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The identification of the inorganic components in the calcareous corpuscles from Mesocestoides corti

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Title The Identification of the Inorganic Components in the Calcareous Corpuscles from Mesocestoides corti

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The inorganic components in calcareous corpuscles from Mesocestoides corti have been identified by use of powder x-ray diffraction and emission spectroscopy. Different methods were compared for the isolation of the calcareous corpuscles from the residual tissue of the Mesocestoides corti. Previously reported and newly developed methods for the isolation are discussed.

Analyses by powder x-ray diffraction for in vivo samples which had been heated at various temperatures ranging from room temperature to 1000°C are reported.

The qualitative emission spectrographic analyses for in vivo and in vitro corpuscles are reported for normal systems and systems designed to study the ion uptake of the corpuscles. The relationship between the corpuscles' composition and the parasites' environment is discussed. A method for the quantitative emission spectrographic analysis was initiated for the percent calcium and magnesium. There is a linear relationship between the intensities and the percent calcium or magnesium

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TABLE OF CONTENTS

INTRODUCTION

The calcareous corpuscles of Mesocestoides corti consist of inorganic compounds bound by thin concentric spheres of organic material. The function of the calcareous corpuscles is not known. They have been considered skeletal substances or excretory products that serve to bind the respiratory carbon dioxide, store an excessive uptake of calcium from the food material, or store an alkali reserve to neutralize metabolic acids. These views do not have experimental foundation at present.

It is known that the cestodes are found in the liver and eventually die. The residual tissue of the parasite is then digested by the host and the calcareous corpuscles come in contact with the tissue of the host's liver. It has been shown that at this time sarcoma or cancer of the liver is induced. Therefore, it is very important to know the chemical composition of the corpuscle.

The research objective of this thesis is the chemical identification of the inorganic components of calcareous corpuscles as normally occurring metabolic products produced by the parasite Mesocestoides corti.

This study will be limited to the calcareous corpuscles found in the larval or tetrathyridial stage of the parasite. It is important to note that reproduction of the larval stage of Mesocestoides corti is by asexual multiplication through binary fission and budding in the

host animal. This leads not only to the *in vivo* but also the *in vitro* cultivation of the tetrathyridium of *M. corti* and allows the obtaining of sufficient amount of the sample for analysis under complete control of the physio-chemical environment.

HISTORICAL BACKGROUND

Several investigators have reported the occurrence of sarcoma found in animals infected with parasites. Bogliolo (5) has written a review on the early work with emphasis on Schistosomiasis. This review states that an average incidence of liver sarcoma was 1% in experimental animals infected with Schistosoma mansoni and 10% in natural human infection. Another review on the natural occurrence of liver sarcoma produced by parasitimia was written by Berman (2). It reports that in a group of East African natives where 88% of the population was infected with helminthes, 34% also had developed primary liver cancer. The occurrance of naturally induced sarcoma has been reported in association with the following parasitic groups: Ascaris, Trichuris, Ancylostoma, Schistosoma, Fasciola, Taenia, Mesocestoides, and Echinococcus.

It is apparent that there is an association between the development of cancer in the host and the infection of a host with a given group of parasites. According to Smyth (15) and von Brand (16) the highest ratio of liver cancer and parasitimia occurs with infections by flukes (trematodes) and tapeworms (cestodes) while cancer is rarely observed with infections by round worms (nematodes). The one important common characteristic of trematodes and cestodes is the presence of calcareous corpuscles. It has been suggested by Smyth (15) and von Brand (16) that cancer develops after the corpuscles are deposited, that is, when the parasitic organism ages and dies.

X-ray diffraction has been commonly used for the identification of inorganic crystalline material found in biological samples. Calcareous deposits have been identified in several sites, such as the urinary tract by Prien (13), the aorta by Parsons (12) and Yu (20), the pineal glands by Earle (8) and synovial fluid by Bachman (1) and Kohn (11).

These calcareous deposits derived from glandular and serum material have mainly been composed of microcrystalline hydroxyapatite, $Ca_5(OH)(PO_4)_3$. The x-ray patterns have broad and diffuse Bragg reflections that indicate a crystalline size of the order of 100 millimicrons (Bachman (1)).

Isolation of the calcareous material is usually accomplished by a hot alkaline digestion to remove residual organic material prior to x-ray diffraction studies. In 1965, Bachman (1) reported an identification of an untreated sample of calcareous corpuscles.

Von Brand et. al. (17, 18, 19) studied some of the chemical characteristics of the calcareous corpuscles from parasites by chemical and x-ray analysis. They showed that the corpuscles are composed of metals such as calcium and magnesium; mineral-related compounds like calcium carbonate, brucite ($Mg(OH)_2$), and hydroxyapatite; and also an abundance of mucopolysaccharides, free amino acids, glycogen, and steroids. The x-ray diffraction patterns were not given but the resulting conclusions were stated for samples heated from 300°C to 900°C. At these various temperatures, von Brand (19) has reported hydroxyapatite, brucite, calcium carbonate, calcium oxide, magnesium oxide, dolomite, and whitlockite. It is important to note that the above samples were

isolated by a hot alkaline digestion. Von Brand found brucite absent in samples digested with ethylenediamine, but brucite was observed in samples treated with potassium hydroxide.

Epprecht, Schinz, and Vogel (9) have reported inconclusive evidence for the identification of calcareous deposits by x-ray studies. They reported the calcium deposits from Taenia saginata and Fasciola hepatica to be hydroxyapatite and from Taenia saginata ignited to 900°C to be a mixture of hydroxyapatite and brucite. No crystalline material was found in the corpuscles isolated by an ethylenediamine digestion from the parasites Taenia taeniaeformis, Cysticerus, Fasciolaris, or Taenia saginata. It has been considered by Epprecht, Schinz, and Vogel (9) and suggested by von Brand (17, 18) that the in vivo mineral component of the corpuscles is amorphous, and that heating in alkali solutions results in recrystallization of hydroxyapatite and brucite.

