slight Cd peak.

Note for all of figure 1, the abscissa is measured in Kiloelectron Volts and the ordinate is total number of counts (generally for 100 to 200 seconds)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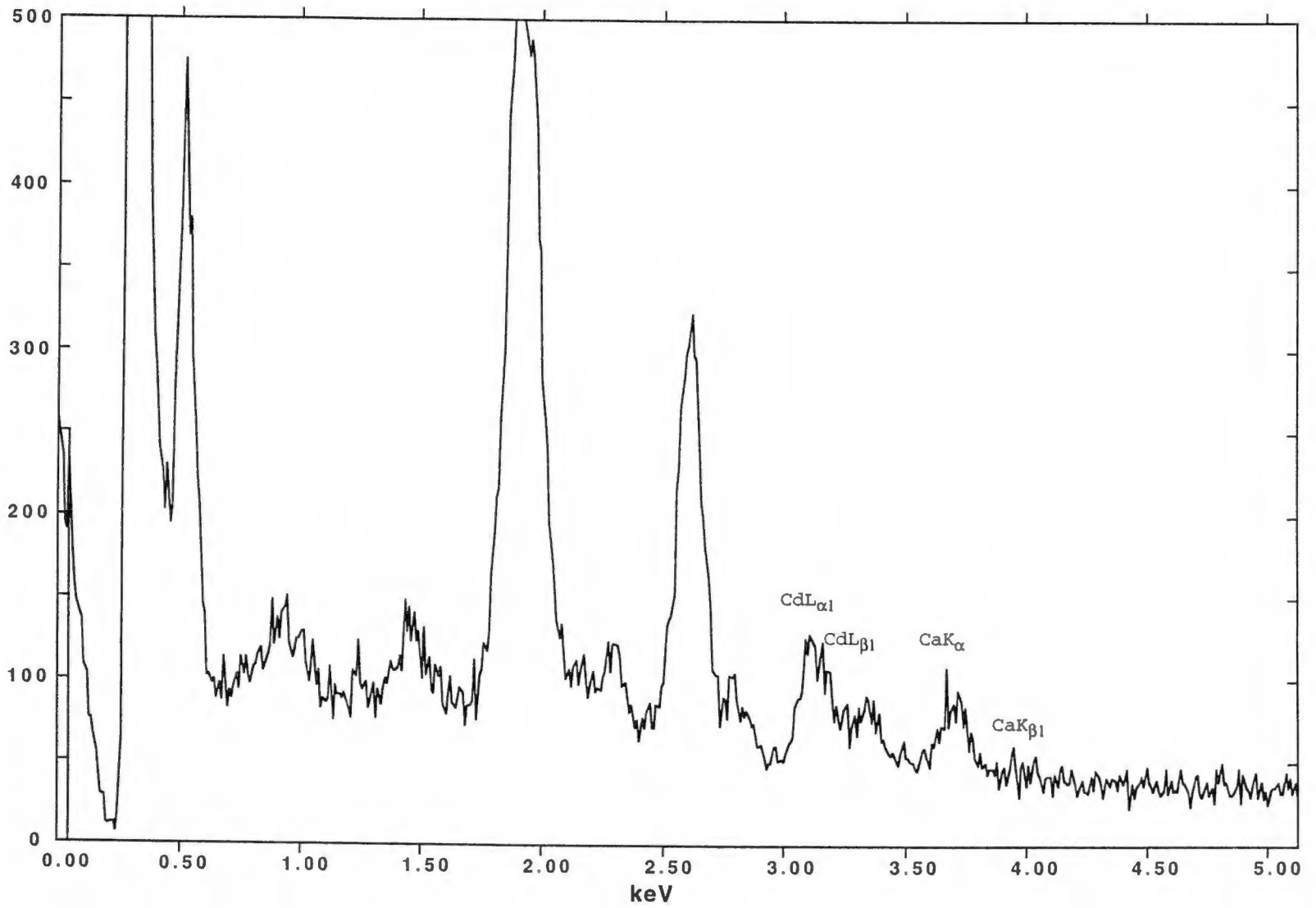
277
208
139
69
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 1b. Spectra of cell wall of cell treated with 10 mM $CdCl_2$ showing significant Cd and Ca peaks.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400
300
200
100
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 1c. Spectra of cytoplasm in control cell. No Cd detected.

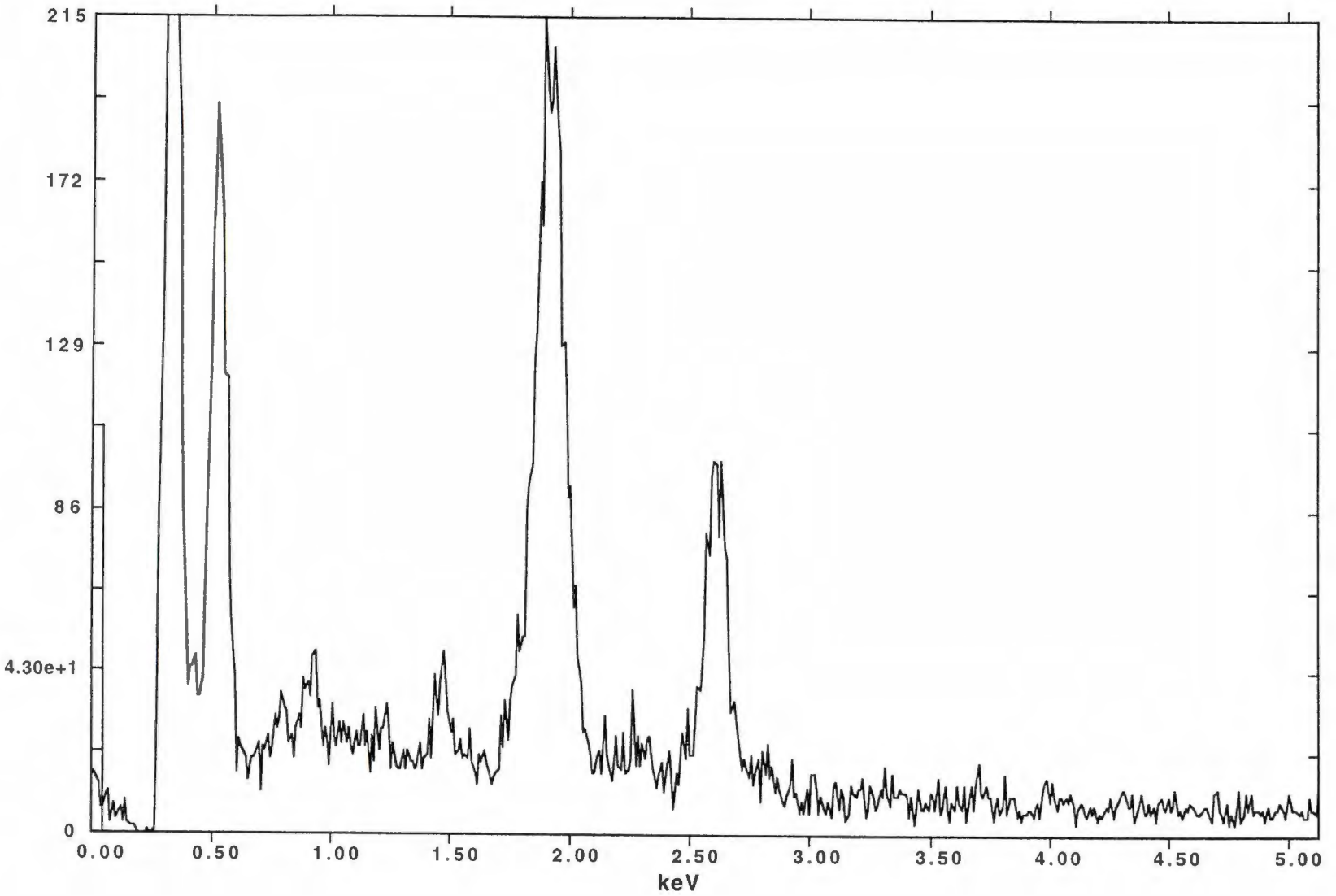

215
172
129
86
4.30e+1
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV

Figure 1d. Spectra showing granule in cytoplasm of control cell. Slight Cd peak shown.

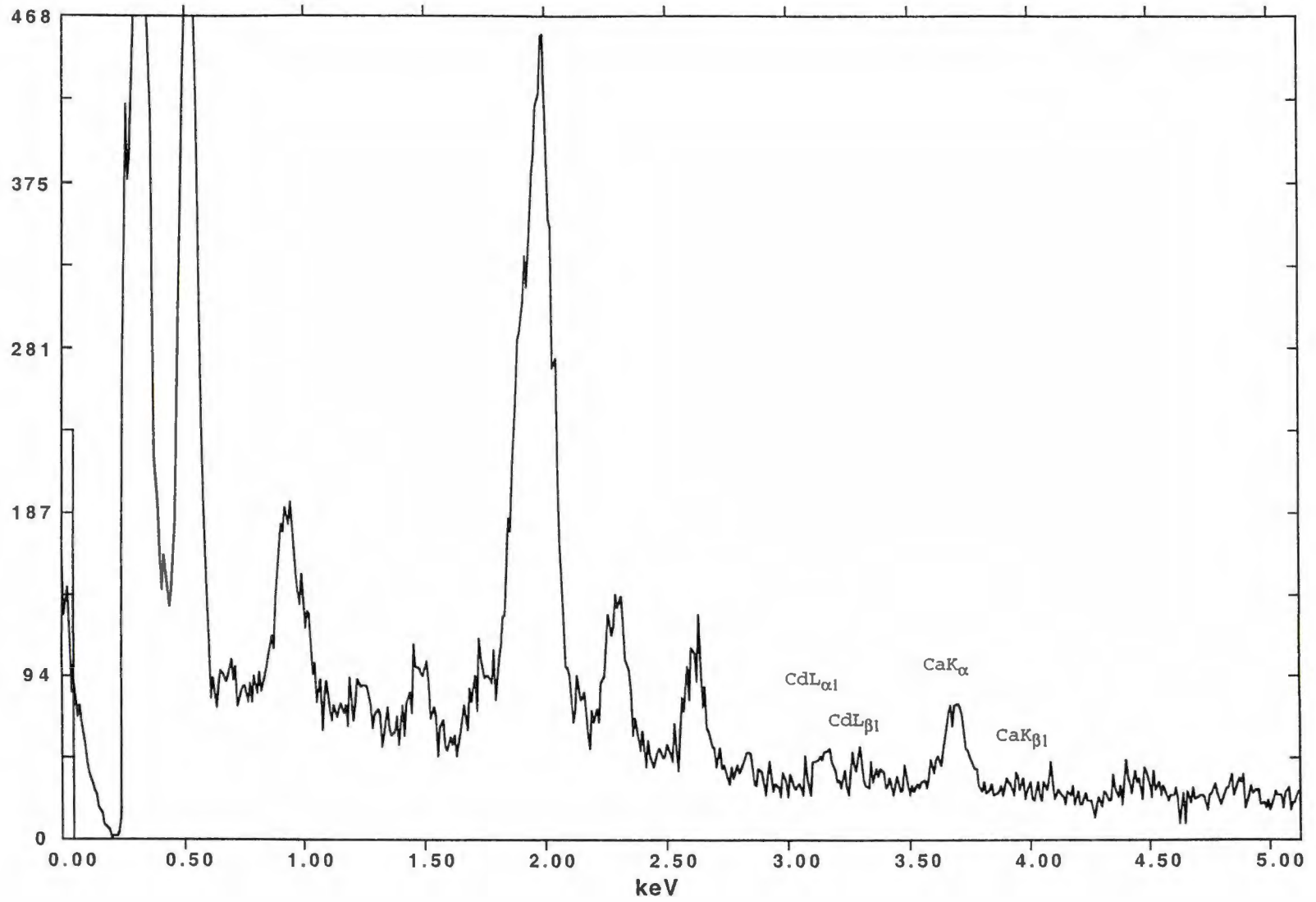

468
375
281
187
94
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 1e. Spectra of cytoplasm in treated cell showing significant Cd peak.

