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Intestinal granules found in the parasitic nematodes Ancylostoma caninum, and Oesophagostomum radiatum

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The parasitic nematodes *Ancylostoma caninum* and *Oesophagostomum radiatum* were collected and analyzed for intestinal inorganic granules. Three means of identification were utilized to determine the composition granules, including birefringence, x-ray diffraction and energy dispersive

spectrometric (EDS) analysis. Initial x-ray diffraction results of the two worms showed a calcium sulfide presence within the worms. Closer examination of the granules within *Ancylostoma caninum* however, utilizing EDS analysis revealed their composition to be zinc sulfide. These results concur with those of Rogers (1940) and Clark (1956) who found zinc sulfide granules in several species of *Strongylus*. The ZnS granules seem to be a result of a detoxification function that binds excess zinc and sulfhydryl groups present from the ingestion and breakdown of dietary blood meals.

INTESTINAL GRANULES FOUND IN THE PARASITIC NEMATODES *ANCYLOSTOMA CANINUM*, AND *OESOPHAGOSTOMUM RADIATUM*

by

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INTRODUCTION

There is considerable confusion surrounding the exact composition of intestinal cell granules found in parasitic nematodes. This confusion adds to the problems of understanding the larger picture of the function of these granules, because only in knowing the composition of the granules will the reason for their existence be understood. The function of the granules has special significance in helping us understand different aspects of the nematode's metabolism and life cycle so that potential treatment of the parasitized host can counteract the actions of the worm.

There are many variables involved in identifying the intestinal granule composition. There seem to be different kinds of granules, depending upon the species of worm and the location within the digestive tract of the worm. There is also the possibility that previous experimental techniques have been inaccurate, and a few published works have represented this information. The granules examined in this study are very small (less than one micron in diameter), their numbers within the intestine are limited, and the nematodes themselves are difficult to cultivate. Histochemical tests working with these minute and difficult parameters have produced variable results.

I set out to isolate and analyze the intestinal pigmented granules found in the canine hookworm *Ancylostoma caninum* and the cattle roundworm *Oesophagostomum radiatum*. I utilized three methods to analyze the granules. The first procedure was microscopic analysis of the intestines of the worm using a polarizing microscope. A characteristic property of most

crystalline materials is to divide the light passing through the crystal into two rays: the ordinary and extraordinary rays. The course of the extraordinary ray is deviated from that of the ordinary ray and a polarizing lens is able to detect this deviant ray. The observed effect is called double refraction or birefringence.

X-ray diffraction was the second technique utilized to more quantitatively identify the crystals. This method permits the study of the crystalline order of materials because x-rays penetrate much further through objects than do other forms of radiation. The unknown samples are irradiated with x-rays and the resulting diffraction patterns recorded on film are representative of their respective crystalline structures. These film patterns are then compared to those of known compounds, and identifications are made. Known diffraction patterns from common elements and compounds are referenced and readily available.

The third technique utilized was energy dispersive spectrometric analysis (EDS) in the form of a microprobe associated with a scanning electron microscope (SEM). Energy dispersive spectrometric analysis (or micro-elemental analysis) irradiates the unknown substance with electrons, and characteristic x-rays result from the sample. These x-rays are then converted back to electrons within the microprobe and the total energy level is measured. This energy level is distinctive for the specific element involved. The integrated computer software identifies the resulting peaks and automatically labels the peaks with their respective element. The result is then ready for interpretation. Utilizing these three methods unequivocally identifies the composition of the intestinal pigmented granules of the hookworm *Ancylostoma caninum* to be at least in part zinc sulfide. Possible

functions of the granules are discussed in the text, though it seems most probable that the granules serve a detoxification function to counteract high levels of metallic ions, specifically zinc, that are ingested through their large dietary intake of host blood.

REVIEW OF THE LITERATURE

Intestinal cell granules, found in a various assortment of nematodes, have been a source of intrigue since the turn of the century. The exact composition and function of these cell inclusions have not yet been agreed upon though, and so these granules continue to be researched. Askanzy (1896), Looss (1905), and Faure-Fremiet (1912), were the first to recognize these crystals. They looked at what they termed reddish brown, weakly birefringent, 'sphaerocrystals' located in the anterior portion of the intestines of *Ascaris, Strongylus, Ancylostoma,* and *Trichuris* nematodes. Their conclusions were that the granules were insoluble in water, sodium hydroxide, acetic acid, ethyl alcohol and xylol. They were also not affected by saliva, gastric and pancreatic enzymes. They proposed that the crystals were probably the result of hemoglobin resorption, even though blood consumption by these nematodes had not previously been documented.

Von Kemnitz (1912) with *Strongylus* spp., was the first to suggest that the granules were something other than hemoglobin when he called them zymogen granules, protein precursors found lodged inside the intestinal cells. Quack (1913) also using histochemical studies (in studies of *Toxocara canis*), was the first to discover calcium in the granules and surmised them to be gypsum with the formula $CaSO4 \cdot 2H2O$.

During this same time period, Cobb (1914) found similar granules in the intestinal cells of free living nematodes. In the intestinal cells of *Rhabditis monhystera,* and numerous other species, Cobb found crystallized, bire-

fringent spheres approximately 1-3 microns in diameter. The crystals were said to be found in large groups surrounding the centrally located nuclei of the intestinal cells. They constituted a large fraction of the mass of the cell, however they were slowly soluble in water and insoluble in alcohol, glycerin, xylol, and oils. Cobb assumed these granules to be carbohydrate food storage units even though with starvation he showed no decrease in the number of granules. Cobb named these granules 'rhabditin' (from the original nematode genus in which they were found). He also made a note that with the crystals of rhabditin there often occurred other granular bodies of a different nature, though he added nothing further on these other granules.

The pathogenicity of selected intestinal worms (by the 1920's) was being traced to the blood sucking characteristics of the parasites. Without any direct evidence, Looss (1905) proposed that worms (such as *Ancylostoma*) assimilated red blood cells which became the source of the pigmented granules. In 1911, Ashford and Igaravidez showed the existence of blood in the intestinal tract of *Ancylostoma caninum* . This finding supported the hypothesis that the intestinal granules were possibly composed of hemoglobin, or a breakdown component of it. It was in 1926 that Beller and Balozet independently observed and investigated the hookworm *Bunostomum* in sheep and traced the pathology to anemia, induced by the blood sucking of the intestinal worm (Lucker 1946). By the 1930's, Wells (1931)and Nishi (1933) actually observed blood being sucked by hookworms in an *in vivo* canine model. Red blood cells were found in the intestines of the swine lung nematode *Metastrongylus* by Hung in 1926.

Hoeppli (1927) could not find erythrocytes in*Ascaris*. This initially distinguished *Ascaris* as a non-blood sucking parasite. Hoeppli's conclusion

was supported by Lievre's work in 1934 which found no hemoglobin in the intestines of *Ascaris* using spectroscopic analysis.

The presence and absence of blood in the intestines of several parasites was documented by the reports listed above, and researchers recognized an association between this blood feeding mechanism and the presence of the pigmented granules. Initially , Cobb (1920) noticed the relationship between 'carnivorous' nematodes and more numerous free living species. He noted that birefringent granules are approximately twice as numerous in 'the less common carnivorous nemas' than they are for the 'herbivorous nemas'.

Evidence of the association of the granules with a blood sucking lifestyle was presented in 1930 by Tornquist. He pointed out that adult *Camalanus*, which was known to be a blood sucking parasite, possessed the intestinal crystals while adult *Cucullanus*, a non-blood sucking parasite showed no production of the crystals. Tornquist hypothesized that a direct association existed between granules and ingested blood. Giovannola (1936) noted the same relationship. He observed 'black, residual substances' in the intestinal lumen of *Ancylostoma* and *Necator* hookworms, however none were found in the *Ascaris lumbricoides* worm. He considered this to be disintegrated blood (hematin) resulting from the feeding habits of the adult hookworms. Chitwood and Chitwood (1950) pointed out that adult oxyurids and thelastomatids, which are not blood feeding nematodes, as well as the first three stages of *Strongylus* and *Camalanus,* which are non-blood sucking larval stages, are devoid of these granules. Thus, Chitwood and Chitwood further substantiated Tornquist's proposal.

A relationship existed between blood feeding and the occurrence of the crystals, and researchers began looking for elements found in the composition

of blood that might also occur in the granules, even though a hemoglobin and/ or a hematin granular composition could easily be expected with this hypothesis. Chitwood and Chitwood (1933) first postulated that iron was somehow loosely bound with gypsum in the composition of the granules found in *Strongylus equinus.* Hsu (1938) found erythrocytes in the lumen of *Ancylostoma caninum* and demonstrated iron in the pigmented granules found in the intestinal cells. Rogers (1940) was the first to conclude that larger amounts of zinc and sulfur were present in the granules of *Strongylus* spp., probably in the form of zinc sulfide. He used basic histochemical tests, and agreed that a low level of iron was also present. Clark (1956) using x-ray diffraction patterns, unequivocally showed the presence of beta zinc sulfide as the makeup of these granules in three species of *Strongylus*. This result was later reiterated by Lal and Kumar (1984), however it was not clear if Lal and Kumar were repeating this finding or stating their own experimental results. The zinc findings also concurred with the association between blood feeding and the granules, since zinc is found as an essential constituent of over 100 mammal metallo-enzymes, many of which are found as enzymes in blood and tissues (Sugarman, 1983).

The advent of the electron microscope brought new and improved experimental techniques into play. Objects with dimensions in microns could now be easily observed and their locations pinpointed. Elemental analysis was developed in conjunction with the electron microscope and could be done more precisely and on a microscopic scale. This was a different approach and possibly a marked improvement over the variable results obtained from histochemical tests.

Browne, Chowdhury and Lipcomb (1965) used light microscopy,

histochemical tests and electron microscopic analysis to determine the makeup of pigmented granules from *Ancylostoma caninum*. Histochemical studies using Gomori's method for staining ferric iron failed to reveal iron in the intestinal cells of the worm. However, with bleaching and staining with Prussian blue, the granules did stain positive for iron. The contradictory results led the authors to believe that the iron was masked in some way.

Under the electron microscope, the granules concentric configuration and close association with other structures suggests that they are either modified or synthesized in the cell. A close association with RNA also suggested that they are formed during the process of protein metabolism. Their abundance in the cell led the authors to believe that the granules or a metal-protein complex giving rise to them are stored in the same way that other cells store fat or carbohydrate in readiness for growth. The authors made no mention of the granules possibly being waste product material of no use to the organism, or whether the granules seem to increase with age of the organism.