It has been shown that there is no simple chemical composition to calcareous systems. Earle (8) has reported that ashed extracted crystals from human pineal glands yields a calcium to phosphorus (C/P) ratio of 1.59, which is below the calcium to phosphorus ratio of 1.67 for pure hydroxyapatite. Brown, Smith, Lehr, and Frazier (6) have shown that octacalcium phosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$, with a C/P ratio of 1.33 forms intra-crystalline mixtures with hydroxyapatite, $Ca_5(PO_4)_3OH$, but the crystallographic methods are unlikely to distinguish between the two compounds. This mixture could explain why a value of 1.59 for the C/P ratio reported by Earle (8) was lower than the C/P ratio for pure hydroxyapatite.

Hydrolysis and dehydration of octacalcium phosphate causes gradual conversion to hydroxyapatite. Brown, Smith, Lehr, and Frazier (6) found that anhydrous dicalcium phosphate, $CaHPO_4$, and, at higher temperatures, calcium pyrophosphate, $Ca_2P_2O_7$, is sometimes formed. Magnesium in very low concentrations markedly inhibits the hydrolysis of octacalcium phosphate and may be important in the growth and development of hard tissues as reported by Brown, Smith, Lehr, and Frazier (6). It also reacts directly upon all metabolism in general.

Evans (10) developed a medium (NCTC 109) that Berntzen (3) used to develop methods for the in vitro growth of Mesocestoides corti. This procedure is similar to the work reported by Berntzen (4) in 1966.

Emission spectroscopic analysis of minor components found in calcareous corpuscles were reported by von Brand (19) in 1967. The analysis included calcareous corpuscles for Ligula intestinalis, Taenia crassiceps, Raillietina cesticillus, and Mesocestoides corti. This analysis covered thirteen different elements.

METHODS AND EXPERIMENTAL DATA

I. X-Ray Diffraction Studies of In Vivo Material

The x-ray diffraction technique consists of transferring the prepared calcareous material to a Lindeman glass capillary tube (0.5 mm diameter). The filled capillary was sealed with cement and aligned in the powder camera on the General Electric XRD-5 x-ray unit. Copper K x-radiation was used and one half of the film was covered with nickel foil to filter out the K_β x-radiation. After exposure, the x-ray film was processed in the usual manner, with a final rinse in Kodak Photo-Flo 200 to prevent water spotting. The d-spacings on the film were measured, and identification of the crystalline components are made using the ASTM powder data file (14) or other published patterns. Quantitative measurements of line intensities are made with the Photovolt TLC Densitometer feeding into a Photovolt Linear/Log Varicord 43 Recorder. The sample sizes available were not sufficiently large to warrant use of the powder diffractometer.

Before the x-ray diffraction analysis, the residual tissue material from the tetrathyridium of Mesocestoides corti must be separated from the calcareous corpuscles that are to be analyzed. Three methods of digestion were compared to determine which method should be used for analysis. Samples of in vivo calcareous corpuscles were obtained from Swiss albino mice which had been infected with M. corti tetrathyridium for three and one-half to four months. A sample of the tetrathyridium was ground on the Spex Mixer/Mill for twelve minutes. The sample was

divided into three equal parts and digested with ethylenediamine, potassium hydroxide, and trypsin, respectively. The ethylenediamine and potassium hydroxide digestions were identical to the procedure used by von Brand (19). The trypsin digestion consisted of stirring the sample for one and one-half hours in 200 ml 0.85% NaCl solution containing 1.0% trypsin (1:250) and adjusted to a pH of 7.2 with sodium bicarbonate. After digestion, the sample was washed twice with saline solution (0.85% NaCl), once with water, once with absolute alcohol, twice with ethyl ether, and air dried.

The three samples of digested corpuscles were placed in a muffle furnace and heated at 300 $\pm$ 10°C for eighteen hours. The powder pattern was obtained and the crystalline material was identified as: (1) from the potassium hydroxide digestion - magnesium hydroxide ($Mg(OH)_2$); (2) from the ethylenediamine digestion - calcium sulfate ($CaSO_4$), calcium sulfate hemihydrate ($2CaSO_4 \cdot H_2O$), magnesium oxide (MgO), calcium magnesium carbonate ($CaMg(CO_3)_2$), and calcium magnesium iron(II) carbonate ($CaMg_{2/3}Fe_{1/3}(CO_3)_2$); and, (3) from the trypsin digestion - calcium sulfate, calcium sulfate hemihydrate, magnesium oxide, calcium magnesium carbonate, and calcium magnesium iron(II) carbonate. See Appendix I and Appendix II for comparison of d-spacings.

After concluding that the trypsin digestion was the best method, corpuscles were analyzed at varying temperatures. Trypsin digested samples of the tetrathyridium with no heating and those subsequently heated at 100°C and 200°C gave a diffuse pattern characteristics of amorphous materials and the components were unidentifiable.

After heating at 400°C and 500°C the trypsin treated corpuscles had calcium magnesium carbonate and calcium magnesium iron(II) carbonate as the major constituents. The measured d-spacings and their comparisons are listed in Appendix III.

Heating above 650°C caused considerable change in the nature of the crystalline material, with hydroxyapatite and calcium oxide as major components. The observed pattern for magnesium carbonate probably arose from atmospheric carbon dioxide reacting with magnesium oxide, which is a more probable component at this temperature. See Appendix IV for a comparison of d-spacings.