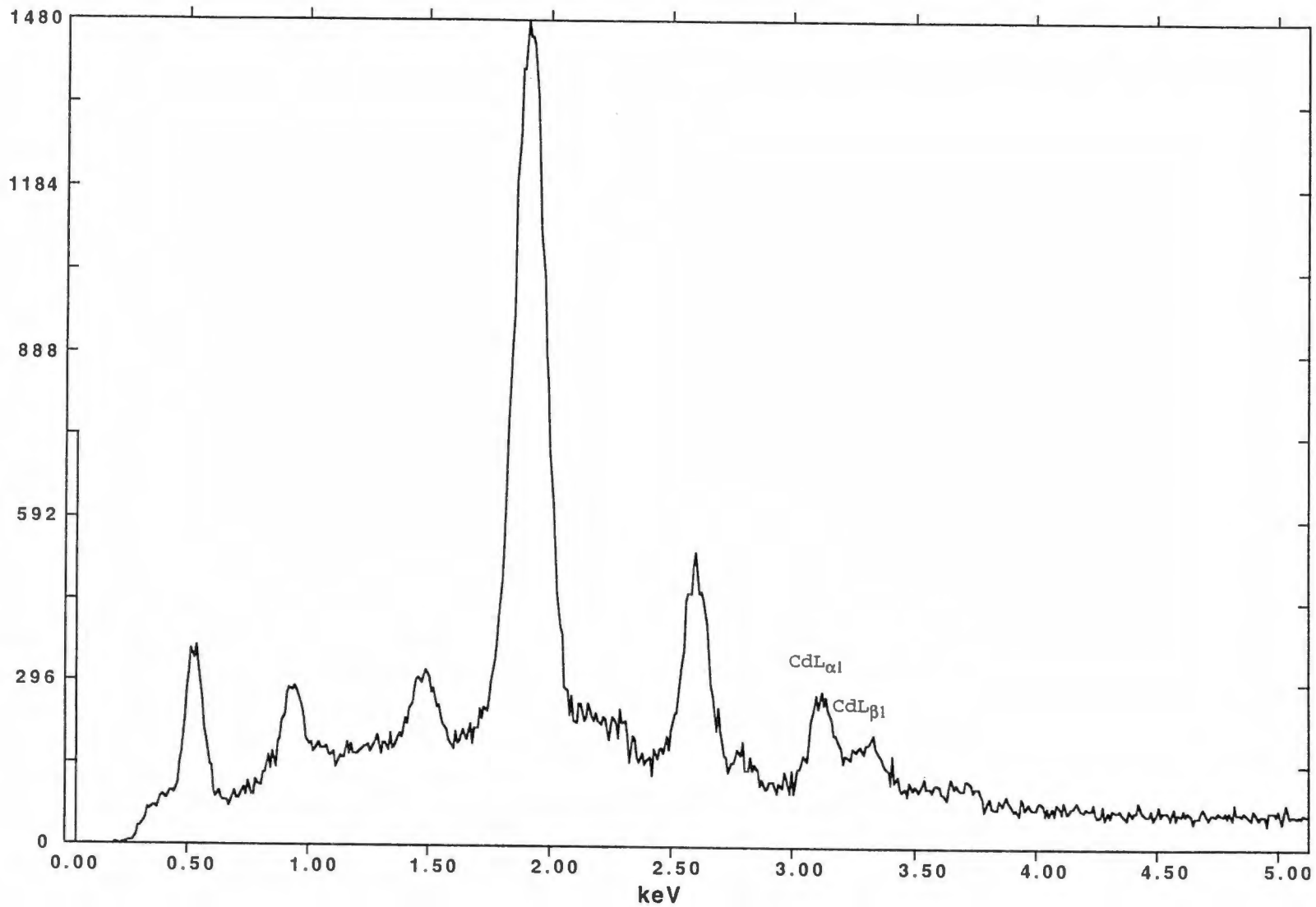

1480
1184
888
592
296
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$

Figure 1f. Spectra of euchromatin in control cell. No Cd detected.

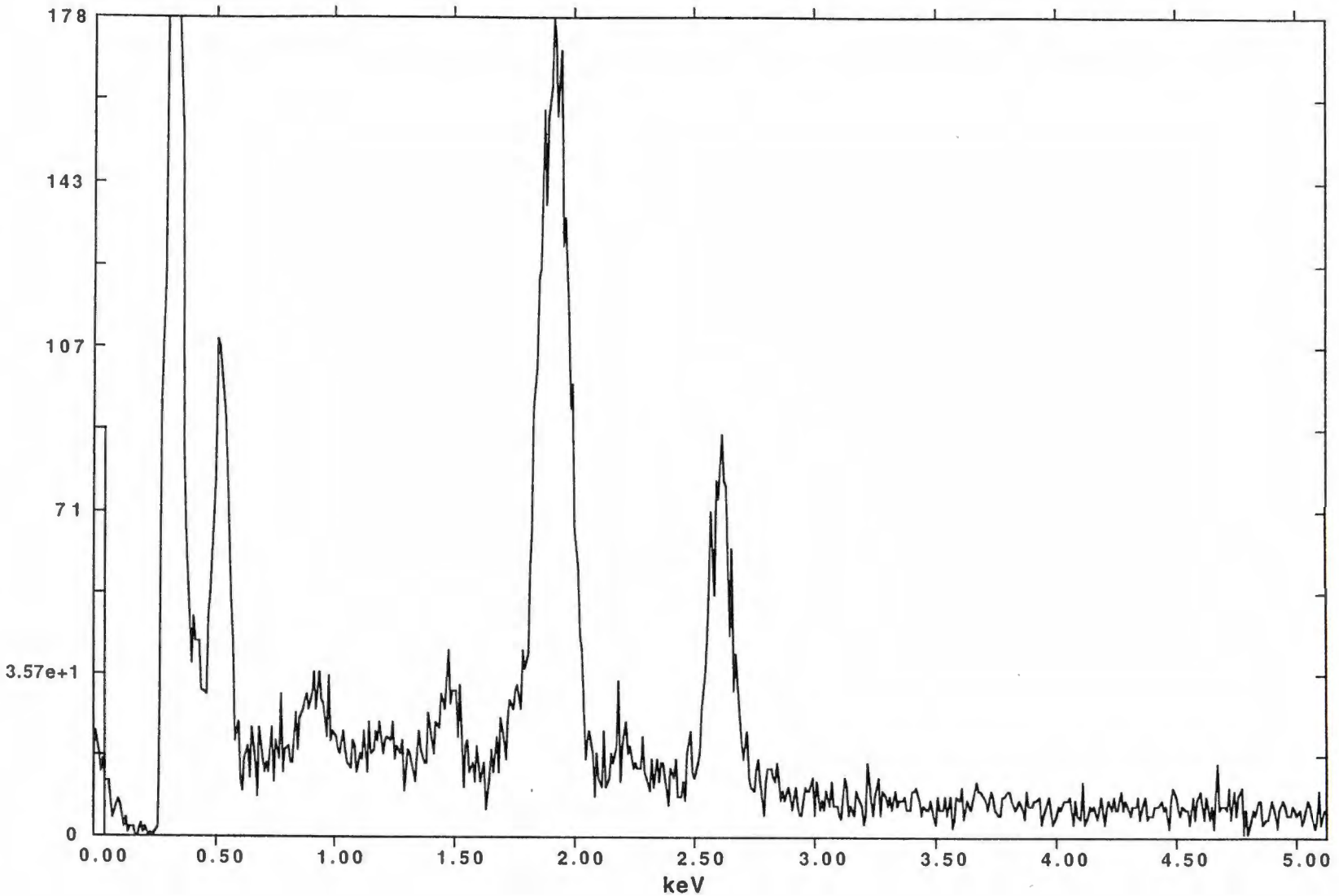
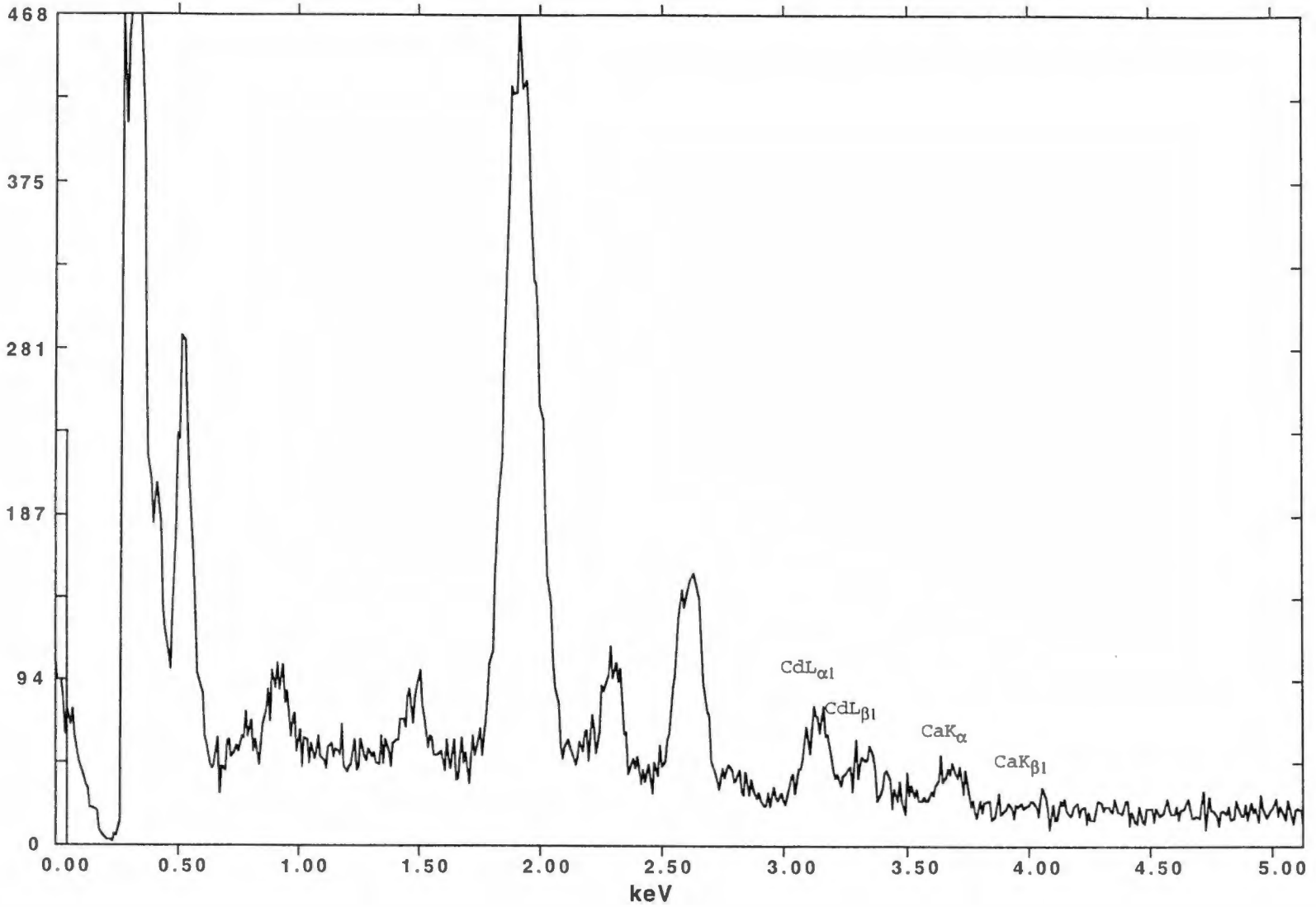

178
143
107
71
3.57e+1
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV

Figure 1g. Spectra of euchromatin in treated cell nucleus. Cadmium localized at this site.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375
281
187
94
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 1h. Spectra of Polyphosphate body in control cell. Cadmium was detected here. When a peak to background ration was compared, the sample was found to show a Cd peak that was 44% above background.