Lee (1970) employed histochemical, autoradiographic and electron microscopy to study the nature of the granules found in the anterior portion of the intestines of *Ancylostoma caninum*. Lee observed that each granule consisted of two parts: a core and an outer shell. The core material was extractable with sodium hydroxide while the shell was resistant. Starvation of the worm caused diffusion of the core through the porous shell and then degeneration of the core into small particles. Histochemical studies indicated the presence of a specific protein with iron incorporated into it that was experimentally found to be derived from host hemoglobin.

Radioactive iron (Fe-59) was used to label erythrocytes *in vivo* and *in*

vitro. Radioactivity appeared in the region of the pigmented granules. Autoradiography used in conjunction with electron microscopy, showed the radioactivity to be associated with only some of the pigmented granules. Histochemical tests were done on deparaffinized sections incinerated for four to eight hours, and treated with Prussian blue. This procedure gave a positive reaction for iron, which appeared as a bright greenish blue reaction at the location of the pigmented granules. The Perl's method for iron identification, followed by hydrogen peroxide bleaching resulted in very marginal reactions with only a few positive cases for iron. The contrasting histochemical results again make the case for the iron being masked in some way, or a lack of precision within the histochemical tests. Lee concluded that pigmented granules in *Ancylostoma caninum* are not entirely inorganic in nature. He based this conclusion on the partial susceptibility of the granules to the enzyme pepsin, suggesting the presence of a protein. Pepsin is a gastric enzyme that digests proteins into their respective amino acids.

The autoradiography technique upon *Ancylostoma caninum* provided strong evidence for the derivation of iron granules from host hemoglobin. The more recent findings of iron positive pigmented granules in the intestinal cells of the blood feeding *Dirofilaria immitis* (Lee and Miller 1969) and the absence of granules from the larval stages of *Ancylostoma caninum* (Lee 1970) all but proved Tornquist's proposal in 1930 that the presence of this iron-containing pigment or granule is related to the parasitic worms dietary intake of blood.

Lee (1970) presumed the granules functioned either as a nutrient storage and dissimilation system in which protein and iron are the main components or they involved a hydrolytic function where the pigment

inclusions had some enzymatic function. This hypothesis had been first put forth by Weinstein (1966) working with *Nippostrongylus brasiliensis* , who related the pigmented granules to lysosomes. Lee noted that hydrolytic activities were often associated with food vacuoles, pigmented inclusions and other metabolic products.

Jenkins, Erasmus and Davies (1976) also used the electron microscope to study the intestinal cell inclusions of two blood feeding parasites, *Trichuris suis,* and *Trichuris muris*. The authors first noted that the analysis of the composition of the inclusions is limited by the size of the granules, the quantities available for study, and mostly by unreliable histochemical tests for inorganic elements. They concluded that with the advent of the x-ray analysis adaption to the electron microscope, reliable, specific judgements could be made.

The intestinal cells of *Trichuris* contained what the authors termed 'numerous, spherical inclusions' varying in size from 0.25 microns to 0.8 microns, and possessing a concentric lamellar arrangement. Using an elemental analysis adaption to the transmission electron microscope, they found the elemental composition to be largely calcium and phosphorous with traces of magnesium, iron and potassium. The elements magnesium, calcium and iron were not detected in the adjacent cytoplasm indicating the granules differentially accumulated these ions.

A different intestinal granule, apparently unrelated to those previously focussed upon have also been studied in recent literature. Martin and Lee (1975) with *Nematodirus battus* found large, hexagonal crystals in the lumen of the intestine which appeared to be associated with immunity of the nematode within the lamb, and not correlated with the age of the

nematode. The granules ranged in size from 5 - 40 microns in diameter, and were located in the posterior portion of the intestine, increasing in size as they progressed in the posterior position. Using chemical and x-ray elemental analysis, the authors concluded the substances to be lipo-protein with a very high presence of sulfur in the crystals. However, the accelerating voltage used in the experiment did not appear to be high enough to excite elements of higher atomic number, such as zinc (atomic number 30). Martin and Lee assumed the crystals to be metabolic end products of an abnormal metabolism in immune damaged nematodes.

Bird, Walker and Major (1977) observed crystals much like those looked at by Martin and Lee. The crystals were found in the intestinal cells of *Haemonchus contortus* and in the intestinal lumen of *Ostertagia ostertagia*. *Haemonchus* contained crystals located in the distal intestinal cells. The crystals were irregular, hexagonal rod shaped and approximately 3-15 microns in diameter. *Ostertagia* produced much larger crystals, 25-50 microns in diameter, and the crystals were found in the intestinal lumen. The authors assumed the crystals to be products of a degenerative process. Elemental analysis revealed the crystals contained protein and sulfur and were composed in a uniform composition. There was no core of different refractive index. The crystals of *Ostertagia* possessed a higher content of sulfur and were birefringent while those of *Haemonchus* were not birefringent.

MATERIALS AND METHODS

Two species of parasitic nematodes were collected and studied in reference to the intestinal inclusions found within their intestinal cells. The two species: *Oesophagostomum radiatum* found in the colon of cattle, and *Ancylostoma caninum* found in the small intestine of the dog, are both blood feeders in their adult stages.

Oesophagostomum radiatum

Oesophagostomum radiatum were collected at the veterinary research facility on the Oregon State University campus. Six cattle were killed and their intestinal organs were removed. The contents of the large intestine and cecum were collected and the intestinal lining was gently scraped clean. Using a 100 mesh screen, the contents (approximately two gallons) were filtered for nematodes yielding one hundred adult *Oesophagostomum.* The worms were bottled fifty to a vile which were filled with chloroform. The samples were drained in twenty four hours and refilled. Samples from the first vile were removed in four days and dried in an incubator at 37 degrees C.. The samples were pulverized with a small mullite mortar and pestle. These ground worms were then loaded into a thin, boron micro-tube specifically designed for x-ray diffraction use. The diameter of the tube was 0.7 microns (sample #1).

Specimen from the second vile were placed in groups of four, six, and ten, with the worms oriented in the same direction, and dried in that

configuration. These samples (#2, #3 &#4) were then mounted on brass stubs with Duco-cement. Each of these representations of *Oesophagostomum* were then mounted in the x-ray diffraction camera, and irradiated. The x-ray powder camera was a Norelco (model #52019/0), made by the Philips Electronics Company and owned by Physics department of Portland State University. It was irradiated at fifty kilovolts, twenty milliamperes, and variable exposure times. In the case of whole worms (vile #2), the x-ray beam was focussed upon the anterior portion of the worms (just below the esophagus) to increase the probability of the beam interacting with the crystals. This whole worm irradiation method was taken from Clark (1956) on his work with *Strongylus* spp..

Ancylostoma caninum

Species of *Ancylostoma caninum*, a blood sucking hookworm found throughout the jejunum of its canine host were the second species used for experimentation. Five thousand infective third stage larvae (L3) were administered to a dog via a cutaneous mesh patch adhered to a shaved area on the back of the dog. The hookworm larvae were donated by Byron L. Blagburn of Auburn University. A fecal sample was taken to identify the presence of any other parasitic infection, and a slight *Toxocara canis* infection was noted, though not deemed serious enough to influence my experimentation.

Hookworm egg counts were monitored throughout the infection utilizing a sucrose flotation assay. The assay utilized Sheather's sucrose solution to float less dense nematode eggs to the surface after centrifugation. This process was repeated three times to ensure an accurate representation

of eggs present. The egg count peaked on the eighth week, registering 1300 eggs per gram feces, with the simultaneous *Toxocara* infection at about 175 eggs per gram feces. One week after these counts were taken, the worms were harvested and immediately frozen at -20°C. The total number of *Ancylostoma* frozen was 137, along with 73 *Toxocara* worms.

Several worms were initially taken out of deep freeze and viewed under a light microscope utilizing a polarizing lens to observe the presence of birefringence. The worms were not manipulated in any fashion for this process (which was also true for the *Oesophagostomum*). The second analytical method, however, required much preparation of the worms. Four completely independent *Ancylostoma samples* were prepared for x-ray diffraction analysis.

The first manipulation entailed a whole worm analysis. Six complete worms were combined, again one on top of another, dried, and mounted onto a brass stub which was placed in the x-ray powder camera. The particular sample was titled *Ancylostoma* #1, and was irradiated twice using two different exposure times of four and a half, and six hours to produce two separate diffraction patterns. The second manipulation of *Ancylostoma caninum* for x-ray powder diffraction (*Ancylostoma* #2), involved grinding up six adult worms using the mortar and pestle. The sample was finely ground and then loaded into the thin boron tube specifically designed for x-ray diffraction work. The powdered sample in this tube was irradiated twice for six and ten and a half hours. The third preparation (*Ancylostoma* #3), involved dissecting six *Ancylostoma* intestines which were then placed in a dissecting slide containing water, sucked up into a capillary tube, and left to settle out. The tube was then plugged at one end with Duco-cement and left

to dry. One week later, the little water that was left was pipetted out and the six intestines were irradiated within the tube. The fourth *Ancylostoma* preparation (*Ancylostoma* #4), consisted of collecting the anterior portion of fifteen intestines. The intestines were grouped together, dried and then irradiated at two separate exposures, for six and a half, and twelve hours.

Ascaris suum samples were also studied as a negative control (parasitic nematode known not to possess the intestinal granules). The nematode was collected at the Oregon Chief slaughterhouse in North East Portland. The ascarids were found in the small intestines of swine and were collected at the site and placed into a saline solution. In the laboratory, the nematodes were washed with deionized water, drained, and immediately dried. Two of the adult ascarids were then ground by the mortar and pestle, and loaded into the boron x-ray powder microtubes. The sample was then irradiated for six hours.

Sphalerite, a naturally occurring mineral composed of beta zinc sulfide was utilized as a standard in which to compare diffraction results. The sample was donated by the Geology department of Portland State University. A small enough piece was obtained to be crushed in the mortar and pestle. It was then finely ground so that it too could be loaded into a boron microtube and irradiated. This particular sample was exposed for one hour and three hours time periods.