II. Emission Spectrographic Studies of In Vivo Material

A. Qualitative Analysis Studies

Elemental analysis by emission spectroscopy of the calcareous corpuscles was obtained by the use of an ARL Emission Spectrograph. Five to ten milligrams of calcareous corpuscle from the digestion of *Mesocestoides corti* tetrathyridium was mixed with approximately an equal amount of spectroscopic graphite powder and placed in a one-quarter inch diameter graphite electrode (necked crater = 3/22" deep). The one thousand volt potential arc was applied for the life of the electrode, usually from two to five minutes. A standard iron spectrum was taken on the same film for calibration of wave lengths. This film was developed in the usual manner. The measurement of the spectral lines and the identification of the corresponding elements were performed on the ARL Viewer-Comparator.

To study the metal ions present in the corpuscles, samples of tetrethyridium were taken from C3H Mai mice which had been infected with M. corti for three to five months. The residual organic material was removed either by wet-ashing with a nitric acid - sulfuric acid mixture or by a trypsin digestion, and ignited at 800°C for thirty minutes. The results are listed in Appendix V.

The possibility of host-specific effects on the composition of the corpuscles was investigated by changing from C3H Mai mice to Long Evans rats with four month infections. This is to determine if a specific composition is related to a specific host. The comparison is given in Appendix V.

Von Brand et al. (19) reported strontium in calcareous corpuscles obtained from M. corti; however, it was absent in our emission spectrographic analysis. The possibility of ion concentration in the corpuscles by the tetrathyridium was investigated. Swiss albino mice were given a dilute strontium chloride solution (1.0 gram Sr/liter) in their drinking water during their two and one-half month infection. The results of the analysis, after trypsin digestion, shows significant uptake of strontium and are given in column six of Appendix V.

B. Quantitative Analytical Studies

A procedure for the quantitative analysis of calcium and magnesium in calcareous corpuscles has been initiated by the use of a log sector wheel in the ARL Emission Spectrograph and using the Joyce-Deeley flying-spot integrating microdensitometer to measure line intensities. Stan-

dard samples containing calcium, magnesium and iron were prepared as described in Table I. Although this section of the analytical procedure is concerned only with analysis of calcium and magnesium, the samples were prepared containing iron so that the standard set of films would be available for anticipated investigation of corpuscles for the percent of iron.

Each standard sample was mixed with 900 milligrams of lithium carbonate internal standard on the Spex Mixer/Mill for six minutes. A one milligram portion is placed in an electrode and covered with ten to twenty milligrams of spectographic graphite. The arc is applied for the life of the electrode or burned completely and the spectrum is taken by the use of a log sector wheel and filters allowing twenty-five percent transmittance.

TABLE I

STANDARD SAMPLES FOR QUANTITATIVE ANALYSIS OF CALCAREOUS CORPUSCLES

$CaCO_3$ (mg)	$MgSO_4$ (mg)	Mohr's Salt* (mg)	Total Weight (mg)	%Ca	%Mg	%Fe
0	100	0	100	0	20.2	0
10	20	70	100	4.0	4.0	10.0
20	60	20	100	8.0	12.2	2.8
30	40	30	100	12.0	8.0	4.3
40	10	50	100	16.0	2.0	7.1
60	30	10	100	24.0	6.0	1.4
100	0	0	100	40.0	0	0

* $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$

Reference lines for calcium (3179 A), magnesium (2781 A), and lithium (3233 A and 2741 A) were selected for the construction of a standard curve because the lithium line was close to the corresponding standard line of calcium or magnesium, and the lines were of moderate intensity. The sector caused the width of each line to vary along its length, giving each line a characteristic wedge shape. The wedge was divided lengthwise into five equal parts for examining, the integrated intensity of each portion was measured, and the film background subtracted from each integrated intensity. The five separate wedge intensities were added to determine the total relative integrated intensity of the selected line. A linear function was obtained for the standard curve by plotting the log of the ratio of the intensity of the calcium or magnesium wedge to the intensity of the lithium wedge as the ordinate, and the log of the percent of calcium or magnesium, respectively as the abscissa. This yields a linear function of the type:

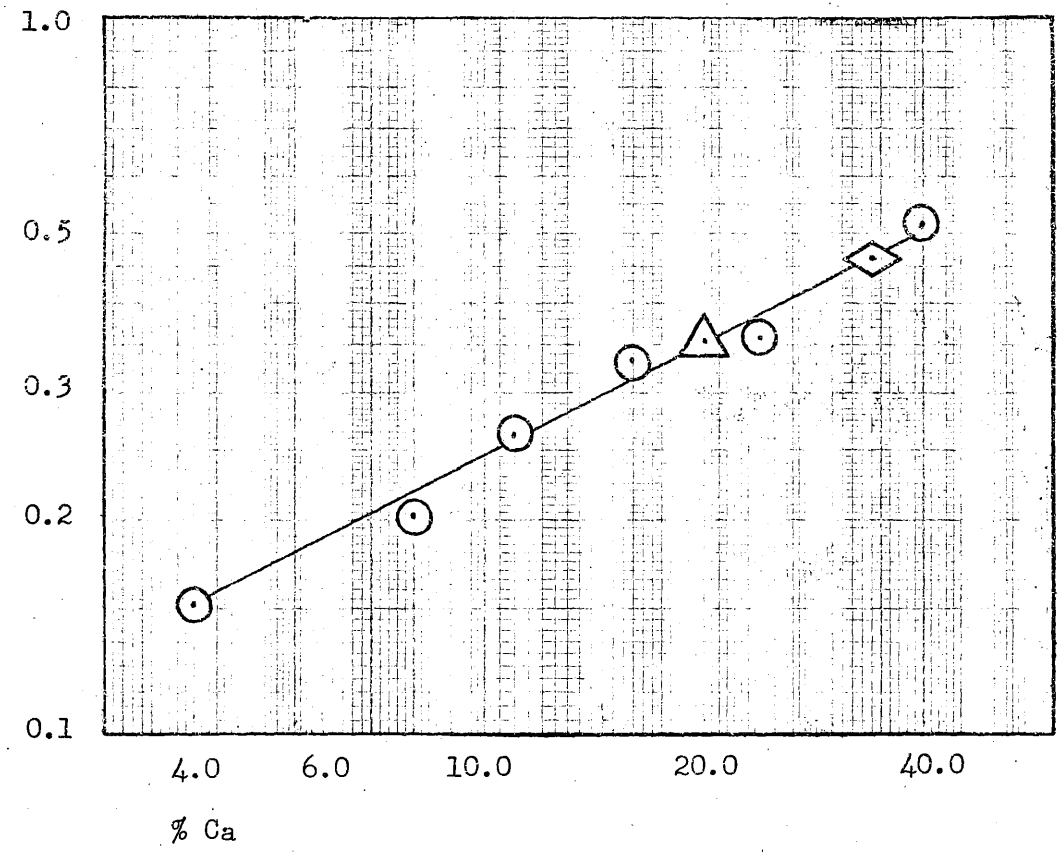
$$\log \frac{I_{Ca}}{I_{Li}} = m \log C_{Ca} + b$$