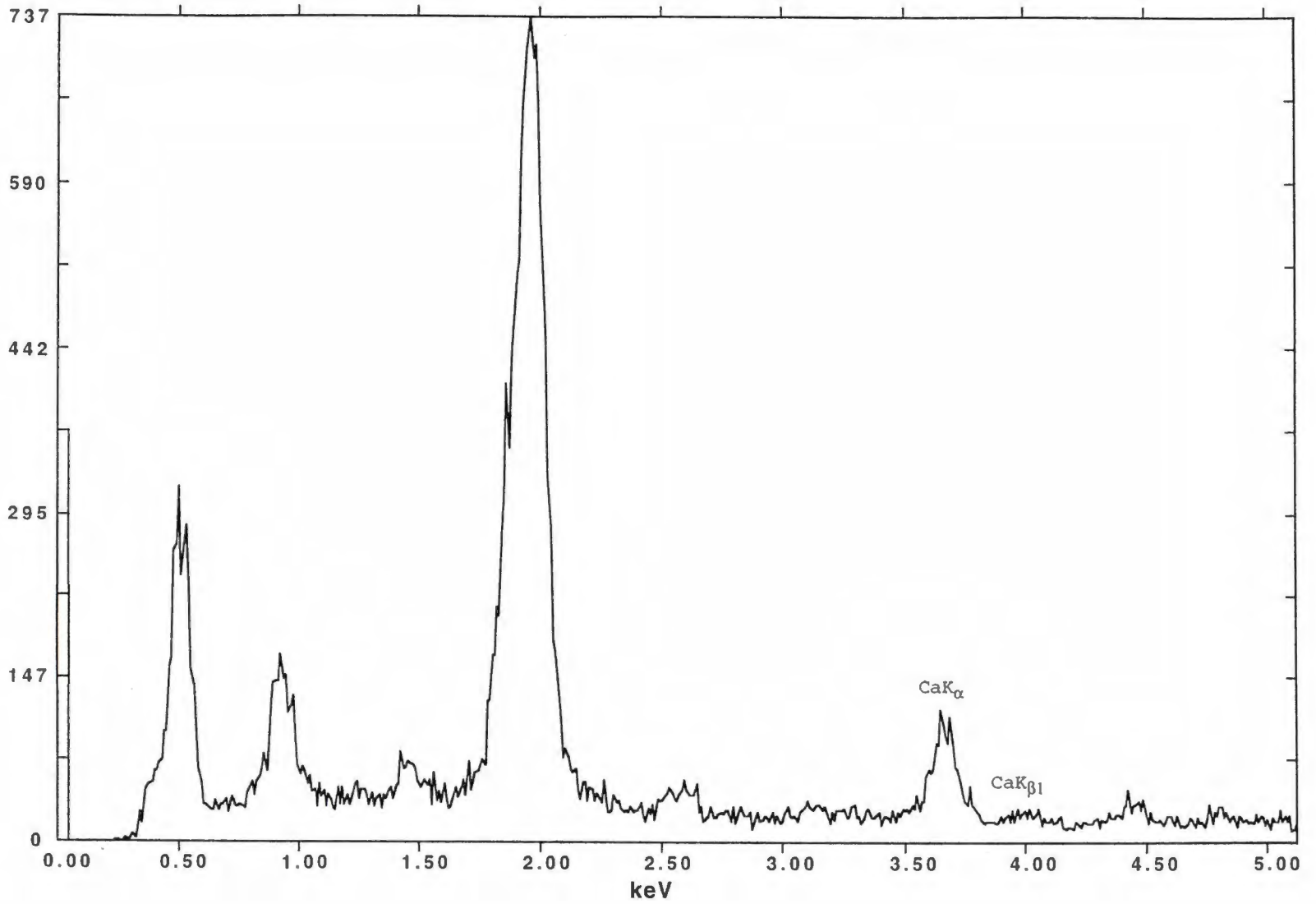
737
590
442
295
147
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CaKα
CaKβ1

Figure 1i. Spectra of Polyphosphate body in control cell showing no Cd.

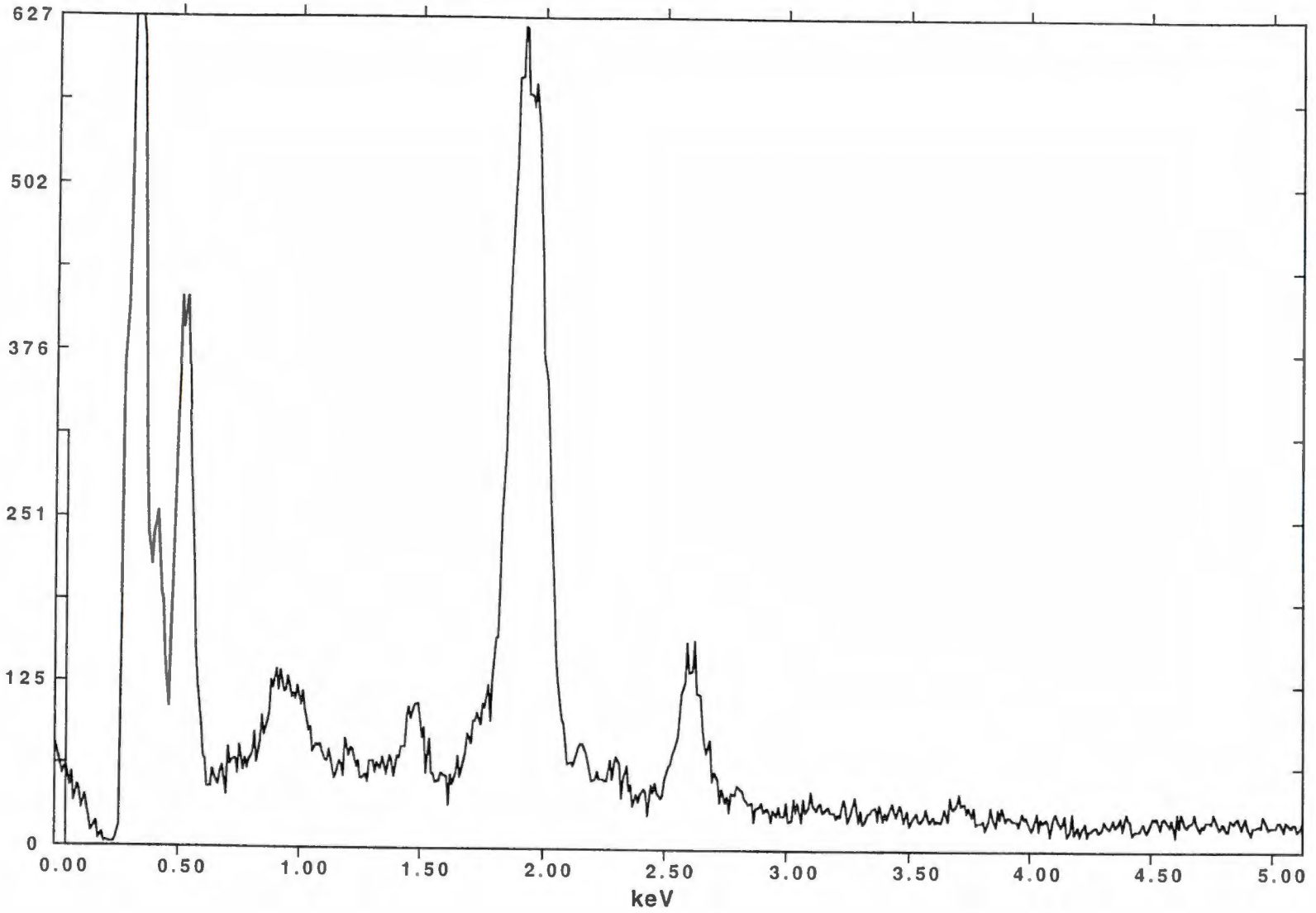
627
502
376
251
125
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV

Figure 1j. Spectra of polyphosphate body in 14 day p.i. treated cell showing Cd.

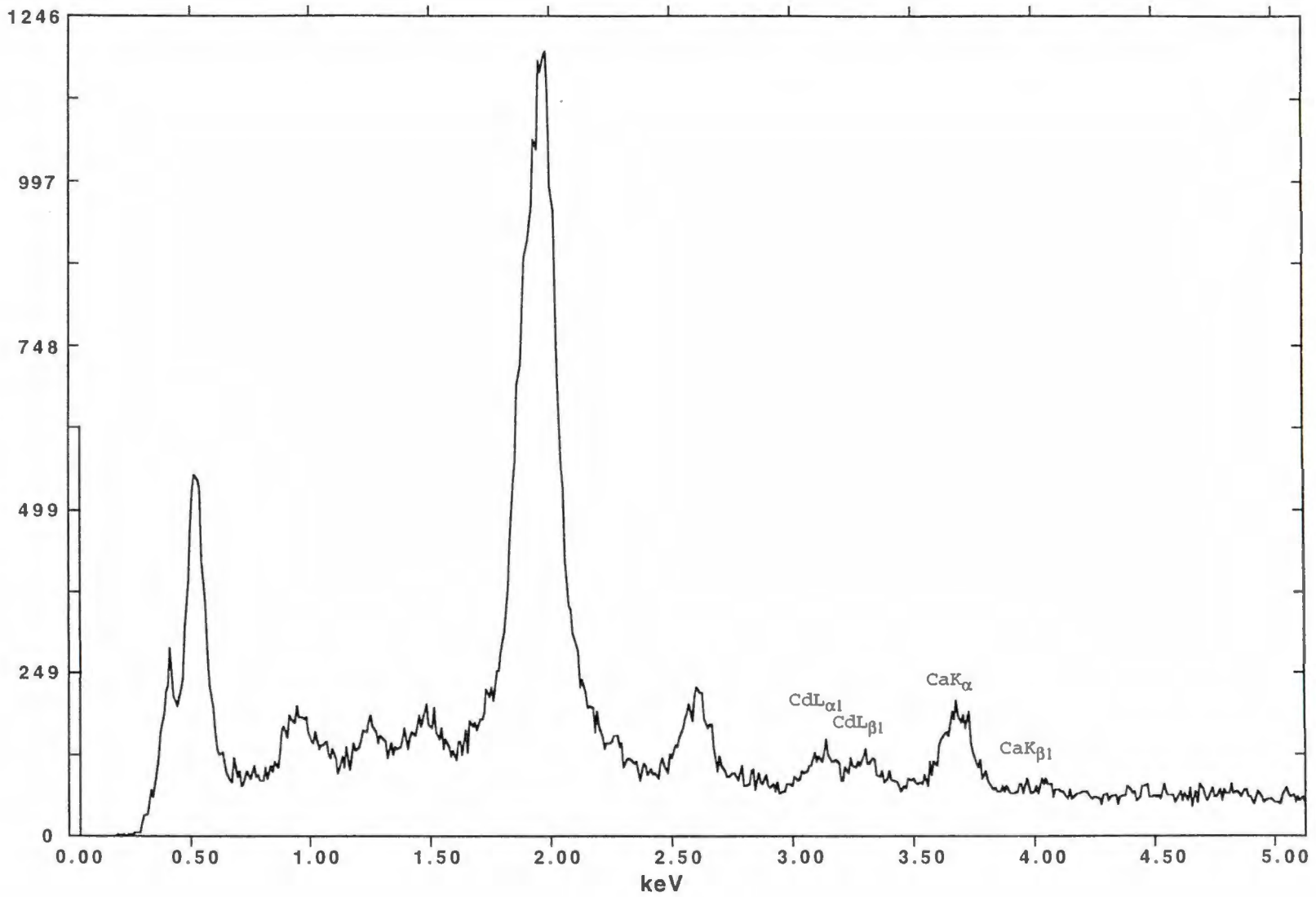
1246
997
748
499
249
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 1k. Spectra of polyphosphate body in 19 day p.i. treated cell showing Cd.