X-ray diffraction patterns from irradiated specimens were exposed on Kodak direct exposure diagnostic film (DEF-392) and developed in the dark room, mounted upon a light board, and measured precisely with a pair of calipers. Measurements were then converted to working equivalents, termed D-spacings. D-spacings are the interplanar spacings (in angstroms) for the

angle 2θ converted from the radius, in centimeters, of the given spectrum. Each line is subjectively measured on its intensity (arbitrarily giving the brightest line a 100% measurement), and these two data points are used in comparing x-ray diffraction results. (Intensities will be written following the appropriate D-spacing, in parentheses). This information was used in analyzing the different spectrums.

Developing time for the x-ray diffraction patterns followed a temperature:developing time curve with colder temperature baths receiving longer developing times. Identically irradiated samples were developed at different points on the curve to verify the curves validity, and to see if the temperature of the developer had any effect upon identical D-spacings. The last control was a capillary tube filled with Duco-cement. These materials are frequently used and should be analyzed to see if any crystalline structure exists within them.

Energy Dispersive Spectrometric Analysis (EDS)

The frozen *Ancylostoma caninum* had to be specially prepared for the electron microprobe analysis. Six intestines were dissected out and mounted onto a precisely machined aluminum stub, adapted for the ISI SS40 scanning electron microscope (SEM). The SEM utilized was also the property of the Portland State University Physics Department. The EDS microprobe was a Link 10,000 with a silicon detector. The platform on which the specimens were placed was at 45 degrees and the accelerating voltage was at 20 kilovolts (kv). The high voltage is necessary to excite elements with high atomic numbers such as zinc. However, resolution was much clearer at lower voltages, and so five or ten kv was utilized for photographs.

The first elemental analysis was on anterior intestines of *Ancylostoma caninum* that had not been teased apart, but rather left whole upon the aluminum stub. The exterior surface of the intestines was scanned and an elemental description was taken. This first scanning survey focussed upon the intestines in general and anything out of the ordinary protruding from the intestines. Pictures were taken to compare with the elemental description. This output came in the form of area graphs consisting of the x-axis, representative of the elements characteristic energy, and the y-axis, representative of radiation counts. Specific peaks were analyzed by the computer software, and automatically labeled with the appropriate element (representative of the atomic number). All curves showed some level of background noise that was estimated by a normal curve and subtracted from peak values (Dash, personal communication). This manipulation accurately depicted the size of the peaks, though only K alpha energies were utilized in representing the data. K beta energies and corrections for K beta overlap were not transcribed. No strict quantitative levels were being sought, only qualitative measurements of existing elements. An example of the original data and the analytical method utilized to produce the graphs herein is found in the appendix.

Upon completing the scanning survey, these intestines were teased apart with a micro-scalpel. The individual granules were then focussed upon by the SEM and photographed. Their elemental compositions were analyzed simultaneously. The electron beam was focussed upon a field approximately ten microns in diameter. In this initial granule survey, five different areas were scanned of which three were simultaneously photographed.

The next objective was to directly compare the intestinal granule

composition with a known similar material under identical conditions. A finely ground sample of sphalerite was placed on the same aluminum stub as were the intestines. The sphalerite did not adhere as well as the moist intestines did and had to be mounted. Two samples were prepared for mounting: one utilizing double stick tape and the other using colloidal graphite. The complete stub was then carbon coated (plated) to ground the specimens. The sphalerite (on double stick tape) was initially photographed and then analyzed at four distinct places to observe if the output varied. The beam was then moved and focussed upon the intestinal samples for a photograph and analysis.

Zinc sulfide powder (mw=97.45) was then mounted to the same stub on double stick tape, and carbon coated. The sample was then analyzed at two distinct locations. These were directly followed by two more analyses of both sphalerite (on colloidal graphite) and the intestinal granules. A third analysis was done of one specific granule; concentrating the electron beam directly into the granule, as opposed to scanning a field of granules.

RESULTS

Birefringence

Crystalline birefringence was positive for the granules in the intestines of both *Oesophagostomum* and *Ancylostoma*. This led directly to x-ray powder diffraction testing and eventual EDS elemental analysis.

X-ray Powder Diffraction

Oesophagostomum radiatum. The first set of x-ray diffraction patterns obtained were from *Oesophagostomum radiatum*. This species first sample (#1) was irradiated for six and a half hours and produced spectrum diameters of 6.33cm, 9.03cm, and 11.15cm. These distances convert to D-spacings of 2.83 (100%), 2.01 (80%) and 1.65 (60%), respectively. The sample #1 also showed two wide, non-distinct bands with approximate D-spacings of 9.50 and 4.23.

Oesophagostomum radiatum sample #2 was irradiated for six hours. The results were non-determinable (spectrum lines were not present). The sample was possibly negative for granules, though the film may have been developed incorrectly.

Oesophagostomum radiatum sample #3 was irradiated onto two separate film segments, the inner layer being a layer of x-ray film exposed at a 180 degree angle for a five hour period. The outer layer was irradiated for the same five hour period, but was a longer piece of film covering a 360 degree angle. The data for the first five hour exposure produced D-spacings

and intensities of 2.86(100%), 2.03(80%), 1.65(60%). Wide, non-distinct bands were again present at approximately 4.37 and 10.52. The first layer was the only layer to produce a spectrum. The second layer of film possessed bands but they were too light to measure.

Oesophagostomum radiatum #4 was irradiated for seven hours and provided a high resolution of bands. The five distinct bands were measured to be 2.90(100%), 2.05 (70%), 1.68(30%), 1.29(20%), and 1.45(10%). The two wide, amorphous bands were again present however, measurements were not recorded. Table I contains a compulation of *Oesophagostomum* results.

TABLE I

X–RAY POWDER DIFFRACTION MEASUREMENTS
OF FOUR SEPARATE SAMPLES OF
OESOPPHAGOSTOMUM
RADIATUM.

SAMPLE *Oesophagostomum radiatum*	D–SPACINGS (ANGSTROMS)	RELATIVE INTENSITY
#1	2.83 2.01 1.65 (Non-distinct bands at 4.23 and 9.50)	100 80 60
#2	Non-determinable	
#3	2.86 2.03 1.65 (Non-distinct bands at 4.37 and10.52)	100 80 60
#4	2.90 2.05 1.68 1.29 1.45 (Non-distinct bands present, but not recorded)	100 70 30 20 10

<u>*Ancylostoma caninum.*</u> *Ancylostoma caninum* sample #1 (whole worms) was the first of the hookworm samples to be exposed to the x-rays. Irradiation for

six hours showed poor resolution with only two bands present. These bands possessed D-spacings of 2.90(100%), and 2.00(70%). Two non-distinct bands were again present at 4.07 and 9.30. A four hour irradiation produced results of 2.84(100%) and 2.00(70%), with two more diffuse bands produced at approximately 4.37 and 10.39.

Ancylostoma caninum sample #2 (ground worms) was irradiated for six and ten and a half hours. Six hour exposure yielded two bands, 2.84 (100%) and 2.00(70%). However, ten and a half hour exposure yielded four bands: 2.82(100%), 2.02(70%), 1.25(30%), and 1.64(25%). The six hour sample was repeated to verify the results and identical spectra were recorded. Six *Ancylostoma caninum* intestines were then dissected out to more specifically irradiate the granules within *Ancylostoma caninum* (sample #3). A nine hour irradiation was first done and no bands appeared on the film. The x-ray beam was readjusted to focus upon the small sample and exposed again for five and a half hours. D-spacings were recorded at 1.96(100%) and 1.89(60%). The second spectrum (1.89) was extrapolated due to the presence of only one line on the x-ray pattern.

Ancylostoma caninum sample #4 increased the number of dissected intestines to fifteen. The first irradiation of #4 lasted six and a half hours and produced four spectra at 3.07(100%), 1.89(50%), 1.20(50%) and 1.61 (30%). After the twelve hour irradiation, the D-spacings read 3.07(100%), 1.90(75%), and 1.63(50%). The third irradiation utilized two layers of x-ray film, again for varying exposures. The inner layers produced four spectra: 3.08(100%), 1.90(50%), 1.64(25%) and 1.21(5%). The outer layer produced three bands with spacings at 3.09(100%), 1.89(50%), and 1.64(30%). Table II contains a compilation of *Ancylostoma* results.

TABLE II

X–RAY POWDER DIFFRACTION MEASUREMENTS
OF FOUR SEPARATE SAMPLES OF
ANCYLOSTOMA CANINUM.

SAMPLE *Ancylostoma caninum*	HOURS EXPOSED	D–SPACINGS (ANGSTROMS)	RELATIVE INTENSITY
#1a	6	2.90 2.00 (Non-distinct bands present at 4.07 and 9.30)	100 70
#1b	4.5	2.84 2.00 (Non-distinct bands present at 4.31 and 10.40)	100 70
#2a	6	2.84 2.00 (Non-distinct bands present, but not recorded)	100 70
#2b	10.5	2.82 2.02 1.25 1.64 (Non-distinct bands present, but not recorded)	100 70 30 25
#3a	9	Non-determinable	
#3b	5.5	1.96 1.89	100 60
#4a	6.5	3.07 1.89 1.20 1.61	100 50 50 30
#4b	12	3.07 1.90 1.63	100 75 50
#4c	8	3.08 1.90 1.64 1.21	100 50 25 5
#4d	8	3.09 1.89 1.64	100 50 30

Controls and Standards. The third group of x-ray diffraction patterns were those of the controls and standards of the same possible composition as the unknowns. Published diffraction pattern results came from the Powder Diffraction File, Inorganic Volume. This file lists all the diffraction patterns from which comparison of unknown patterns can be made. The first pattern transcribed was that of beta zinc sulfide (ZnS), and five D-spacings were given: 3.12(100%), 1.91(51%), 1.63(30%), 2.71(10%), and 1.24(9%). The mineral sphalerite (obtained from the Portland State Geology Department)

was ground down and irradiated to produce the following spectra: 3.10(100%), 1.90(80%), 1.62(70%), 1.78(50%), and 1.35(30%). The file was then checked for similar results to those that were seen and two compounds emerged: Calcium sulfide (CaS) with a spectra of 2.85(100%), 2.01(70%), 1.64(20%), and 1.27(20%); and KZnFe3 with a spectra of 2.87(100%), 2.03(60%), 4.06(40%), and 1.66(30%).

Controls were done to check the accuracy of the technique, and to guarantee the diffraction patterns were actually those of the worms. Table III contains a compilation of the results from these controls, and also the measurements of known standards. The first control irradiated intestines of

TABLE III

X–RAY POWDER DIFFRACTION MEASUREMENTS OF CONTROLS AND CLOSEST KNOWN STANDARDS.