Where: I_{Ca} = intensity of the calcium wedge,
I_{Li} = intensity of the lithium wedge, and
C_{Ca} = percent of calcium in sample.

The calcium and magnesium standard curves are shown in Figures I and II respectively. The sample from normal <u>in vivo</u> mice gave a value of 34% for calcium (as $CaCO_3$) and 14% for magnesium (as $MgSO_4$). The sample from mice with strontium drinking water gave values of 20% for calcium (as $CaCO_3$), and 6% for magnesium (as $MgSO_4$). Because there was insufficient time to obtain enough tetrathyridium for another sample, the experiment could not be repeated.

FIGURE I

STANDARD CURVE FOR CALCIUM

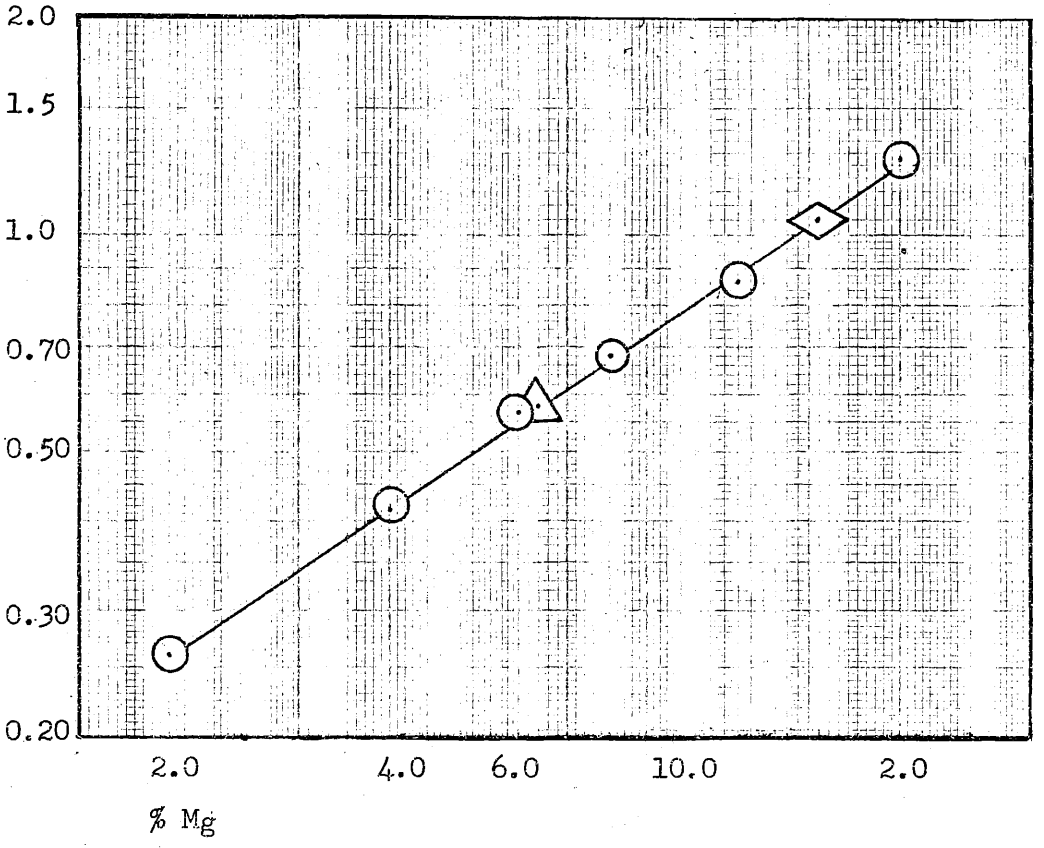


◇ = In vivo calcareous corpuscles from normal mice

△ = In vivo clacareous corpuscles from mice with strontium in their drinking water

FIGURE II

STANDARD CURVE FOR MAGNESIUM



$\frac{I_{Mg}}{I_{Li}}$

= In vivo calcareous corpuscles from normal mice

= In vivo clacareous corpuscles from mice with strontium in their drinking water

III. Emission Spectrographic Studies of In Vitro Material

Mesocestoides corti were grown in vitro for further study using the NCTC 109 Medium developed by Evans (10) and in vitro procedure developed by Berntzen (3). The NCTC 109 Medium was purchased from Grand Island Biological Company, Grand Island, New York, diluted to 800 ml with distilled water, buffered with sodium bicarbonate to a pH of 7.2, sterilized by millipore filtration, and 200 ml of horse serum added. The tetrathyridium was obtained from Swiss albino mice and placed in the in vitro culture system. The cultures were grown at 37°C for three to six days.