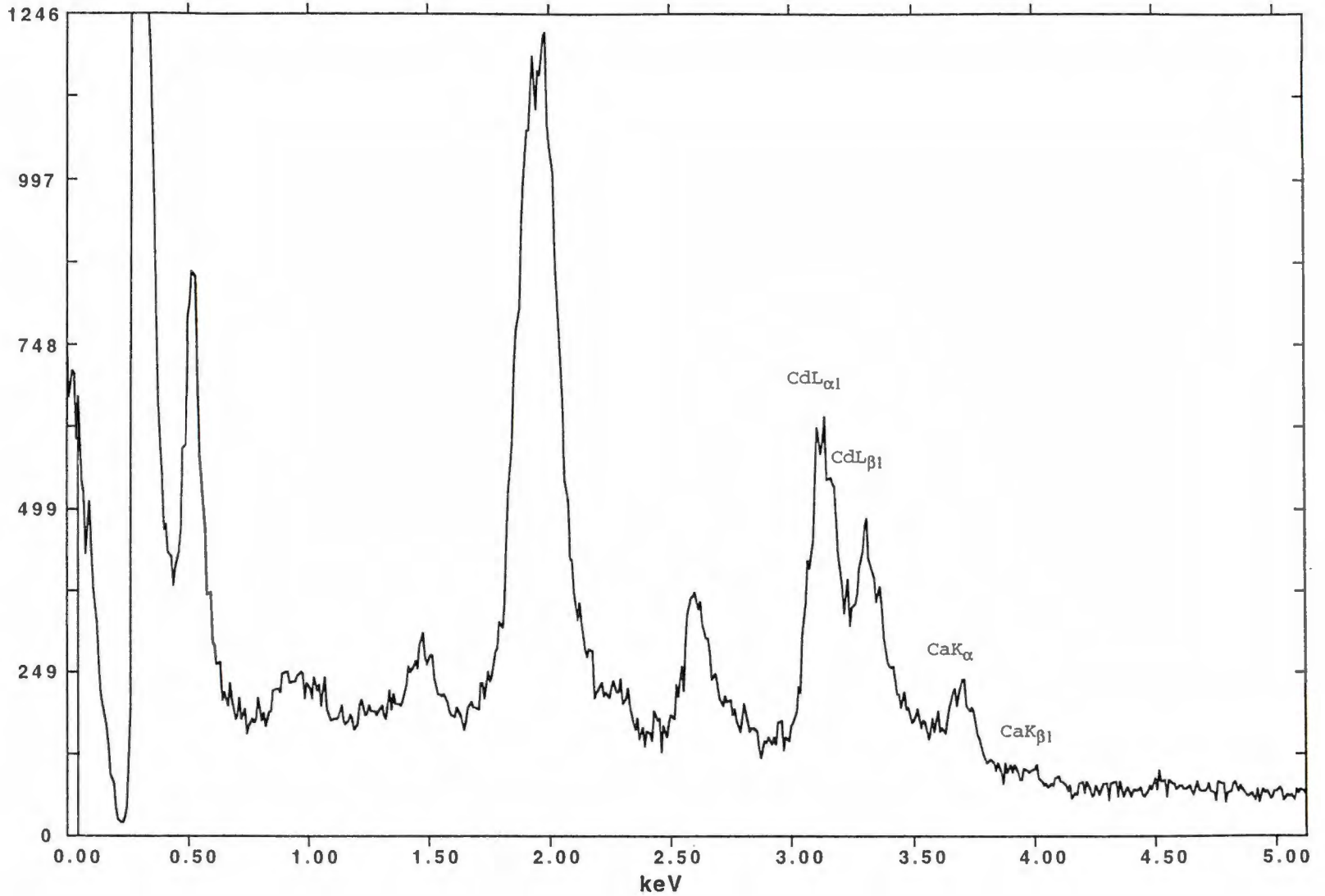
1246
997
748
499
249
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
keV
CdL$_{\alpha 1}$
CdL$_{\beta 1}$
CaK$_{\alpha}$
CaK$_{\beta 1}$

Figure 2. Electron micrograph of bacteroid (B) within symbiosome (Sy) in a control cell. Polyphosphate bodies (P) are in the center of the bacteroid. bar = 0.15 μm

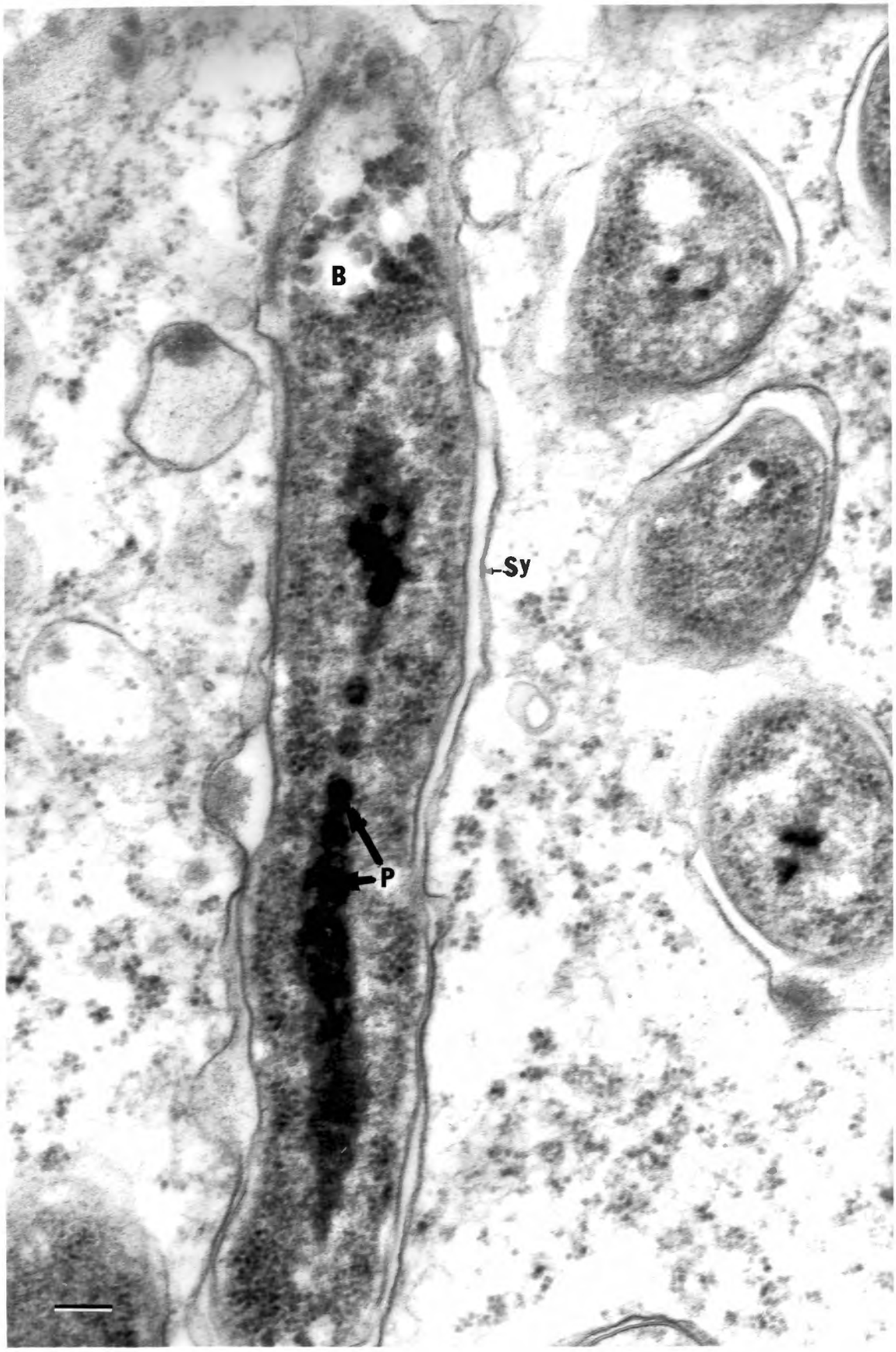
B
Sy
P

Figure 3. Electron micrograph of two 14-day-p.i. untreated infected cells. The nuclei (N) in the top cell are surrounded by several small vacuoles (V). The cell wall (Cw) and numerous symbiosomes, each with only a single bacteroid, are seen. These cells are still young as judged by their total sizes and comparatively few symbiosomes. Mitochondria (M) are just under the plasma membrane. bar = 1 μm

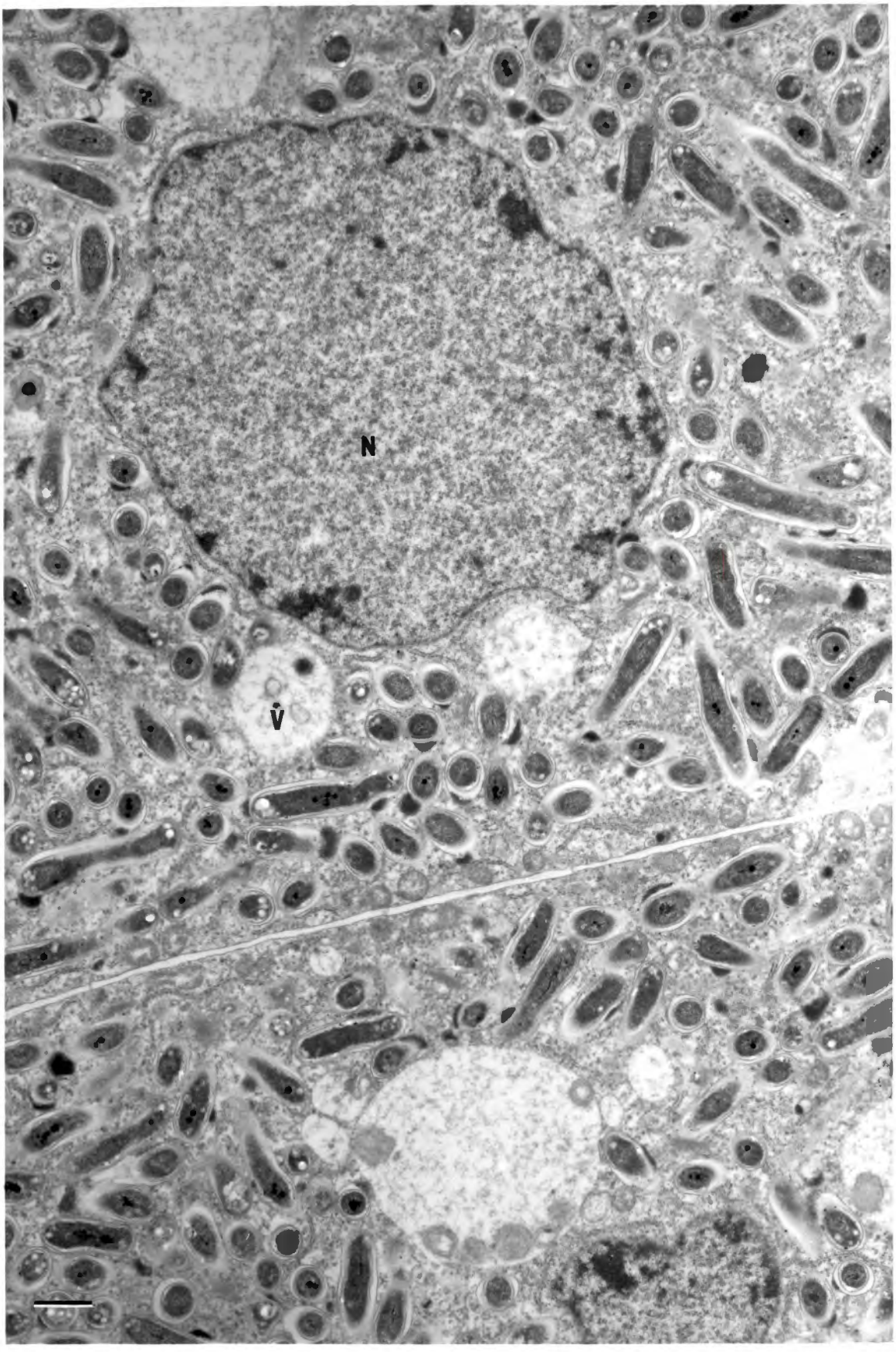
N
V

Figure 4. Electron micrograph of 14-day-p.i. infected cells treated with 10mM $CdCl_2$. The top cell contains a typically large and centrally located nucleus (N). Note the prominent nucleolus (Nu) and numerous clumps of heterchromatin (H). Numerous mitochondria are seen, some not close to the plasma membrane. These are also young early infected cells. Granules (G) can be seen in the cytosol as well as the vacuole (V). bar = 1 μm