SAMPLE	D–SPACINGS (ANGSTROMS)	RELATIVE INTENSITY
*CaS**	2.85	100
	2.01	70
	1.64	20
	1.27	20
*$KZnFe_3$**	2.87	100
	2.03	60
	4.06	40
	1.66	30
	1.43	30
*ZnS**	3.12	100
	1.91	50
	1.63	30
	2.71	10
	1.24	9
SPHALERITE (beta ZnS)	3.09	100
	1.91	80
	1.63	70
	1.78	50
	1.35	30
CAPPILARY TUBE and DUCO CEMENT	(No spectra-1 non-distinct band present)	
ASCARIS SUUM	(No spectra- 2 non-distinct bands present)	

a parasitic nematode (*Ascaris suum*) which was known not to contain these granules. This produced no distinct diffraction patterns. A capillary tube with Duco-cement (the adhesive utilized in the experiment) was irradiated and also produced no distinct diffraction patterns.

Temperature changes in the development process were also observed for their possible production of variability among diffraction patterns. A zinc sulfide powder sample was irradiated three times under exact conditions and developed at variable temperatures. The results showed little variance with slightly better precision at the colder temperatures, and longer developing times. Intensities, observed subjectively, also varied slightly (Table IV).

TABLE IV

ZNS X-RAY POWDER DIFFRACTION MEASUREMENTS
AT VARYING DEVELOPMENT TIMES
AND TEMPERATURES.

	Developing Temp: Time		
D-Spacings in Order of Appearance	17°:6.75 min.	20°:5.0 min.	25°:3.25 min.
	1.63 (70%)	1.62 (60%)	1.63 (70%)
	1.77 (20%)	1.76 (30%)	1.77 (30%)
	1.91 (90%)	1.91 (90%)	1.90 (90%)
	1.96 (80%)	—	—
	2.94 (60%)	2.93 (80%)	2.92 (60%)
	3.12 (100%)	3.11 (100%)	3.13 (100%)
	3.31 (50%)	3.29 (70%)	3.36 (50%)

Energy Dispersive Spectrometric Analysis (EDS)

Energy dispersive x-ray analysis provided immediate and clear compositional data. The outer surface of the intestines, during scanning,

showed high levels of sulfur, phosphorous, calcium, potassium, silicon, and chloride, with concentrations decreasing in this respective order. Aluminum was also present , though considered a product of the mounting stub used in the experiment (figure 1).

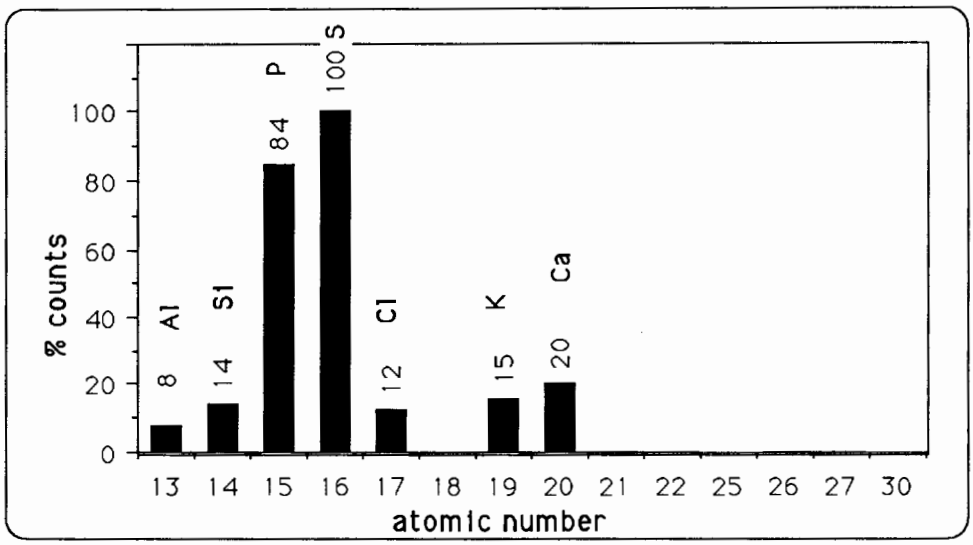

Figure 1. Elemental (EDS) analysis of intact intestines.

A particular protrusion was also observed and analyzed among the scanning of the outer surface. The particle (approximately 3 microns in diameter) produced an extremely high level of sulfur, phosphorous, and silicon, with smaller amounts of calcium, potassium, iron and zinc (figure 2 and 3). Aluminum was also identified in the output .

A completely different elemental analysis emerged after teasing open the intestinal cells and analyzing the intestinal granules. Sulfur and zinc dominated the elemental output in a proportion of about three counts sulfur to one zinc. Phosporous, chloride, calcium, and copper were also recorded at very low levels (figures 4 and 5).

A photograph of the cytoplasmic granules (which seem to coat the

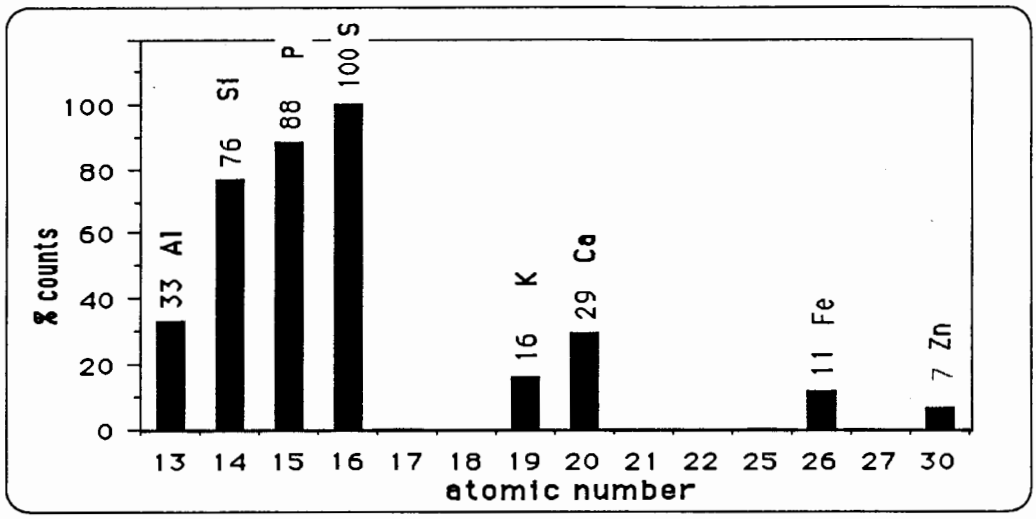

Figure 2. Elemental (EDS) analysis of surface protrusion.

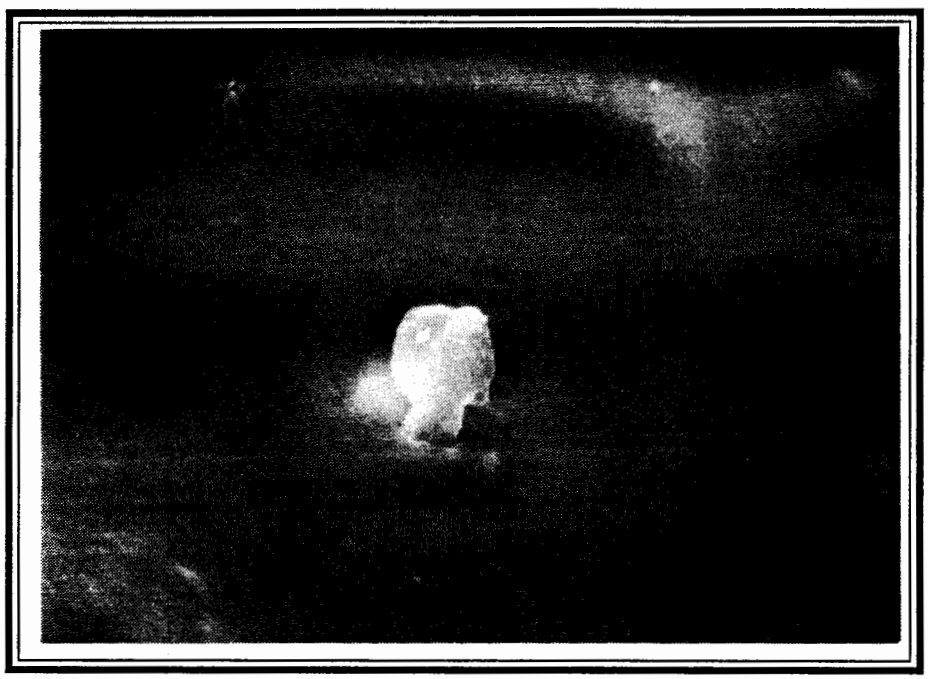

Figure 3. Electron micrograph of surface protrusion (4,000x).

inner intestinal wall) was taken. The circular to oval granules (approximately 0.5-0.8 microns in diameter) arrange themselves into groups forming confluent layers in the area they are found. A second elemental

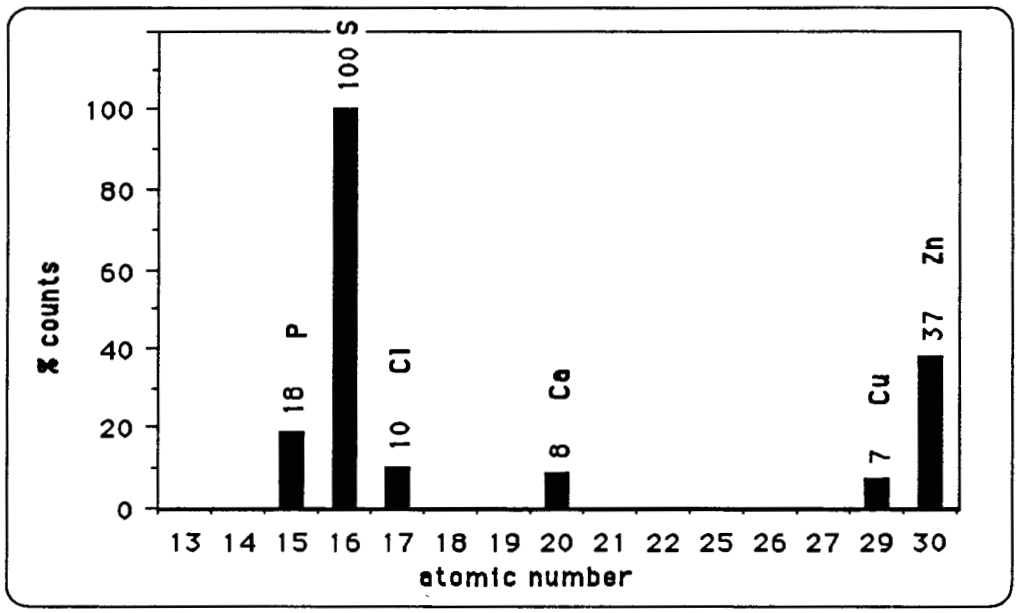

Figure 4. Elemental (EDS) analysis of selected intestinal granules.