In studying the ability of M. corti to concentrate metal ions in the calcareous corpuscles, selected ions were introduced into the in vitro growth medium. The initial dilution of the medium was done with 800 ml of solution containing one gram of metal per liter. The compounds used in this study were selected because they were toxic to man, or because they contained ions chemically similar to ions found in the corpuscles; strontium chloride, beryllium chloride, uranyl acetate, and arsenic trioxide were selected. After the in vitro cultivation of the tetrathyridium in these cultures for three to six days, the corpuscles yielded emission spectra containing lines for strontium, beryllium and arsenic, respectively. However, numerous light lines caused interference in the spectrum, and uranium could not be positively identified. Appendix VI has the details of the emission spectrographic analysis.

A fifth medium, made from a solution containing one gram each of strontium, beryllium, and uranium per liter, was used in the in vitro

growth of M. corti for five days and the results are given in Appendix VI. This experiment showed uptake of these metals by the corpuscles, with uranium again not being positively identified because of interference.

DISCUSSION

In the past, the isolation of calcareous corpuscles from parasites like Mesocestoides corti has prevented a clear analysis of their chemical composition. A good example of this problem is shown by the work reported by von Brand (19). When equal portions of the same sample are taken and digested with potassium hydroxide, ethylenediamine, and trypsin to give different results, it is evident that both recrystallization processes and changes in chemical composition can be occurring.

Appendix I shows that the sample digested by potassium hydroxide has a powder pattern with magnesium hydroxide as the main constituent; however, the ethylenediamine and trypsin digestion methods gave a sample with a different pattern characterized by the absence of magnesium hydroxide. Even though ethylenediamine digestion is not as harsh as the potassium hydroxide digestion, the sample treated by ethylenediamine gives a partial x-ray powder pattern that was not completely identifiable. When the trypsin digestion method was employed, the samples gave a more complete pattern, as indicated by the presence of low intensity lines. This indicates that the ethylenediamine digestion was inefficient and did not give a full spectrum of lines for the minor components. The absence of the lines of low intensity is due to residual tissue from the M. corti which acts to reduce the density of corpuscles in the sample.

This inefficient isolation with ethylenediamine as used by von Brand (19) and others limits the amount of crystalline material in the sample that will diffract the x-rays. The result is not only a loss of

the low intensity lines, but an incomplete identifiable spectrum. The comparison of the pattern from the ethylenediamine digested sample and the pattern from the trypsin digested pattern in Appendix I shows the differences. The potassium hydroxide digestion is not acceptable for powder diffraction analysis because it changes the chemical composition. The ethylenediamine digestion can be used because there is no sign of chemical change; however, trypsin digestion is the most successful since it appears to completely remove residual tissue and yields a more complete powder pattern.

The enzyme, trypsin, is the preferred chemical to be used for digestion of the residual tissue for numerous reasons. It is not only a mild digestion that differentiates the organic material from the inorganic material, but it is selective for specific peptide bonds. Since the digestion proceeds at a pH of 7.2, no acid-base reactions are likely to occur. The removal of the residual tissue of the tetrathyridium surrounding the calcareous corpuscles is achieved in a normal proteolytic manner.

It is important to note that trypsin is an enzyme of the pancreas, and acts very similar to the process of digestion of the M. corti by the host after it has died. When the parasite is in the host in an internal organ like the liver, the parasite eventually dies. The removal of the residual tissue is accomplished by the lysis of cells due to the action of the protective system of the host. This is essentially accomplished by the presence of cells in the blood system which break down the residual tissue by enzyme action. The net effect

is the depostion of the inorganic substance known as the calcareous corpuscles. Trypsin is preferred for the digestion of the tetrathyridium for its similarity to the lysis reaction in the host and for its efficiency in obtaining a pure sample.

The analysis of the trypsin treated corpuscles at various temperatures shows changes in chemical composition. Von Brand (19) reported an unidentifiable pattern for the calcareous corpuscles from M. corti that had been isolated by an ethylenediamine digestion and heated at 300°C.

In this investigation $CaMg(CO_3)_2$ and $CaMg_{2/3}Fe_{1/3}(CO_3)_2$ were present in samples heated at 300°C. These compounds cannot be differentiated by the use of x-ray powder diffraction. The qualitative emission spectrographic analysis shows the presence of iron, manganese and magnesium, indicating that there is isomorphic replacement of magnesium by iron to give a mixture of the two dolomites, $CaMg(CO_3)_2$ and $CaMg_{2/3}Fe_{1/3}(CO_3)_2$, in the corpuscles. Manganese frequently enters the crystalline lattice and isomorphically replaces magnesium. In those samples which gave a positive emission spectrographic analysis for manganese, there probably has been incorporation of manganese into the dolomite lattice by this isomorphic replacement of magnesium. Also at 300°C, the diffraction pattern showed $CaSO_4$ and $2CaSO_4 \cdot H_2O$; however, in the corpuscle, before heating, $CaSO_4 \cdot 2H_2O$, the stable room temperature form of the hydrate, would be expected.

Heating at 400°C and 500°C gave no change in the analysis. The majority of the lines were those of the $CaMg(CO_3)_2$ pattern; however,

the strong lines of the pattern had become broad and diffuse. Appendix III gives the pattern for the material heated at 400°C and 500°C.

Von Brand (19) reported the presence of $CaCO_3$ and MgO in the samples of calcareous corpuscles from M. corti that had been heated at 450°C. Previously, in Appendix II we reported the presence of MgO in a trypsin treated sample that had been heated at 300°C; however, at 300°C, 400°C, and 500°C we reported $CaMg(CO_3)_2$ not $CaCO_3$. The difference between von Brand reporting $CaCO_3$ and our analysis of $CaMg(CO_3)_2$ can be explained by the environment of the host or the isolation process.