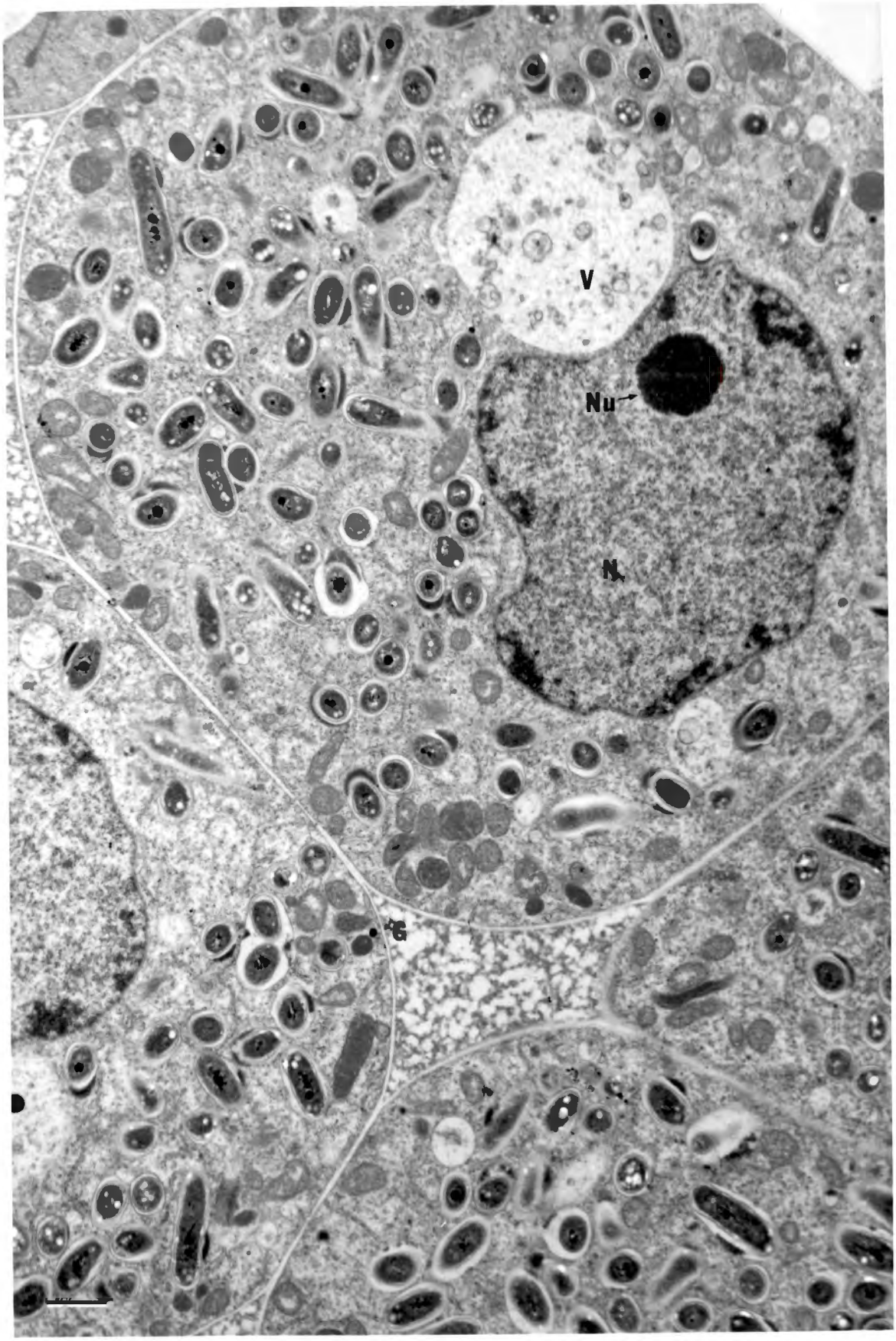
V
Nu
N

Figure 5. Electron micrograph of three 19-day-p.i. infected cells in a nodule from a plant treated with 10 mM $CdCl_2$. Symbiosome membranes are no longer around the bacteroids (B). Note the abnormally large vacuoles (V) with fibrillar material (M), which contained no metal component when examined with X-ray microanalysis. Part of an uninfected cell is included (U). bar = 1.5 μm

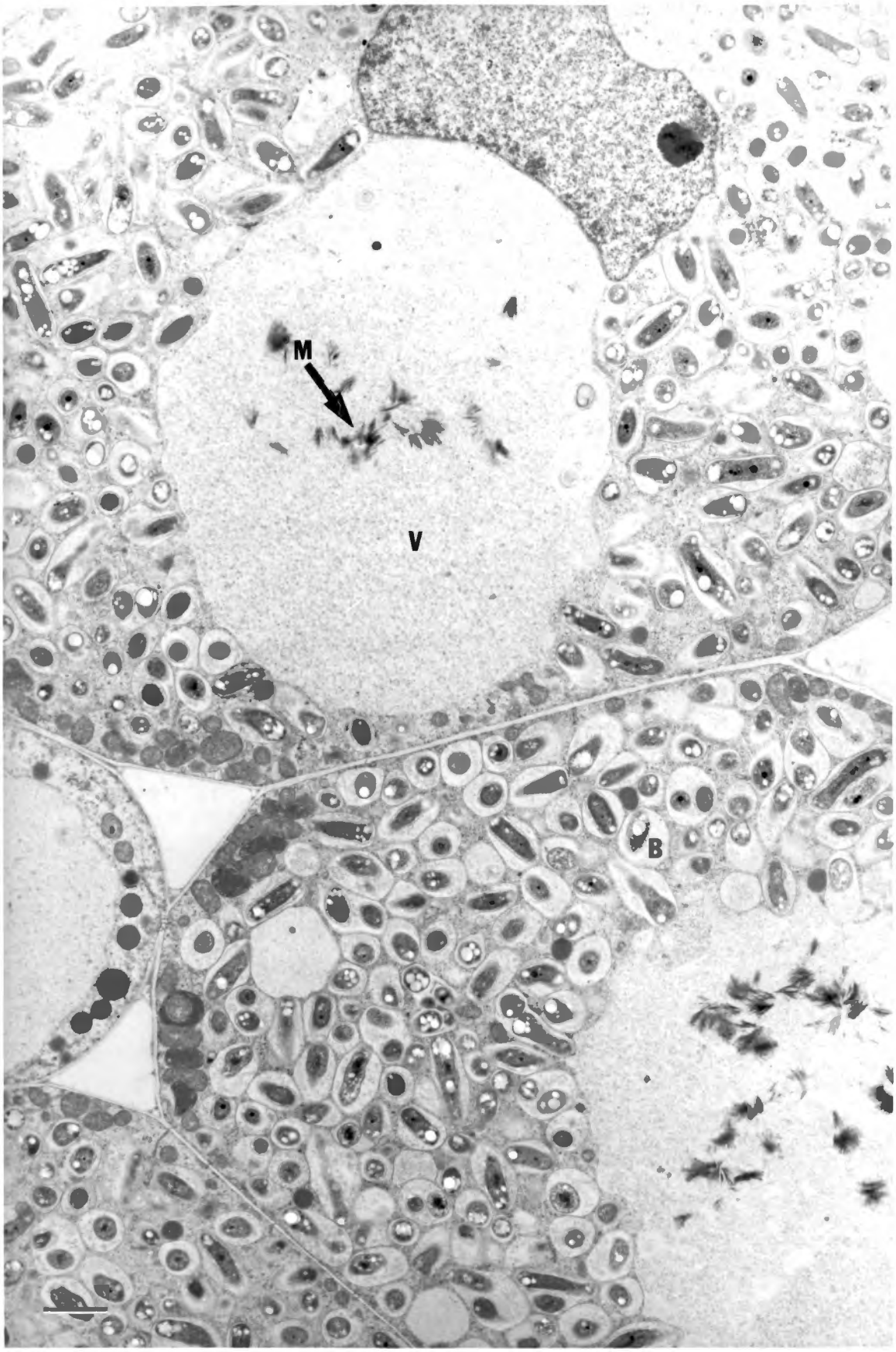
M
V
B

Figure 6. Electron micrograph of a 19-day-p.i. cell treated with 10 mM $CdCl_2$ showing further signs of degradation. Note the large symbiosome space (Sy) around the bacteroids (B), and areas (A) of the cytoplasm devoid of symbiosomes and sometimes may or may not be densely packed. bar = 1.3 μm