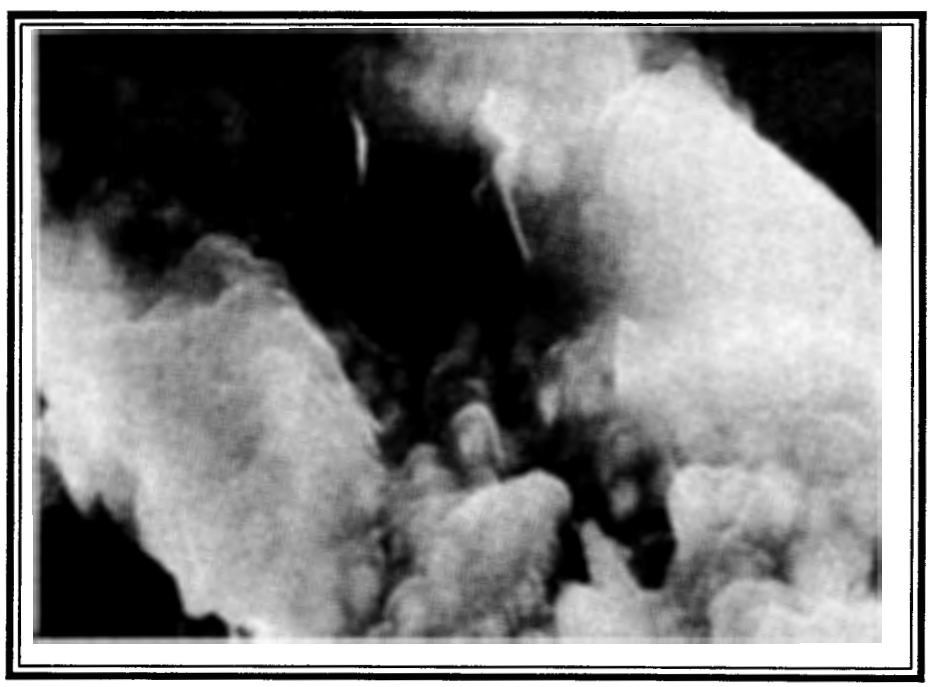

Figure 5. Electron micrograph of selected intestinal granules (6,500x).

analysis of this particular arrangement of granules produced a sulfur to zinc ratio of over four counts to one, respectively, with much more prominent levels of phosphorous, and aluminum, and lower levels of calcium, and iron (figures 6 and 7).

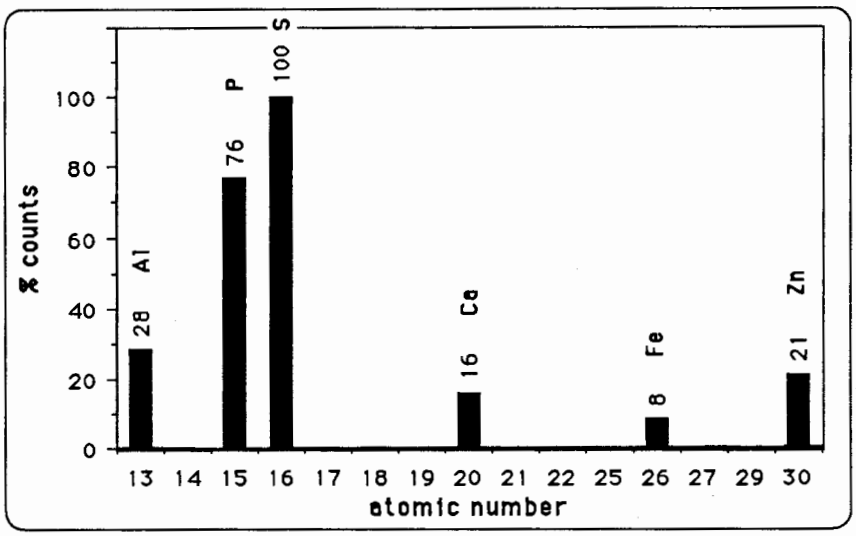

Figure 6. Second Elemental (EDS) analysis of selected intestinal granules.

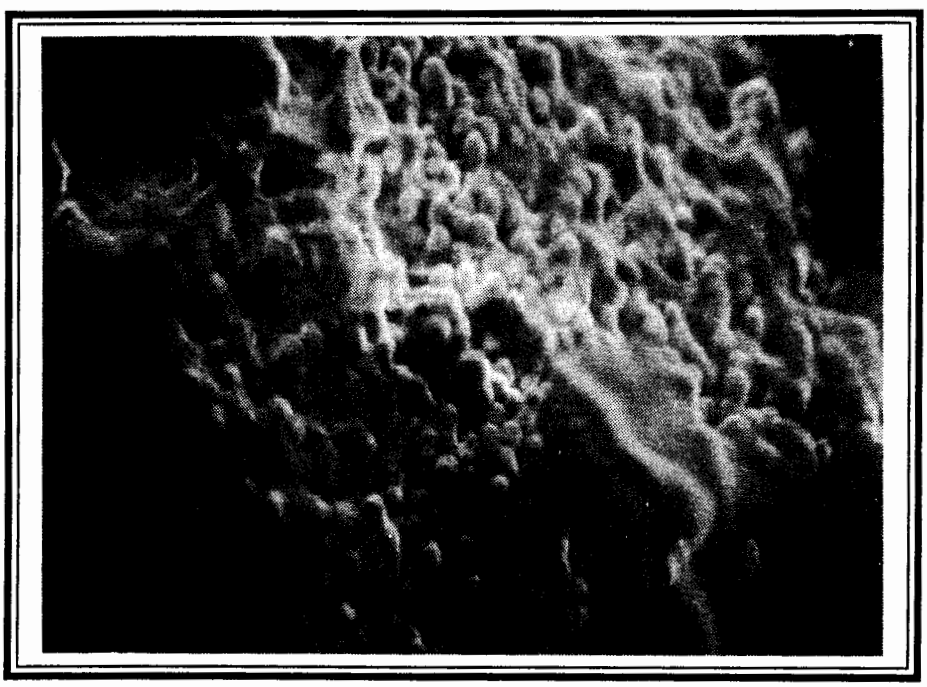

Figure 7. Second electron micrograph of selected intestinal granules (4,500x).

The next steps involved the addition of the two standards, ground sphalerite and zinc sulfide powder, to directly compare their elemental output with that of the intestinal granules. Sphalerite initially gave a sulfur to zinc ratio of less than two counts to one with traces of iron, and aluminum (figure 8).

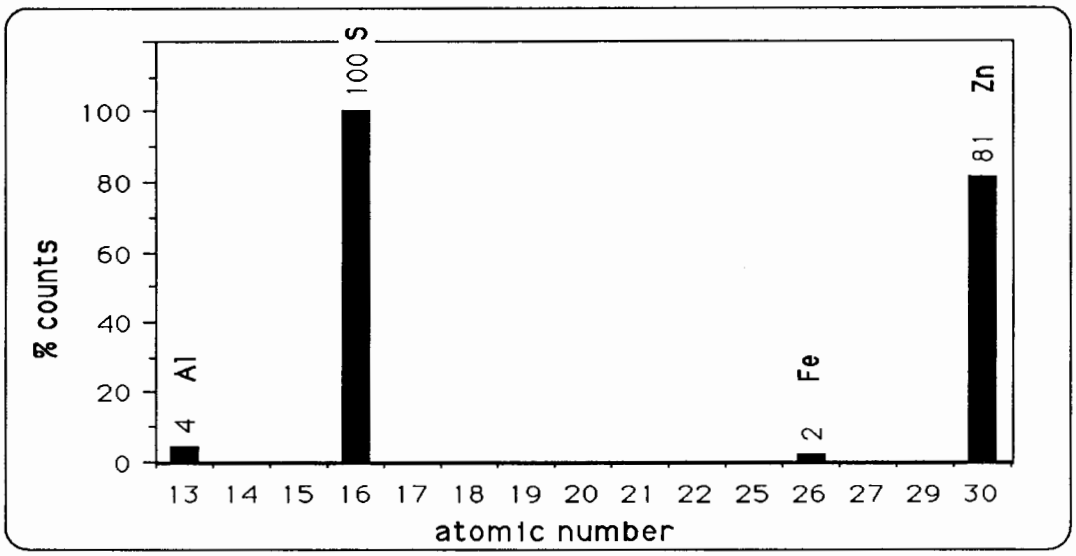

Figure 8. Elemental (EDS) analysis of sphalerite mounted onto two-sided tape.

Changing the direction of focus of the x-ray beam into sphalerite gave a reversal of major peaks producing a zinc to sulfur ratio of approximately six counts to one. The resulting trace levels again revealed iron, and aluminum, though calcium was absent (figure 9).

Upon observing the ground sphalerite, it was clearly observed that the material was in no way a homogeneous material, rather it was large crystalline structures as seen under six thousand magnification (figure 10).