The data at higher temperatures reflects recrystallization and chemical decomposition of the inorganic matter present in the original calcareous corpuscles. At 600°C to 1000°C the carbonates have decomposed leaving calcium oxide and magnesium oxide as given in Appendix IV. Magnesium carbonate decomposes at 540°C; the magnesium carbonate pattern also observed probably resulted from atmospheric carbon dioxide reacting with magnesium oxide during sample manipulation. At these temperatures, hydroxyapatite undergoes recrystallization. It is important to note that the hydrolysis and dehydration of octacalcium phosphate causes gradual conversion to hydroxyapatite. Brown, Smith, Lehr and Frazier (6) found that calcium hydrogen phosphate ($CaHPO_4$) is sometimes formed, and at high temperatures calcium pyrophosphate ($Ca_2P_2O_7$). They also report that in the presence of hydroxyapatite, calcium hydrogen phosphate and calcium pyrophosphate are very difficult to detect; calcium hydrogen phosphate was detected by the single-crystal method but not by x-ray powder diffraction. It is possible that calcium hydrogen

phosphate has been present and been overlooked in many apatitic materials. Magnesium in very low concentrations markedly inhibits the hydrolysis of octacalcium phosphate (Brown, Smith, Lehr, and Frazier (6)).

The possibility must be considered of isomorphic replacement in hydroxyapatite, similar to the replacement in the $CaMg(CO_3)_2$. The isomorphous compound, Voelekerite ($Ca_5(PO_4)_3O$), could exist (Dana (7)) and the x-ray pattern would not be measurably different by powder methods.

Von Brand (19) reported hydroxyapatite, calcium oxide and magnesium oxide for the material heated at 900°C. Unfortunately, he did not report any d-spacings and a data comparison can not be made.

The qualitative emission spectrographic analysis of calcareous corpuscles from in vivo M. corti has shown calcium and magnesium to be the major constituents. In fact, the major constituents of the corpuscles are calcium and magnesium compounds. The work reported by von Brand (19), and given in Appendix V, does not report magnesium, calcium, and phosphorus, because they are major components. These elements are expected because they were found in compounds reported in the x-ray studies. The strong lines for carbon are a result of the use of graphite electrodes.

Silicon was observed as a minor constituent. For this reason silicon was examined closely to determine if it was introduced from the glass containers or from the original environment. Samples of the tetrathyridium obtained from mice were handled in a silicon-free system

and the analysis of the corpuscles gave a positive test for silicon. Therefore, the silicon was incorporated into the corpuscles from the original environment.

Certain minor constituents reported by von Brand (19), and shown in Appendix V, were verified. For example aluminum, copper, and sodium; however, we also found lithium and chromium which were not reported by von Brand (19). Lithium was found in at least one sample and chromium in the majority of our samples. Von Brand also reported the absence of boron, manganese, and nickel, but our samples gave a positive test in most cases. These inconsistencies indicate that there is no simple composition for the corpuscles.

The most significant portion of Appendix V is the comparison of the strontium analysis. Von Brand (19) reported strontium but our normal in vivo samples show no sign of strontium. The main difference might well have been a result of the environment. Although von Brand's laboratory is located in Maryland, his samples are obtained from Los Angeles, California, which uses Colorada River water known to contain trace amounts of strontium. The tap water in Portland contains no detectable strontium. From these observations, we decided to investigate the ability of the corpuscles to concentrate strontium by giving the mice dilute strontium chloride. The strontium found in these in vivo samples show that the inorganic composition of the corpuscle depends on the environment of the parasite and is directly related to the environment of the host.

From x-ray analysis, calcium, magnesium, manganese, iron, and phosphorus could be present in the form previously suggested; but the remaining ions reported in Appendix V are incorporated into the corpuscle in a form not yet determined; this may be other compounds or further isomorphic replacement.

For a better controlled environment, the tetrathyridium was grown in vitro with the NCTC 109 Medium. With the addition of arsenic, beryllium, and strontium to the medium, the corpuscles were able to concentrate these ions. Like the samples from mice that were drinking water containing strontium, this further confirms the relationship between the composition of the corpuscles and the host's environment. These compounds were chosen because of their toxicity or their similarity to the compounds in the corpuscles. Beryllium and arsenic are very toxic chemicals to the host, but the parasites do not seem to have toxic effects from them. Because the normally white corpuscles grown in the uranium medium were yellow-colored, they appeared to have incorporated uranium. A positive identification could not be made due to spectrographic interference consisting of numerous low intensity lines in the spectrum from other components coinciding with the uranium lines. The uranium spectrum has no strong lines which can be used for unambiguous identification.

At present, no ions have been introduced into the medium that the corpuscle does not seem to incorporate. It is possible that tetrathyridium may be able to concentrate all metal ions.

The quantitative analytical procedure by the use of the emission spectrograph has not been fully perfected. There is a linear relationship between the log of intensity and the log of percent composition. The normal *in vivo* sample gave an analysis of 34% calcium (as $CaCO_3$) and 14% magnesium (as $MgSO_4$). The *in vivo* sample containing strontium gave an analysis of 20% calcium (as $CaCO_3$) and 6% magnesium (as $MgSO_4$) showing significant reduction caused by isomorphic replacement by strontium.

The standard curve for magnesium shows that the method could be very useful. After the standard curve has been calibrated the time and preparation for analysis is relatively small and simple. It is impossible to use standard wet methods because of interferring ions; likewise, the flame photometer would not only have problems with interference, but the samples are difficult to dissolve

This analytical method shows promise, but must be perfected. With further development, it will be possible to quantitatively analyze for other elements like beryllium and strontium to find percent present in the corpuscles. At present, the actual validity of the quantitative analysis is not known. The complete development of the analysis will require many additional samples of the corpuscles, each of which requires time for the *in vitro* or *in vivo* growth.

CONCLUSION

The chemical analysis of the inorganic material found in calcareous corpuscles is very complex. Analysis by powder x-ray diffraction is limited and inadequate for determination of the complete composition of the corpuscles. Qualitative emission spectroscopy supplements the x-ray powder diffraction techniques and has shown that the composition of the corpuscles is dependent upon the environment of the parasite. The introduction of a quantitative emission spectrographic analysis has added knowledge to the other information gained by other analytical methods; however, the technique is in the initial stages of development.