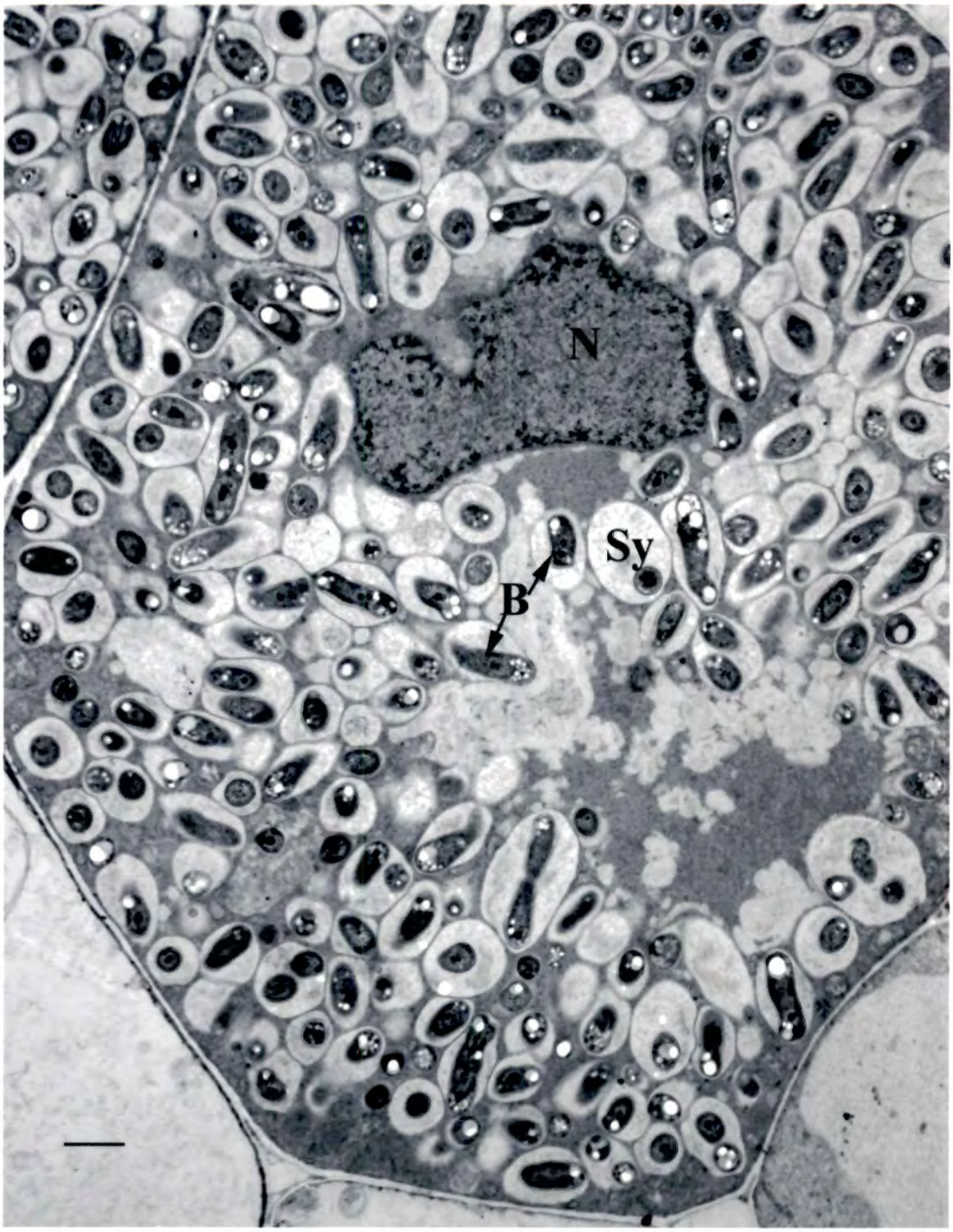
N
Sy
B

Figure 7. Electron micrograph of an area from a 28-day-p.i. infected cell that had been treated with 10 mM $CdCl_2$. Bacteroids free from the symbiosome. Note that the bacteroids are arranged into arrays and not enclosed in a membrane. This is an unstained and a much thicker section than others with the result that superimposed bacteroids may be seen (B). It may be more appropriate to refer to the organisms as "bacteria" because organisms released from symbiosomes and found in senescing cells may be able to return to the bacterium form.

bar = 0.4 μm

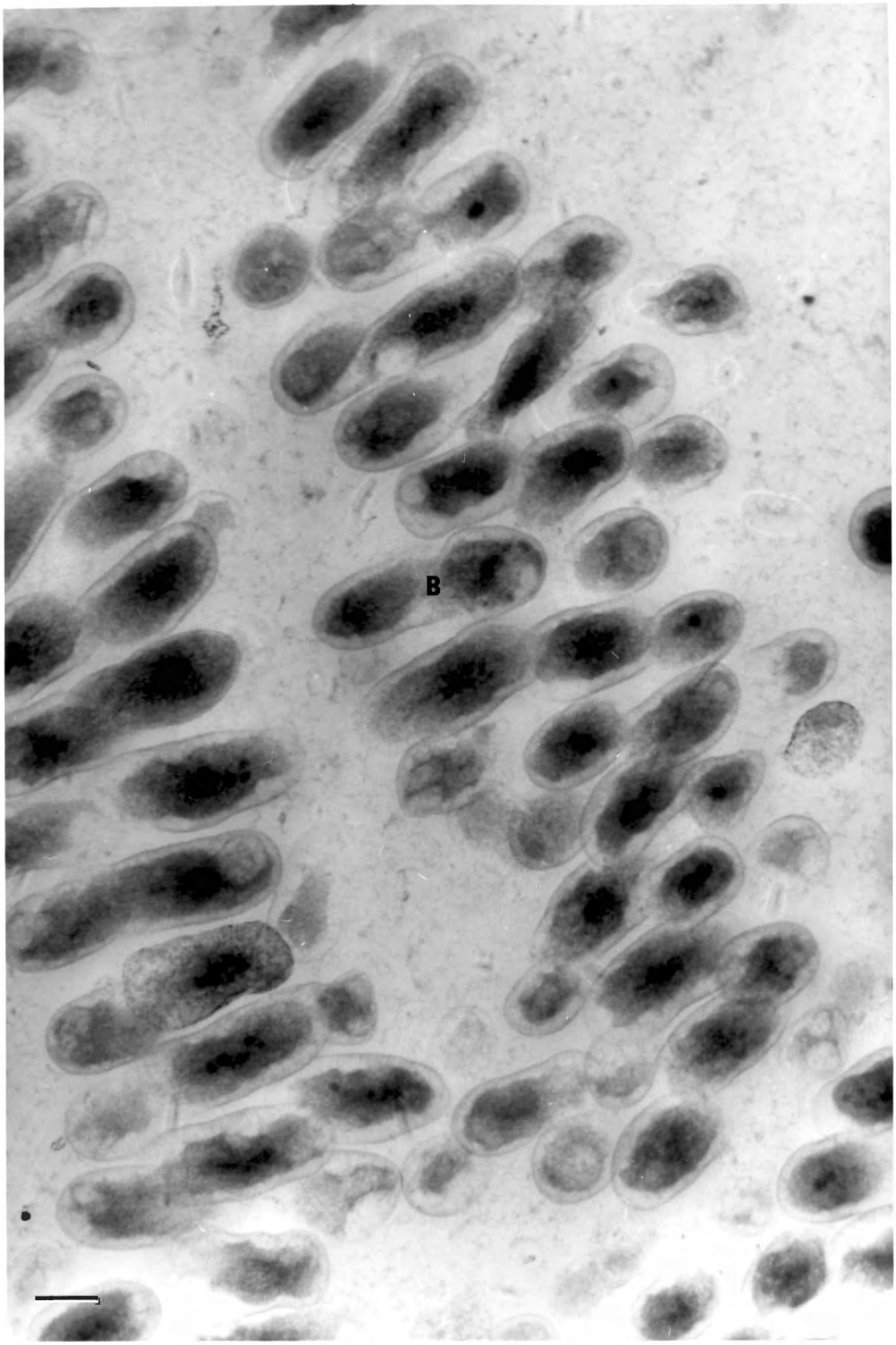
B

Figure 8. Electron micrograph of an area from a 28-day-p.i. infected cell. Lower magnification than Figure 7. bar = 2 μm