Under the same vacuum conditions, the electron beam was once again focussed upon the intestinal granules to qualitatively compare peak ratios. The granules produced a sulfur to zinc ratio of over two to one with

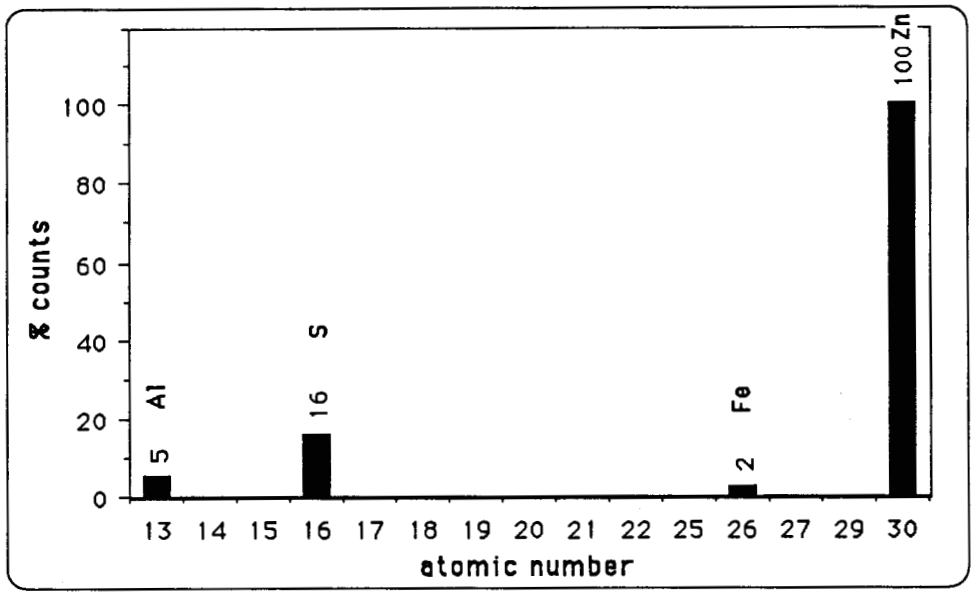

Figure 9. Second elemental (EDS) analysis of sphalerite on two-sided tape.

Figure 10. Electron micrograph of ground sphalerite (6,000x).

prominent phosphorous, calcium, and iron peaks (figure 11).

A representative photograph of the granules was taken to contrast the

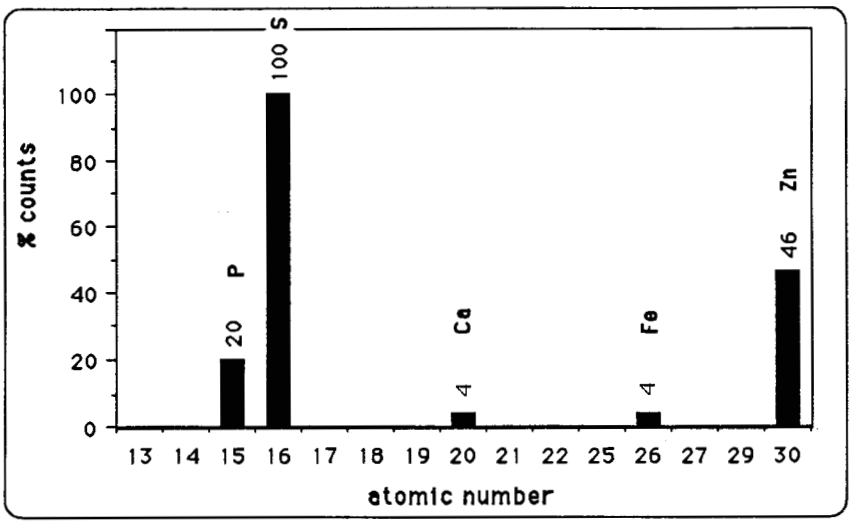

Figure 11. Third elemental (EDS) analysis of selected intestinal granules.

difference of appearance between sphalerite and the intestinal granules at 6,000 magnification (figure 12).

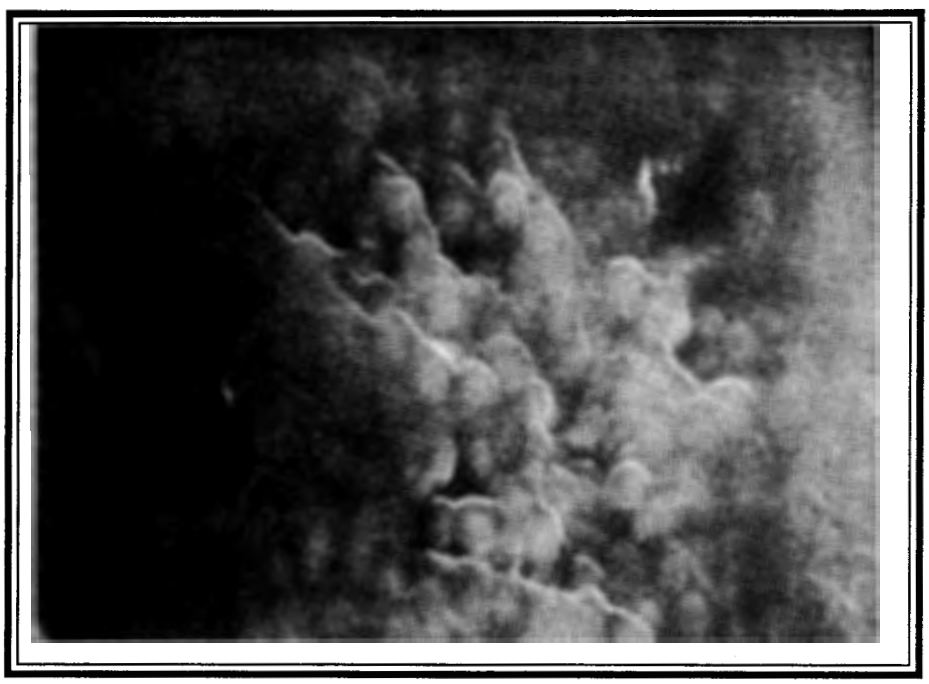

Figure 12. Third electron micrograph of selected intestinal granules (6,000x).

The next step to conclusively analyze the standards available was to mount zinc sulfide neutral powder to the stub and analyze it under the exact conditions of the unknown and qualitatively compare the peaks. ZnS powder gave two clean peaks of sulfur and zinc in a ratio of a little over two counts to one. No other peaks were present in the spectrum verifying that no other elements exist in the ZnS powder (figure 13).

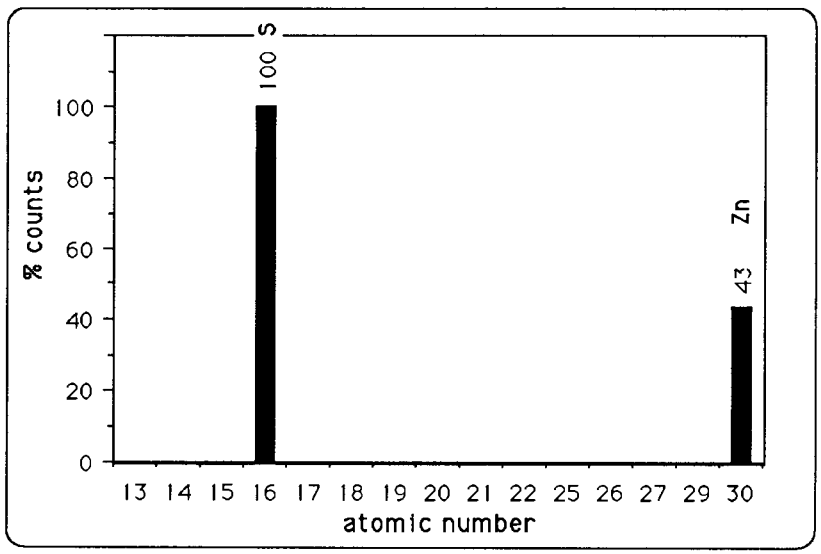

Figure 13. Elemental (EDS) analysis of zinc sulfide powder.

The electron beam was then moved to the sphalerite sample (upon colloidal graphite) and the output produced the sulfur to zinc ratio of a little less than two counts to one, with prominent silicon, aluminum, and iron peaks. This sphalerite sample mounted on the graphite was much more consistent than the sphalerite sample (analyzed previously) mounted on the double stick tape (figure 14).

The third set of analyses was performed upon a single intestinal granule under these same conditions, and during this same time frame. As before, sulfur and zinc produced peak ratios of approximately two counts to

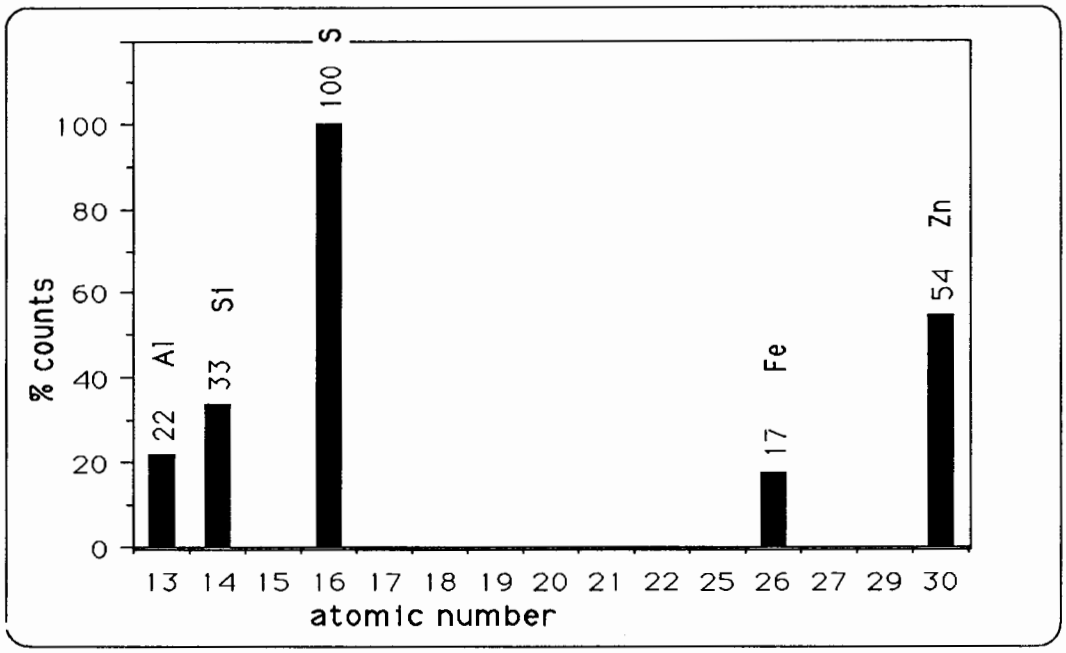

Figure 14. Elemental (EDS) analysis of sphalerite on colloidal graphite.

one, with minor peaks of phosphorous, calcium and iron. The x-ray beam was focussed just off center of the core of the 0.8 micron oval inclusion (figures 15 and 16).