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APPENDIX I

X-Ray Patterns of Calcareous Corpuscles Heated at 300°C

KOH-digested		ASTM 7-239*		(en)-digested		Trypsin-digested	
d	I	d	I/I_0	d	I	d	I
4.82	vs	4.77	90			6.70	w
3.44	w			6.03	s	6.03	vs
3.07	w					3.82	vw
2.79	m			3,46	s	3.47	vs
2.72	m	2.72	6			3.18	w
2.37	vs	2.36	100			3.00 }	s
1.927	w			2.88	s;vb	vb }	
1.876	w					2.86	m
1.798	vs	1.794	56	2.70	m	2.67	m
1.575	vs	1.573	36			2.50	w
1.495	m	1.494	18			2.33	m
1.373	m	1.373	16	2.26	s	2.27	s
1.311	m	1.311	11			2.18	w
1.86	m	1.183	9	2.12	s	2.11	s
1.146	w					2.05	w
1.116	w	1.118	1	1.994	s	1.998	s
1.092	w	1.092	3	1.894	m	1.897	m+
1.034	m	1.034	5	1.806	m	1.798	m+
1.007	m	1.007	7			1.730	w
0.9473	m	0.945	8	1.66	w	1.665	m
0.9094	m	0.9085	3			1.611	w
0.8930	m	0.8923	2	1.546	w	1.546	w+
0.8645	m	0.8643	5	1.501	w	1.492	m
0.8164	w	0.8156	3	1.450	w	1.450	m;vb
0.7880	w	0.7865	3	1.517	w	1.413	m
						1.373	w+
*$Mg(OH)_2$						1.264	m

APPENDIX II

X-Ray Patterns of Calcareous Corpuscles Heated at 300°C

Corpuscle Pattern		1* ASTM 11-78		2* ASTM 12-88		3* ASTM 4-829		4* ASTM 6-226		5* ASTM 14-453	
d	I	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o
6.70	w										
6.03	vs-1									6.01	95
3.82	vw							3.87	6		
3.47	vs-2							3.498	100	3.46	45
3.18	w							3.118	3	3.21	2
3.00	s vb									3.00	100
2.86	m	2.89	100	2.899	100			2.849	33	2.80	50
2.67	m	2.67	10	2.68	3					2.70	2
2.50	w	2.54	8	2.55	2			2.473	8		
2.33	m							2.328	22		
2.27	s							2.21	20		
2.18	w	2.19	30	2.197	6			2.18	8		
2.11	s	2.07	5	2.02	3	2.106	100	2.2086	9	2.13	10
2.05	w	2.07	15	2.067	1						
1.998	s							1.993	6		
1.897	m+	1.804	20	1.852	1			1.869	15		
1.798	m+	1.786	30	1.812	6						
1.730	w							1.748	10		
1.665	m							1.648	14		
1.611	w	1.567	8	1.569	1			1.594	3		
1.546	w+	1.545	10	1.548	1			1.564	5		

APPENDIX II (CONT.)

Corpuscle Pattern		1* ASTM 11-78		2* ASTM 12-88		3* ASTM 4-829		4* ASTM 6-226		5* ASTM 14-453	
d	I	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o
1.492	m	1.496	1	1.50	1	1.489	52	1.490	5		
		1.465	5	1.449	3						
1.450	m;vb	1.445	4	1.468	1						
1.413	m	1.431	10	1.436	1			1.418	1		
1.373	w+	1.389	15	1.391	1			1.365	1		
1.264	m	1.269	2	1.273	1	1.27	4	1.277	5		

1*=$CaMg(CO_3)_2$

2*=$Ca(Mg_{2/3}Fe_{1/3})(CO_3)_2$

3*=MgO

4*=$CaSO_4$

5*=$2CaSO_4 \cdot H_2O$

APPENDIX III

X-Ray Patterns of Calcareous Corpuscles Heated at 400-500°C

Corpuscle Pattern		$CaMg(CO_3)_2$ ASTM 11-78		$Ca(Mg_{0.67}Fe_{0.33})(CO_3)_2$ ASTM 12-88	
d	I	d	I/I_o	d	I/I_o
4.15	w				
		4.03	3		
3.75	m	3.69	5	3.70	3
3.30	m				
3.01-2.89	vs;vb	2.89	100	2.90	100
2.70	w	2.67	10	2.69	3
		2.54	8	2.55	1
2.43	ms	2.41	10	2.41	3
2.21	ms	2.19	30	2.19	6
2.11	w	2.07	5	2.07	1
2.03	m	2.02	15	2.02	3
1.863	w	1.848	5	1.852	1
1.806	m;vb	1.804	20	1.812	6
		1.786	30	1.792	6
1.563	w;b	1.567	8	1.569	1
		1.545	10	1.548	1
1.486	w;b	1.496	1	1.501	1
		1.465	5	1.468	1
		1.445	4	1.449	3
		1.431	10	1.436	1
		1.413	4	1.416	1
1.392	w	1.389	15	1.391	1
		1.335	8	1.341	1
		1.297	2	1.300	1
		1.269	2	1.273	1
		1.238	5	1.241	1
1.214	vw	1.202	3	1.205	1
		1.168	4	1.171	1
		1.144	2	1.144	1
1.123	vw	1.123	5	1.126	1
				1.112	1
		1.096	3	1.099	1
		1.068	1	1.066	1
1.015		1.008	4	1.010	1
		1.001	5	1.003	1