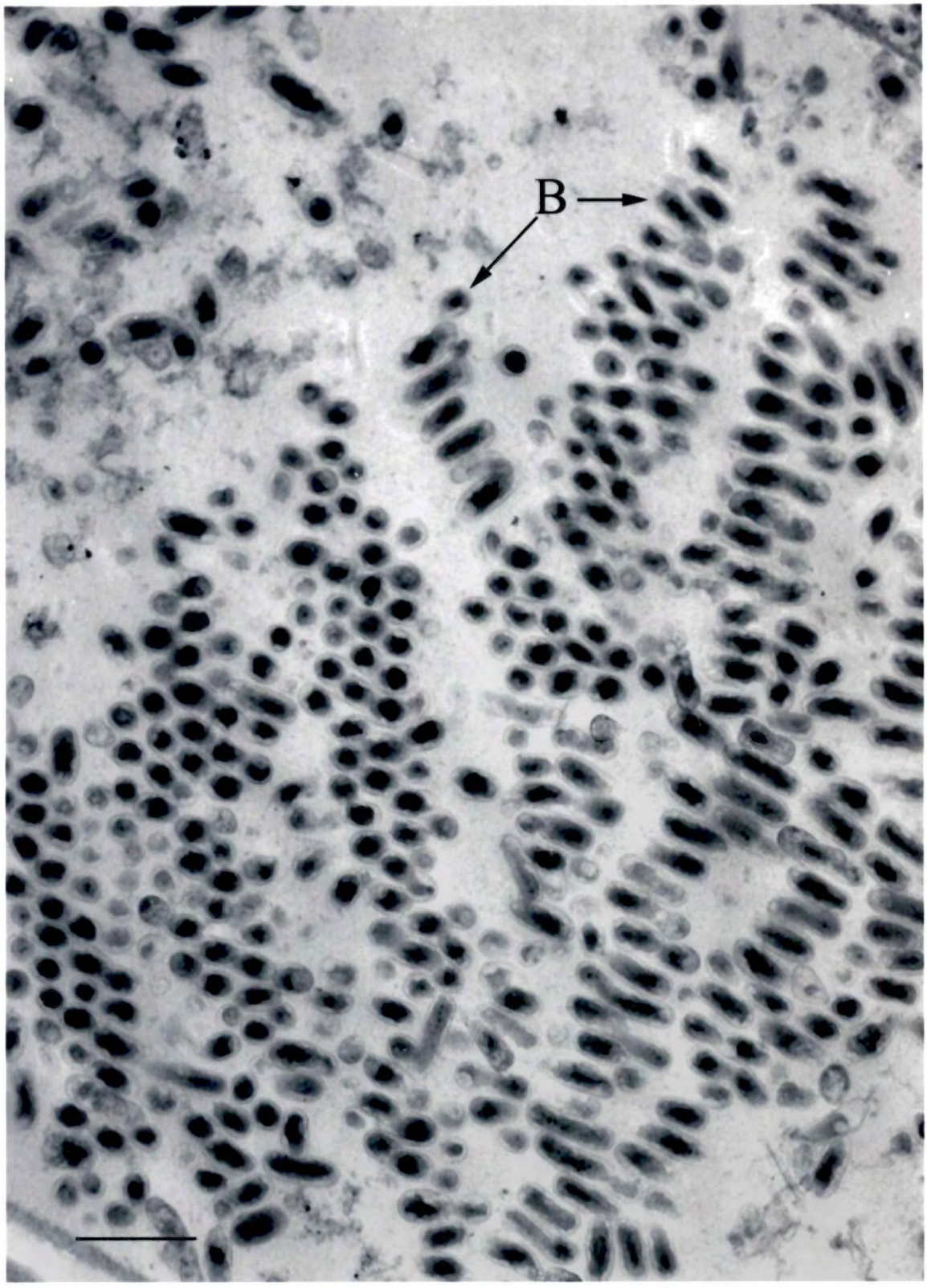
B

Figure 9. Electron micrograph showing cytosolic granules (G). bar = 0.2 μm

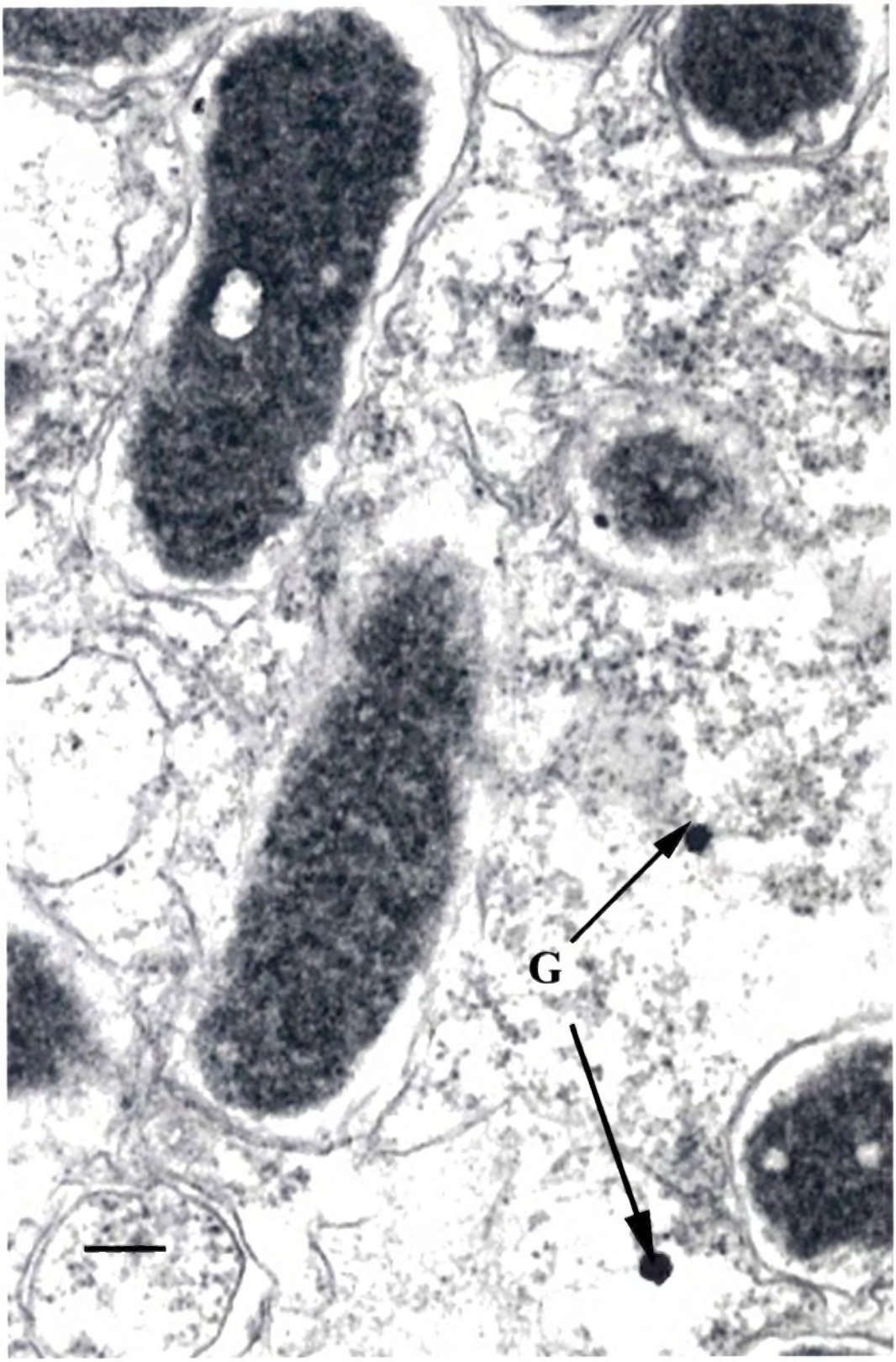
G

Table 2. Relative frequency of Cd present based on days p.i.

	Days p.i.	
	14	19
Cell Wall	1.5% (1/65)	20% (13/65)
Cytoplasm	5% (3/67)	24% (16/67)
Nucleus	1.7% (1/58)	22% (13/58)
Amyloplast	11% (3/28)	14% (4/28)
Vacuole	5% (1/22)	14% (3/22)
PPB	35% (9/26)	31% (8/26)

3.4 Discussion

The relevant subjects are organized here in four areas. First, what is the cell biology of Cd pathogenicity in soybean and other legume nodule cells? Second, what is the significance of Cd being found in control-cell structures? Third, does the regular co-localization of Ca and Cd demonstrated in this study indicate a relationship between Cd toxicity and Ca metabolism in soybean nodule cells? Fourth, what is the broader significance of Cd being found in soybean cells?

3.4.1 Cadmium Pathogenicity to Soybean Nodules

This study demonstrates that soybean nodules are extensively damaged by Cd in several microscopically recognizable ways that ultimately result in total disruption of cellular organization. How might nodule cells react to the presence of Cd and what protective mechanisms are used by the cells?

This study focuses on the importance of cellular mechanisms of inactivation by sequestration and attempts to identify the numerous sites that nodule systems use for such detoxification. I believe that when these methods are overtaxed, toxic damage results in cessation of nitrogen fixation, generalized phytotoxicity, and host-cell death.

Pathogenicity is indicated by a study of events over the course of time. Nineteen-day p.i. nodules show much more cellular damage than 14-day p.i. samples which are virtually indistinguishable from their non-treated counterparts. This is an expected observation since older samples should have absorbed a higher Cd load. In addition, Cd effects on cellular chemistry are probably additive, requiring a certain threshold concentration to be toxic. It is indisputable that Cd is present throughout the cells at 14 days p.i., since it appears in the functionally innermost part of the nodule, the PPB.

Cadmium binds to metabolically sensitive sites, e.g., nuclei, cytoplasm and bacteroids, and less metabolically sensitive sites, e.g., cell walls and tonoplasts. Intermediately sensitive sights, e.g., amyloplasts, are also shown to harbor Cd.

Pathogenicity may start with the nuclei, where Cd was found in euchromatin, heterochromatin and nucleoli. Vallee and Ulmer (24) suggest that one of the primary targets of Cd toxicity are the Zn metalloenzymes, including RNA polymerase, DNA polymerase, and proteins containing Zn-fingers (10, 11, 20). It would not be unreasonable to hypothesize that by interfering with transcription and replication of DNA, bound Cd could severely hamper the normal functions of protein synthesis. Unable to produce new proteins, the cell would lose essential functions and eventually succumb to the toxic affects of Cd.

Another metabolically important Cd-binding area is the symbiosome content. I believe this thesis is the first report that Cd binds to the PPB of the bacteroids of nodules. Jensen et al. (9) report the binding of Cd to the PPB of *Plactonema boryanum*. I believe that the findings of this report, in addition to data reported for Al (14) and other heavy metals (9), suggest that the PPB function in detoxification of heavy metals by sequestration. To do so Cd must have traversed the whole nodule, including the infected-cell cytoplasm in sufficient levels to show up in PPB after crossing at least two plant membranes, one cell wall and both membranes of the bacteroid, perhaps entering the bacteroids bound to the small organic acids that serve as their carbon source. Once inside the bacteroid, the PPB then sequesters the metal and prevents it from adversely interacting with proteins in the bacteroid cytoplasm.

Intrasymbiosomal effects of Cd are probably critically important to the viability of the host cells. An important finding of this study is that Cd is detected earliest and strongest in the PPB.

The following sequence of events can be hypothesized for how Cd enters and affects the bacteroids. Cadmium is transported across several membranes including the symbiosome membrane and the two bacteroid membranes to the PPB where it is inactivated by sequestration. However, since the PPB are literally wrapped in bacteroid chromatin, that DNA is also exposed to and binds Cd, so that bacteroid DNA of the chromatin is affected to the extent that DNA function is impaired or inactivated, resulting in metabolic inactivity of bacteroids. By 19 days-p.i., some symbiosomes are beginning to show signs of fusing while others are being disrupted. At some point between 19 and 28 day-p.i., the symbiosome membranes are completely destroyed along with most organelles and the PM. It is not clear whether Cd directly causes the disruption of the symbiosome membrane, but this is likely since Cd has this membrane effect, or if damage results from the indirect involvement of Cd interrupting the function and assembly of symbiosome membrane proteins (both may be occurring). Enzymes similar to those found in lysosomes that are contained within the symbiosome may also play a role (25). It is my contention that certainly by the time the symbiosome membrane becomes disrupted nitrogen fixation is no longer occurring and may have actually stopped at some prior time. The total effect on infected cells is that, first, large amounts of energy are utilized in processing degradation products, and, second, nitrogen fixation is progressively terminated. As this process continues, the infected cells are killed. Though large amounts of Cd remain bound to the PPB, the effects of Cd are not only disastrous to the capacity for nitrogen fixation but also create a large garbage-control burden for the infected cells.

Bound Cd in the cytosol is present in two forms. The first is in cytoplasmic granules, a form in which Cd is likely to be comparatively inert, it is certainly possible that these granules could release Cd back into the cytosol. The formation of granules may be protein-mediated and could represent a type of cellular accommodation to Cd invasion (13, 20). Second, some amount of Cd is undoubtedly present in non-granular form, either

bound to phytochelatins and small organic acids, or as free ions (12, 20). The methods used in my study would probably not detect this Cd since it is dispersed in the cytosol. Such ions are particularly problematic for nodule cells since these ions are able to bind to and cross link cytoplasmic proteins in a non-specific manner and thereby disrupt their function. In fact, Cd's interaction with protein thiol groups is believed to be one of its primary toxic effects on cells (20).

Silverberg et al. (26) reported the binding of Cd to the mitochondria of algal cells. I was unable to detect any such binding, though it may still occur at levels below the sensitivity of the instrument.

Cadmium also binds to some structures which have fewer effects on the metabolic physiology of the cell. These structures include the comparatively inert cell wall, an important detoxifying mechanism, suggested by Wagner et al. (20).

Cadmium was also detected with low frequency in the tonoplast and some smaller vacuoles, typically in the form of granules that appear similar to those found in the cytosol. The comparatively low frequency of Cd detected in the tonoplast is not unexpected since most of this Cd is likely to be in the form of either free ions or as a few atoms bound to individual low molecular weight phytochelatins and other proteins. This dispersed state would remain below the detectability of the technique. In fact, the tonoplast could actually represent the major site of free or lightly bound Cd; Vogeli-Lange and Wagner (19) showed that in tobacco the majority of Cd and Cd-BP were located in the tonoplast.

In summary, Cd appears to be perceived by nodule cells as a massive and multifaceted assault.

Further studies will be useful to improve our understanding of these several modes of detoxification of Cd, the capacity of the plants to detoxify, and the anomalies that result from such mechanisms being used and overtaxed. Still further studies could usefully look at the cell biology of the toxic effects caused by other heavy metals. Toxic effects by

excessive levels of other metals which, in physiologically appropriate levels, are required but in higher concentrations become detrimental. Other studies might focus on numerous hydrocarbon pollutants. Nodules of other legumes should also be studied similarly.

3.4.2 Significance of Cd in Control-plant Nodules

The presence of Cd in PPB and cytoplasm of control plants is an unexpected finding in this study. While nodules have not previously been examined for Cd uptake, background levels have been reported (3) in soybean roots, stems and leaves through the use of radiolabels, centrifugation and chemical extraction. Shacklette (17) states that low concentrations of Cd are probably present in all plants.

What are the possible explanations? First, the mishandling of samples could have resulted in some control plants inadvertently being exposed to Cd or nodules somehow becoming mixed during handling and processing. This is unlikely for two reasons; the samples that showed Cd peaks came from three different experiments grown at different times and represent four different plants, also the Cd levels are below those detected in the treated plants in peak intensity as well as frequency. If they had received treatment or been mislabeled, the Cd levels would have been expected to be the same.

Second, perhaps the nutrient solutions were contaminated with Cd, though this is also unlikely since the media used to grow these plants was made from purified reagent-grade chemicals which should not contain enough Cd to show up in PPB.

The third possibility is that the seeds used contained stores of Cd. I feel this is the most likely source of Cd in the controls. This explanation would follow the pattern indicated for Al in soybean PPB by an earlier study (14) in which Al was localized in the PPB of bacteroids in soybean nodule cells and further localized in ferritin in the amyloplasts of cotyledon cells in the seeds from which those plants had been grown. Unfortunately

time and funding did not allow me to conduct the necessary studies to prove or disprove the presence of Cd in the seeds.