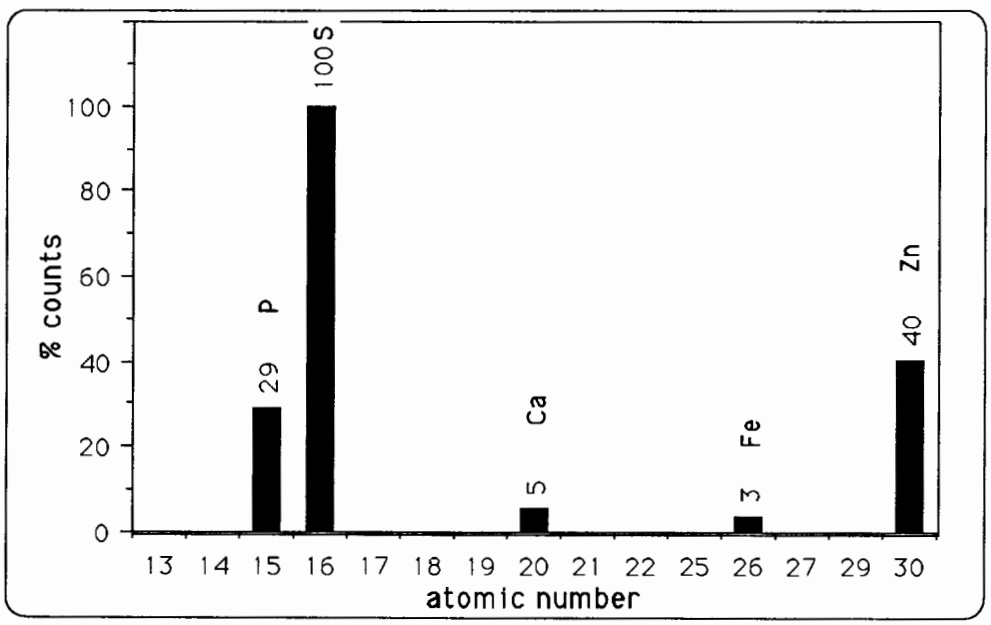

Figure 15. Elemental (EDS) analysis of a single intestinal granule.

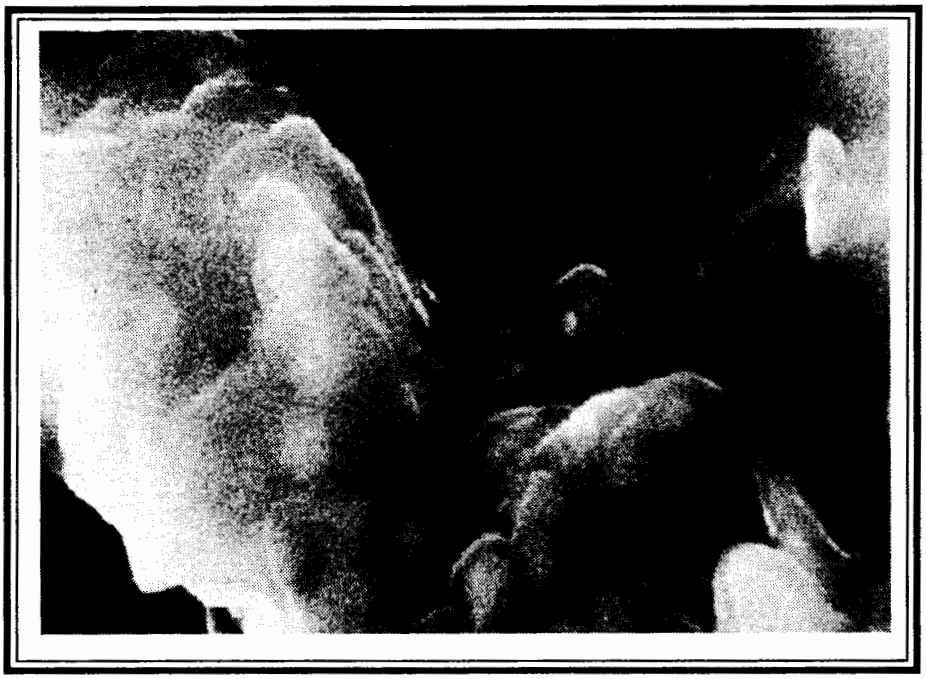

Figure 16. Electron micrograph of a single intestinal granule (8,000x).

DISCUSSION

Granular Composition

It was initially clear that some crystalline structure was present in the nematodes *Oesophagostomum* and *Ancylostoma* when their anterior intestinal portions proved birefringent. X-ray powder diffraction was a logical second step in the identification process. *Oesophagostomum* was irradiated and its spectra matched that of the compound calcium sulfide (CaS) almost exactly. Both Quack (1913) and Chitwood and Chitwood (1938) postulated this presence of calcium in the granules, but in the form of gypsum. In comparing the first four D-spacings of the worms and the standard CaS, only the intensities of the bands varied. *Oesophagostomum* sample #1 produced three D-spacings: 2.83(100%), 2.01(80%), 1.65(60%). Calcium sulfide's first three D-spacings are 2.85(100%), 2.01(70%), and 1.64(20%). However, densities of the darkness of the bands was chosen subjectively and should have been done with a densitometer.

The second irradiation (four whole worms) produced a clear spectrum with no bands visible and was not repeated a second time because sample #3 possessed a larger number (six) of the same nematodes. This assumed that the larger number would produce better resolution. This sample gave results which corroborated with the results of sample #1, though sample #4, an accumulation of ten *Oesophagostomum* gave an even clearer resolution of the crystalline structure than did samples #1 & #3. Five bands were recorded with sample #4 which further verified the composition as CaS. The first

three bands repeated the first three bands of CaS, and the fourth band, 1.29(20%) though very faint, matched the fourth band of CaS (1.27[20%]). Calcium sulfide in some form was present within the nematodes, however the very general means of identification could not specifically point to the intestinal granules as the source of the CaS. Only whole *Oesophagostomum* and ground up whole *Oesophagostomum* were irradiated at this stage.

Ancylostoma caninum were irradiated whole (sample #1) and the resulting resolution was poor, but the spacings immediately indicated a match with the *Oesophagostomum* spacings, and those of CaS. The first two irradiations of *Ancylostoma* gave only two bands, but after the worms had been ground up, a higher resolution in the form of four bands was produced which closely matched up with the spectra of CaS.

A more accurate irradiation was mandatory to locate the position of the crystals in question. *Ancylostoma caninum* intestines were dissected out, grouped and irradiated while focussing in on the exact location of the granules. The first x-ray done on these intestines (sample #3a, #3b) gave poorly defined results. A greater number of intestines were added and *Ancylostoma* sample #4a and #4b gave higher resolutions with increased numbers of bands. This output closely matched the spacings given by the compound zinc sulfide. The next three irradiations of *Ancylostoma* intestines all produced spectra that corroborated with the ZnS spectra. In further matching the output of zinc sulfide, *Ancylostoma* sample #4c recorded a fourth D-spacing identical to that of the ZnS.

Somehow the presence of CaS is lost once the anterior intestine is removed from the cuticle and the rest of the nematode. Within this anterior intestine however, there exists some material with the exact crystalline

structure as ZnS. Only micro-elemental analysis could focus upon the particle in question and analyze its composition.

The EDS microprobe within the SEM is the best method to microscopically analyze the elemental composition in question. The *Ancylostoma* intestines were dissected out and initially placed whole upon an aluminum stub to be analyzed. Outer surface scanning gave the perfect complement to the x-ray diffraction analysis done on the whole and ground up whole worms. Sulfur was found in highest concentrations, followed by phosphorous and calcium. Only trace levels of zinc were present, as was the case with potassium, manganese, silicon and aluminum. Aluminum, being the composition of the stub on which the unknowns are mounted was immediately discarded as a possible intestinal composite. A particular protrusion was focussed upon during the outer surface scanning which produced high levels of sulfur, phosphorous, silicon and calcium. It seems most probable that some form of CaS (possibly gypsum [$CaSo_4$] as postulated by Quack [1913] and Chitwood and Chitwood[1938]) is present in the outer intestinal wall of *Ancylostoma caninum.*

The intestines were then teased open and the intestinal granules in question were immediately observed and analyzed. Two predominating peaks of sulfur and zinc (in a 3:1 ratio) unequivocally showed the composition of the granules to be composed of these two elements (in some form). This corroborated with the work of Rogers (1940) and Clark (1956) who showed the existence of zinc sulfide in the granules. Phosphorous also produced predominant readings in the next set of microprobe analyses. Calcium, iron and copper continued to show low level concentrations in the granules.

Qualitative identification of the composition required analysis of

known standards under the identical conditions as those of the unknowns. Sphalerite, a mineral of ZnS composition produced a sulfur to zinc ratio of 1.5:1.0 with low levels of other elements. These results were not easily repeated due to the non-homogeneous sphalerite composition. Mortar and pestle ground sphalerite was not efficient enough to grind and mix the mineral for electron microscopic analysis. Under the same vacuum conditions though, the intestinal granules produced a sulfur to zinc ratio much like that of the first sphalerite reading (2.1:1.0). The reading also showed lower levels of phosphorous, calcium, iron and copper.

Zinc sulfide powder was the ideal homogeneous standard with which to compare the zinc and sulfur material found in the *Ancylostoma* intestines. This powder (ZnS) produced an extremely clean output with only two peaks visible, those of sulfur and zinc in a 2.5:1.0 ratio, respectively. Immediately following this analysis, sphalerite (on colloidal graphite) produced a sulfur to zinc ratio of 2:1. This was followed by the irradiation of one specific intestinal granule which produced a very similar sulfur to zinc ratio of 2.5:1.0. These qualitative comparisons, in conjunction with the crystalline comparisons leave no doubt that the composition of these 0.8 micron ovals is at least in part, zinc sulfide.

Granular Function

Since the discovery of the intestinal crystals at the turn of the century, numerous hypotheses have been proposed as to the function of these granules. Earliest determinations (Askanzy [1896], Looss [1905], Faure-Fremiet [1912]) regarded the granules as reabsorbed hemoglobin, or waste products from red blood cell metabolism. Chitwood and Chitwood (1950)

reported that the 'sphaerocrystals' ($CaSO4 \cdot 2H20$) of strongylin species were waste material and observed to be excreted from the cells of *Strongylus*. The authors went on to report the 'sphaeroids' of *Rhabdias* and *Ironus* were also observed to be eliminated from their intestines.