APPENDIX IV

X-Ray Patterns of Calcareous Corpuscles

Heated Over 600°C

Corpuscle Pattern		$Ca_5(PO_4)_3(OH)$ ASTM 9-432		CaO ASTM 4-77		MgO ASTM 4-829		$MgCO_3$ ASTM 8-479	
d	I*	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o
8.18	w	8.17	11						
		5.26	5						
4.93	w								
4.06	w	4.07	9						
3.90	w	3.88	9						
		3.51	1						
3.43	m	3.44	40						
		3.17	11						
3.07	m	3.08	17						
		2.81	100						
2.77	vs;vb			2.78	34				
		2.78	60						
2.71	m	2.72	60					2.74	100
2.66	m								
2.62	w	2.63	25						
2.50	w	2.53	5					2.50	17
2.40	vs;b			2.40	100	2.43	10		
2.33	m	2.30	7					2.32	4
2.25	w	2.26	20						
		2.23	1						
2.15	w	2.15	9						
2.20	vs	2.13	3			2.11	100	2.10	43
2.04	w	2.06	7						
2.05	w	2.04	1						
1.994	w	2.00	5						
1.939	mw	1.943	30					1.939	12
1.879	m	1.890	15						
		1.871	5						
1.836	mw	1.841	40						
1.798	mw	1.806	20						
1.772	mw	1.780	11					1.769	3
1.750	w	1.754	15						
1.713	w	1.722	20						
1.697	vs	1.684	3	1.701	45			1.700	34
1.643	m	1.644	9						
1.601	m	1.611	7						
		1.587	3						
		1.542	5						

APPENDIX IV (CONT.)

Corpuscle Pattern		$Ca_5(PO_4)_3(OH)$ ASTM 9-432		CaO ASTM 4-77		MgO ASTM 4-829		$MgCO_3$ ASTM 8-479	
d	I*	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o
1.533	m	1.530	5						
		1.503	9					1.510	4
1.485	s	1.474	11			1.489	52	1.488	5
1.44	s	1.465	3	1.451	10				
1.428	w							1.426	4
1.399	w								
1.383	s			1.390	5			1.371	3
1.343	w							1.354	7
1.327	w							1.338	8
1.312	w								
1.266	m					1.270	4		
1.253	w							1.252	3
1.233	w							1.239	1
1.214	m					1.216	12	1.202	1
1.198	m			1.203	4				
1.186	m							1.180	1
1.162	vw								
1.155	vw							1.158	1
1.145	vw								
1.130	vw							1.130	1
1.119	vw								
1.113	vw								
1.100	m			1.104	4			1.101	1
1.083	m								
1.073	s			1.076	9				
1.060	vvw							1.067	4
1.051	m					1.053	5	1.051	1
1.040	m								
1.030	w								
1.022	w								
1.014	w								

*s=strong, m=medium, w=weak, vw=very weak, b=broad or diffuse line; ASTM I/I_o is relative intensity with 100 maximum.

APPENDIX V

Emission Spectrographic Analysis of Calcareous Corpuscles from Mesocestoides corti

	1*	2*	3*	4*	5*	6*
Aluminum	+	++	+	+	+	+
Boron	-	+	-	-	+	+
Cadmium	-	-	-	-	-	-
Calcium	0	+++	+++	+++	+++	+++
#Carbon	0	+++	+++	+++	+++	+++
Chromium	0	+	?	-	+	+
Copper	+	+	+	+	+	+
Iron	+	+	+	++	+	+
Lead	-	-	-	-	-	-
Lithium	0	+	-	-	-	-
Magnesium	0	+++	+++	+++	+++	+++
Manganese	-	+	?	+	+	+
Nickel	-	+	+	-	+	-
Phosphorus	0	++	++	++	++	++
Silicon	+	++	+	+	++	+
Sodium	+	+	?	+	-	+
Strontium	+	-	-	-	-	++
Tin	-	-	-	-	-	-
Titanium	-	-	-	-	-	-

Carbon lines due to graphite electrode mask any carbon in sample.

+++, ++, + = positive tests; - = negative test; ? = questionable at low concentration due to interference; 0 = not reported.

* Columns (1) Work reported by von Brand, 1967; only minor constituents were reported.

(2) M. corti corpuscles from C3H Mai mice wet ashed and ignited at 800°C for 30 min.

(3) M. corti corpuscles from C3H Mai mice trypsin digested followed by ignition.

(4) M. corti corpuscles for C3H Mai mice trypsin digested followed by sugar gradient separation, and ignition.

APPENDIX V (CONT.)

(5) Same as column 3 with Long Evans rats as host.

(6) M. corti corpuscles from C3H Mai mice, trypsin digested followed by ignition. These mice were given a dilute strontium chloride solution as drinking water during an infection of 2½ months.

APPENDIX VI

Emission Spectrographic Analysis of Calcareous Corpuscles from *In Vitro* *Mesocestoides corti* Grown in NCTC 109 Medium

	1*	2*	3*	4*	5*	6*
Aluminum	+	+	+	+	+	+
Arsenic	-	-	-	-	-	+
Boron	+	-	+	-	+	?
Beryllium	-	-	++	-	++	-
Cadmium	-	-	-	-	-	-
Calcium	+++	+++	+++	+++	+++	+++
#Carbon	+++	+++	+++	+++	+++	+++
Chromiun	+	?	+	-	+	+
Copper	+	+	+	+	+	+
Iron	+	+	+	+	+	+
Lead	-	-	-	-	-	-
Lithium	-	?	-	-	-	-
Magnesium	+++	+++	+++	+++	+++	+++
Manganese	-	+	+	-	+	+
Phosphorus	++	++	++	++	++	++
Silicon	+	+	+	+	+	+
Sodium	+	+	+	+	+	+
Strontium	-	-	?	++	++	-
Uranium	-	?	-	-	?	-

Carbon lines due to graphite electrode mask any carbon in sample.

+++, ++, + = present; - = absent, ? = questionable

* (1) normal medium, six days in culture

(2) medium with uranium, four days in culture

(3) medium with beryllium, five days in culture

(4) medium with strontium, six days in culture

(5) medium with uranium, beryllium, and strontium; five days in culture

(6) medium with arsenic, three days in culture