If the seed is indeed the source of Cd in the control, then the plants producing the seed were exposed to Cd. Cadmium contamination of controls suggests that this may be a wide spread but subtle problem. Low levels of Cd in the seeds of test plants could adversely affect the health of the full grown plant, especially when placed under experimental stress. Soybean researchers should also be aware of the possibility that they are working with plants whose metabolism may be adversely affected by Cd-generated stress that would include but not be limited to the demand for metabolic diversion to produce phytochelatins (12), altered DNA properties due to Cd interactions with nucleic acids (10, 11), Cd cross linking of proteins (20, 24), and reduced nitrogen fixation.

3.4.3 Is there a Relationship between Cd Toxicity and Ca Metabolism?

Cadmium is frequently found in association with calcium in treated plants. In fact, Cd is rarely found without Ca; yet, when the relative distributions of the two ions are compared, they differ. Cadmium was heavily deposited in the PPB and more or less evenly distributed in cell walls, cytoplasm, nuclei, amyloplasts and tonoplasts of treated nodules. If Cd behaved exactly like Ca, it would be expected to mimic Ca distribution but it did not exactly match. Calcium showed a higher distribution to both cell walls and PPB, with more uniform distribution to the other areas. When compared to the distribution of Ca, Cd seems to be treated somewhat differently from Ca in the cell. Perhaps this was a technical artifact in which Cd and/or Ca was removed from the cell wall during processing, or perhaps the way Cd is bound in the cell wall does not permit detection. It is also possible that the cell wall is saturated with Ca, at such high Ca levels that most potential-Cd binding sites are already be filled.

I hypothesized at the beginning of this study that Cd interfered with Ca chemistry. However, I feel the data from this study are inconclusive on this point. The literature seems to suggest that interference with Ca physiology is a major pathway in Cd toxicity. Indeed, there did seem to be some correlation between Cd and Ca localization shown here. Certainly where Cd was found, Ca was almost always present. However, when compared to controls, the distribution and frequency of Ca does not change significantly. While this interaction could prove to be significant, the possibility that the data simply are reflecting the fact that Ca seems to appear in many places irrespective of the presence of Cd cannot be ruled out.

3.4.4 Broader Importance of Cadmium Binding to Soybean Plants

I showed not only that nodule cells are damaged, but that, by the later times of my studies, nodules and plants were generally devastated. The agronomic importance of this finding is obvious. Crop yields would be reduced by the lowering of available nitrogen resulting from the early destruction of the nodule.

Is this a problem for agribusiness? Yes, and it is becoming increasingly so because sewage sludge is finding it way to farm fields as efforts to find useful ways to dispose of this waste are sought.

The problem of disposal of consumer Cd wastes has also been exacerbated by uses such as NiCd batteries in an increasing number of products. Newer battery systems are now being developed that use much less toxic chemicals or, if Ni and Cd are used, batteries are rechargeable, better sealed, and disposed of in more environmentally correct ways. These are valuable ways to reduce Cd pollution.

Cadmium is found in human foods. Cadmium in soybeans can be ingested either by being introduced into products in the human food chain or by direct consumption of

soybean products such a oils, and processed products. Clearly, Cd pollution represents a significant problem to our society.

3.5 MATERIALS AND METHODS

Glycine max bv Bragg seeds, obtained from Dr. Peter Gresshoff, were germinated on damp paper towels in the dark for 4 days. Once germinated, seedlings were placed into plastic growth pouches containing 20 mls of Plant Nutrient Solution (PNS) at pH 6.8 (appendix A1). Seedlings were inoculated with *Bradyrhizobium japonicum* USDA 110 and moved to a moderate-light staging area to grow for 1 day. Seedlings were then transferred to the greenhouse where they were supplied with natural and artificial light (approximately 300 μmol m-1 s-1 total light) on a 16 / 8 hour light/dark schedule. A $CdCl_2$ solution was applied to the growth pouches 6 days post inoculation. Treatments included concentrations of 10 mM, 1.0 mM, 0.1 mM and 0 mM. Water and PNS were alternately added to the pouches as needed.

Nodules were harvested at 14, 21 and 28 days-p.i. Once removed from the plant, a small (ca. 0.5 mm) cross section was cut from the middle of the nodule in the presence of a phosphate buffered 1% glutaraldehyde solution (pH 6.8). These nodule sections were then transferred to a fresh phosphate buffered 1% glutaraldehyde solution (pH 6.8) for 1 to 1.5 hours, after which they were transferred to 1% OsO_4 solution in phosphate buffer for 1 to 1.5 hours. The sections were then dehydrated through an acetone step gradient and transferred into Spurr's embedding resin. For conventional transmission electron microscopy (CTEM) ultra-thin sections (ca. 100 nm) were cut using a Reichert OMU3 ultramicrotome, post-stained with uranyl acetate and lead citrate, and then examined with an Hitachi H-600.

For X-ray microanalysis, semi-thin sections (ca. 200- 300 nm) were cut but not post-stained to prevent peak overlap with Cd. The sections were analyzed using an Hitachi H-800 microscope operating in scanning transmission (STEM) mode at 200 KV. The

microscope was equipped with a Link Pentafet X-ray detector which had a silicon nitride window, at a take off angle of 68°. The X-ray data were processed and analyzed using a Link XP3 pulse processor, a 4pi spectral engine, and Desktop Spectral Acquisition (DTSA) software. A total of 15 plants, one nodule per plant, grown over a two-year period of time in 7 separate experiments were sampled; 412 representative spectra were obtained.

Bibliography

1. Cataldo, D.A., Garland, T.R. and Wildung, R.E. 1983. Cadmium Uptake Kinetics in Intact Soybean Plants . Plant Physiol. 73:844-848.

2. Cataldo, D.A. and Wildung, R.E. 1978. Soil and Plant Factors Influencing the Accumulation of Heavy Metals by Plants. Environ. Health Persp. 27:149-159.

3. Collins, F.W., Cunningham, L.M. and Hutchinson, T.C. 1976. Physiological and Biochemical Aspects of Cadmium Toxicity in Soybean. Part II. Toxicity, Bioaccumulation and Subcellular Fractionation of Cadmium in Soybean Plants Grown at Subchronic to Acute Cadmium Levels. Trace Sub. Environ. Health 10:145-166.

4. Davis, R.D. 1984. Cadmium - a Complex Environmental Problem : Part II Cadmium in Sludges Used as Fertilizer. Experientia 40:117-234.

5. Friberg, L., Elinder, C.G. and Kjellstrom, T. 1992. Cadmium, pp. 1-280.World Health Organization, Geneva.

6. Gairola, C.G., Wagner, G.J. and Diana, J.N. 1992. Tobacco, Cd and Health: A Brief Review. J. Smoking Relat. Dis. 3:1-6.

7. Hinkle, P.M., Kinsella, P.A. and Osterhoudt, K.C. 1987. Cadmium Uptake and Toxicity via Voltage-Sensitive Calcium Channels. J. Biol. Chem. 262:16333-16337.

8. Inouhe, M., Ninomiya, S., Tohoyama, H., Joho, M. and Murayama, T. 1994. Different Characteristics of Roots in the Cadmium-Tolerance and Cd-binding Complex Formation Between Mono- and Dicotyledonous Plants. J. Plant Res. 107:201-207.

9. Jensen, T.E., Baxter, M., Rachlin, J.W. and Jani, V. 1982. Uptake of Heavy Metals by *Plectonema boryanum* (Cyanophyceae) Into Cellular Components, Especially Polyphosphate Bodies: an X-ray Energy Dispersive Study Water Pollution. Environ. Pollu. 27:119-127.

10. Kang, S. and Wells, R.D. 1994. Zinc Destabilizes DNA Watson-Crick Pairs at AGCT. J. Biol. Chem. 269:9528-9532.

11. Langlais, M., Tajmir-Riahi, H.A. and Savoie, R. 1990. Raman Spectroscopic Study of the Effects of Ca, Mg, Zn, and Cd Ions on Calf Thymus DNA: Bindings Sites and Conformational Changes. Biopolymers 30:743-752.

12. Rauser, W.E. 1990. Phytochelatins. Annu. Rev. Biochem. 59:61-86.

13. Rauser, W.E. and Ackerley, C.A. 1987. Localization of Cadmium in Granules Within Differentiating and Mature Root Cells. Can. J. Bot. 65:643-646.

14. Roth, L.E., Dunlap, J.R. and Stacey, G. 1987. Localizations of Aluminum in Soybean Bacteroids and Seeds. Applied. Environ. Microbiol. 53:2548-2553.

15. Ryan, J.A., Pahren, H.R. and Lucas, J.B. 1982. Controlling Cadmium in the Human Food Chain: A Review and Rationale Based on Health Effects. Environ. Res. 28:251-302.

16. Salt, D. and Wagner, G.J. 1993. Cadmium Transport Across Tonoplast of Vesicles from Oat Roots: Evidence for a Cd2(+)/H(+) Antiport Activity. J. Biol. Chem. 268:12297-12302.

17. Shacklette, H.T. 1972. Cadmium in Plants. U.S. Geol. Survey Bull. 1314-C:1-28.

18. Van Bruwaene, R., Kirchmann, R. and Impens, R. 1984. Cadmium Contamination in Agriculture and Zootechnology. Experientia 40:43-52.

19. Vogeli-Lange, R. and Wagner, G.J. 1990. Subcellular Location of Cadmium and Cadmium-Binding Peptides in Tobacco Leaves. Implication of a Transport Function for Cadmium-Binding Peptides. Plant Physiol. 92:1086-1093.

20. Wagner, G.J. 1993. Accumulation of Cadmium in Crop Plants and its Consequences to Human Health. Adv. Agron. 51:173-212.

21. Wagner, G.J. and Yeargan, R. 1986. Variation in Cadmium Accumulation Potential and Tissue Distribution of Cadmium in Tobacco. Plant Physiol. 82:274-279.

22. Weigel, H.J. and Jager, H.J. 1980. Subcellular Distribution and Chemical Form of Cadmium in Bean Plants. Plant Physiol. 65:480-482.

23. Wilson, D. 1997. Fear in the Fields - How Hazardous Wastes Become Fertilizer. Seattle Times July 3:

Appendix

Table A1. PNS (Plant Nutrient Solution) formulation. From Dr. Gary Stacey's lab.

Reagent	Stock Concentration g/L	Final Concentration ml stock/L	Final Concentration
$CaCl_2$	69.4 g	2 ml	1.25 mM
K_2SO_4	108.9 g	0.8 ml	0.5 mM
$MgSO_4*7H_2O$	308.1 g	0.8 ml	1.0 mM
KH_2PO_4	14.0 g	0.8 ml	0.08 mM
K_2HPO_4	35.9 g		0.16 mM
Micronutrients		0.8 ml	
$CuSO_4*5H_2O$	3.1 g		10 µM
$MnSO_4*4H_2O$	0.56 g		3 µM
$ZnSO_4*7H_2O$	0.29 g		0.8 µM
$Na_2Mo_7O_{24}$	0.001 g		0.7 µM
H_3BO_3	0.39 g		5 µM

Tannin stain as described by .Jensen (ref 11 part 2)

Cut fresh sections and place them in a 10% solution of ferrous sulfate with a little sodium carbonate. A blue-green color indicates the presence of tannins; a brown-red color indicates the presence of oxidized tannins.

Vita

Gerry Sexton was born in the summer in Saugus California. He has spent most of his adolescent and adult life in East Tennessee. He was educated at Cocke County High School, and went on to the University of Tennessee and earned a B.A. degree in biology. After earning this degree, he enrolled in graduate school at the University of Tennessee to study electron microscopy and cell biology.

Gerry spent two summers collecting bats in Indiana and Georgia.

A doctoral degree in Zoology was awarded August 1998.

He is now working at Johns Hopkins University Medical School studying cellular trafficking and its applications in the treatment of breast cancer.