Cobb (1920) reported that five or six kinds of doubly refractive granules had been discovered in the course of examining almost 200 nematode species of 40 different genera (calcium sulfide composed one of these types of granules). Cobb stated that the granules fall into two groups: (1) stored food material, ie. rhabditin (carbohydrate), and (2) elimination material, ie. CaS. Giovannola (1936) saw the function of the granules generally as energy and food reserves in nematodes. He proposed that only a few granules consisted of waste products such as those composed of disintegrated blood (hematin).

Rogers (1940) postulated that the granules (which were composed of zinc sulfide and iron) were the result of positive metal ions acting as sulfur acceptors in sulfur metabolism within the worm. This supported his view that there was a slight prevalence of iron sulfide, calcium sulfide and copper sulfide in the granules. The granules, according to Rogers, increased in number with the age of the nematode. In the case of the zinc sulfide granules, zinc was assumed to be absorbed and stored in the gut wall of the nematode. Croll (1976) also stated that the pigmented granules increase with age, but he assumed their function to be simply that - an aging pigment (lipofuchsin) from the peroxidation of lipids and proteins.

It was impossible to determine in this experiment if intestinal granules within *Ancylostoma caninum* increased in number with the age of the worms. The nematodes were collected on the ninth week of culturing within

their canine host, during which time the dog had to be sacrificed. It was then not possible to compare these nematodes with others of longer life spans. Granule accumulation with age has been observed elsewhere within species of *Strongylus* (Clark, personal communication).

Lee (1970) regarded the intestinal granules of *Ancylostoma* to be a specific protein with incorporated iron. He postulated that the granules represented a nutrient storage and dissimilation system in which a protein and iron are the main components. Lee did not exclude the possibility that the granules possess hydrolytic function, or some other enzymatic function, as was proposed by Weinstein (1966).

Martin and Lee (1975) researching hexagonal crystals of *Nematodirus* viewed the crystal function as associated with the development of immunity of the host towards the nematode, and not related to the age of the nematode at all. Bird, et.al (1978) viewed similar hexagonal crystals of *Haemonchus* and *Ostertagia* as by-products of the worms degenerative process.

Zinc Sulfide

The function of the specific zinc sulfide granules being dealt with in this document has yet to be determined. High concentrations of zinc in cestodes, specifically in several areas of the whale tapeworms, *Diphylobothrium macroovatum* and *Diplogonoporus balaenopterae* is attributed to growth functions. Zinc in these tapeworms has also been regarded as an essential constituent of enzymes, and DNA, RNA and protein synthesis. Since few researchers have found zinc to be associated with the granules in nematodes, this relationship has yet to be proposed concerning parasitic nematodes.

Barrett Sugarman in his article, "Zinc and Infection" (1983), outlines how important zinc is to viable organisms. Zinc has been identified as a constituent of over 100 mammalian metallo-enzymes, the most well known being carbonic anhydrase. Most of these enzymes become active with zinc incorporation. Nucleic acid polymerases are other good examples that contain zinc and can be associated with increase levels of DNA and RNA. Zinc also readily binds with sulfhydryl groups present on membranes and at various sites on enzymes that do not normally bind zinc at these locations. It is in this manner that zinc can stabilize membranes and inhibit specific enzymes. Prolonged zinc deficiencies can be detrimental to the organisms also, and in mammals is associated with depressed T-cell lymphocyte function, and decreased wound healing. According to Sugarman; "if it's alive, it needs zinc."

Parasitic infection has been shown in a number of cases to deplete zinc levels from their respective hosts. Symons (1983) showed dramatic drops (17%) of plasma zinc levels in sheep infected with *Trichostrongylus colubriformis* . Eisa, et.al (1972) found that parasitic infections among diseased Egyptians significantly lowers blood zinc (and copper) levels below their normal values. In this study, one of the principle parasites was the hookworm, *Ancylostoma* , which was reported on in this document. Prasad (1970) also reported that hookworm infections in Egyptian subjects caused decreased serum zinc levels. Three other authors, Sandstead (1971), Vallee & Gibson (1949), and Vallee (1954) reported zinc deficiency associations with *Ancylostoma* infections.

Normally, people ingest about 10-15 mg of zinc daily (Sugarman 1983). Some 50% is absorbed by the intestinal cells, of which 20% is secreted back

into the gastrointestinal tract. The zinc that is absorbed is almost all in bound form. Approximately 60% of plasma zinc is bound to albumin, 30% bound to 2-macroglobulin and the remainder to various amino acids.

The adult hookworm *Ancylostoma caninum* binds to the intestinal mucosa of the small intestine (specifically jejunum) of its canine host. Its dietary habits solely include blood meals from this intestinal mucosa. The element zinc is abundantly present both on the intestinal lumen and within the ingested blood meal. Zinc is also essential in the viability of all organisms. It seems probable that the nematode takes advantage of the zinc present in its environment, and sequesters it as zinc sulfide. It is also probable that zinc serves many other functions in that it binds with possibly toxic sulfhydryl groups left over from sulfur metabolism (as postulated by Rogers, 1940). Depleting the host of zinc would also decrease the ability of the host to eliminate the nematode by depressing the host's immune system. The fact that zinc readily binds with sulfhydryl groups present on membranes introduces the fact that zinc sulfide could be stabilizing the intestinal membrane of the nematode. The fact that the granules increase with the age of the nematode, however points to the conclusion that the granules are a by-product of the worm's metabolism.

The other possible explanation is that the large quantity of zinc taken in with the blood meal is toxic and the nematode is actually binding up the zinc to decrease its toxicity. Excess zinc toxicity has been documented in mammals with symptoms of growth reduction, anemia, and bone abnormalities. In humans, hyper-zinc levels are known to cause these and other symptoms including decreased calcium levels, diarrhea, nausea, fever and vomiting (Fox and Jacobs 1986). It seems that with the rich supply of

zinc present within the blood meal, the worm has no need to store up quantities of this or any other of the bloods inorganic elements. The most logical conclusion seems to be that the zinc sulfide combination is an anti-toxic strategy, binding up excess zinc and sulfhydryl groups, left over from the nematodes catabolism of host blood.

Elevated zinc levels are detoxified in mammalian tissues by the presence of a sulfur containing protein, metallothionen. This protein acts as a temporary storage and detoxification system during a rapid incorporation or prolonged occurrence of zinc and other heavy metals (Bremner and Davies 1975). It seems very probable that the zinc sulfide compound found in parasitic nematodes is a result of a similar detoxifying function. Stabilizing the intestinal membrane and utilizing the other benefits of zinc could then be advantagous side effects of this detoxification function.

SUMMARY

The parasitic nematodes *Ancylostoma caninum* and *Oesophagostomum radiatum* were collected and analyzed for intestinal inorganic granules. Three means of identification were utilized to determine the composition of the granules, including birefringence, x-ray diffraction and energy dispersive spectrometric (EDS) analysis. Initial x-ray diffraction results of the two worms showed a calcium sulfide presence within the worms. Closer examination of the granules within *Ancylostoma caninum* ,however, utilizing EDS analysis revealed their composition to be zinc sulfide. These results concur with those of Rogers (1940) and Clark (1956) who found zinc sulfide granules in several species of *Strongylus*. The ZnS granules seem to be a result of a detoxification function that binds excess zinc and sulfhydryl groups present from the ingestion and breakdown of dietary blood meals from their respective hosts.

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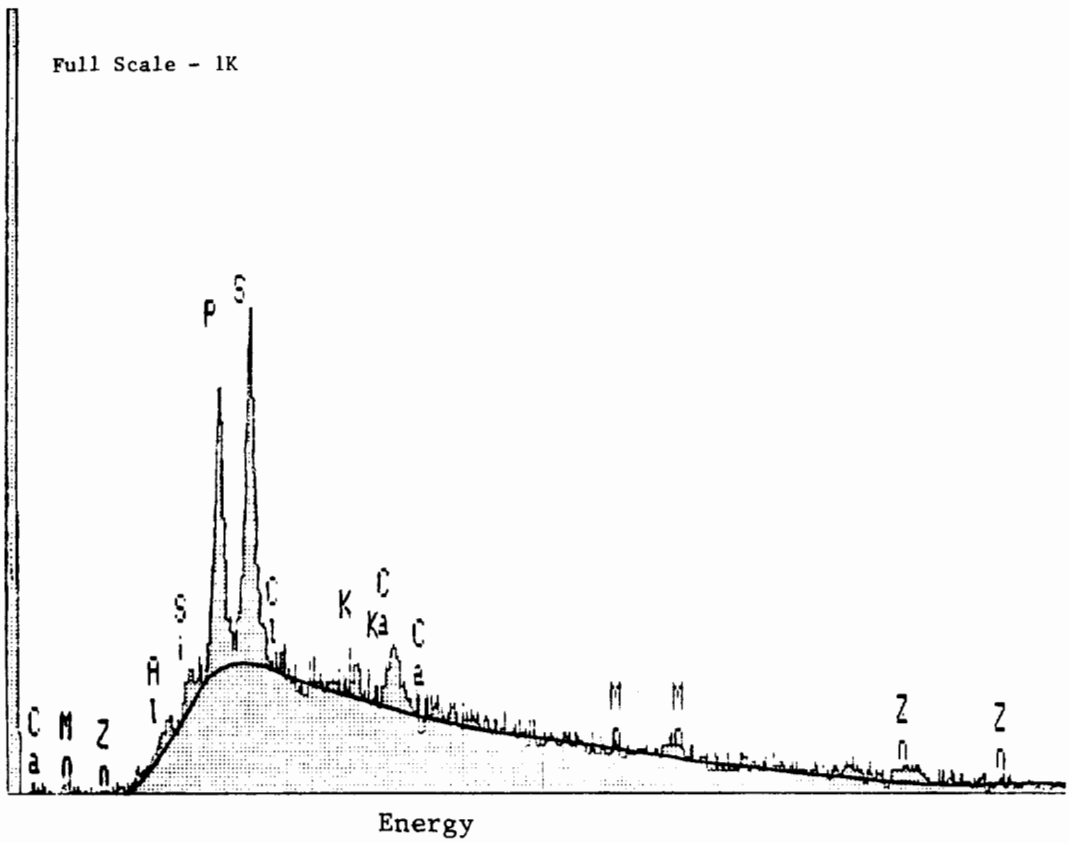
APPENDIX



This is a technical illustration showing the analytical method by which the results described in this document were manipulated. Found above is an example of the original EDS data, with background represented as a solid line (utilizing a French curve). It is this background that is then subtracted from peak heights to produce values illustrated in the figures found herein. This particular output is that of figure 1 in the document.