THE INFLUENCE OF HELMINTH INFECTION ON THE PRESENCE AND LEVELS OF PHOSPHOLIPASE B ACTIVITY IN THE DUODENUM OF THE DOG

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PREFACE

This study was designed to examine the feasibility of the enzyme assay system, phospholipase B as a routine diagnostic tool to evaluate helminth infections in the dog. Its discovery as a basic laboratory aid was made in part by A. Ottolenghi and his co-workers. Thanks to these people and their cooperation our study has been made possible. In addition thanks are given to my advisor Dr. A. A. Kocan and excellant committee members and counselors Dr. H. E. Jordan and Dr. J. T. Homer. Gratitude goes also to my co-worker and fellow graduate student, Harold Laubach whose help I could not have done without. Statistical counseling was provided, with appreciation, by Dr. David Weeks of this university.

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CHAPTER I

INTRODUCTION

Parasitic helminth infections of domestic animals and man are of major medical concern and a source of great economic loss each year in the world. Although these parasite problems are of great medical and veterinary significance, techniques and methods for evaluation of a helminth problem are not presently sophisticated enough to meet the needs. Because of the deficiency in the area of determination and evaluation of parasitic helminth diseases, the development of a quantitative assay system, in this case phospholipase B, which may serve as a chemical index of inflammatory conditions resulting from helninth infections, is warranted.

While this system has not yet been sufficiently tested to be of practical use in routine diagnosis, an examination of its potential is certainly justified. Ottolenghi and Rowland (1) found the system to be an accurate indicator of the presence of selected tapeworm infections (Hymenolepis nana) in mice and rats as seen in the inflammatory response due to the tissue invading worms. The great advantage of this particular enzyme system is that it is a quantitative test which does not depend upon visualizing the parasite or noting the life stage of the worm.

The present study is designed to expand the usage of the enzyme assay system (phospholipase B) as an index of helminth induced inflammation by examining the activity in the proximal one-third of the duodenum of the dog. Based on previously published studies, there is no reason to doubt that phospholipase B is indeed present in normal dog intestine and that a similar causal relationship of intestinal inflammation as induced by helminth infection is also operating with this model. Although baselines will not be established at this time, the storage life of the enzyme in canine intestinal tissue at time periods of 24, 48 and 168 hours at -3 degrees centigrade and -76 degrees centigrade and possible relationship to helminth burdens will be investigated.

Once baseline levels are worked out for each host and parasite model, enzyme activity may well give an accurate indication of parasite worm burdens and severity of infections.

CHAPTER II

LITERATURE REVIEW

The lysophospholipid degrading enzyme, phospholipase B (E.C. 3.1.1.5) has been found in a wide variety of organisms including parasitic protozoans, molds, fungi, bacteria, yeast, grain, vegetables, arthropods, fish and reptiles (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27). Enzyme activity has been extensively studied in rodents with all tissues except adipose tissue of both rabbits and rats proving positive for phospholipase B (28, 29, 30, 31, 32, 33, 34). Enzyme activity has also been reported in human seminal plasma, ascites tumors, in the gray and white matter of the brain, cerebellum, caudate nucleus, corpus callosum and whole blood (28, 35, 36). Phospholipase B activity has been shown in ovine liver, kidney and thyroid tissues, while in the bovine activity is limited to the liver and pancreas (29, 37, 38). Canine tissues have been examined for this enzyme by only a few investigators. Phospholipase B in erythrocytes of the dog was found to reduce the hemolysing power of lysophosphatidyl choline, while a more recent study demonstrated activity in canine heart muscle (39, 40). Only one investigation has dealt with the dog intestine

which noted the complete breakdown of phosphatidyl choline by enzymes present in the small intestine (41). The enzyme responsible was not specifically identified nor located as to region of source in the small intestine. However, due to the chemical reactions achieved by the enzyme reported it is assumed that phospholipase B was present in the canine's small intestine.

Recent studies have focused on the function of the enzyme, phospholipase B, in relation to the lipid metabolism of organisms studied. A leading hypothesis is one of anti-inflammatory function (42). Although lipids or the enzymes involved in lipid metabolism have not been previously considered for this role, it is known that they do constitute a major portion of cellular membranes and the lysophosphatides, known to be highly surface active and cytotoxic in high concentrations, are ideally suited to mediate inflammatory reactions (43, 44).

The presence of phospholipase B activity in homogenates of mammalian (rabbit) polymorphonuclear leukocytes was identified in 1963, but the hypothesis that eosinophils are the carriers for the enzyme was not brought forth until 1967 by Ottolenghi (45) by histochemical demonstration of phospholipase B in rat tissues. A relationship between altered granules of eosinophils and helminth infection, (Moniliformis dubius), in rat jejunum demonstrated that there was yet an unknown role of the enzyme released from these eosinophils. That the helminth infection probably caused the

alterations of the granules was hypothesized by the investigator (46).

Eosinophils have been extensively studied since the late 1800's when an association was reported between eosinophils and the allergic state. In 1907 it was first noted that eosinophils are attracted to the sites of parasitic infection as well (47). The exact role of eosinophils is still questionable but is thought by Bessis (48) to relate to immunologic reactions to foreign material. Eosinophils probably migrate to allergic foci, including areas of parasitic infection and then release the contents of their granules, which may contain phospholipase B. Rytömma (49) suggests that certain chemotactic substances present in the loose connective tissue of the body, bronchial and interstitial epithelium, attract eosinophils as a result of an immune reaction between antigen and antibody. Goetzel, et. al. (50) theorize that eosinophils have a local regulatory function in immediate and subacute hypersensitivity reactions following from their content of factors that inhibit histamine release or its mediator function, and from the presence of high levels of intracellular plasminogen and phospholipase B.

At the cellular level, work by Vaughn (51) and others (52, 53, 54) has shown that the granules of eosinophils are composed of a phospholipid substance with a protein center and deoxyribonucleic acid. Degranulation of eosinophils has been observed in skin and bowel wall of the rat by some type

of coalescence of specific granules without rupture of the cell membrane (49, 55, 56). Histochemical evidence has been provided by Ottolenghi, et. al. (45) that cells staining positive for phospholipase B activity are present in the lamina propria of the intestinal villi and crypts, in the red pulp of the spleen and in the interstitial tissue of the lung, liver and bone marrow of rats. These cells were all determined to be eosinophils by morphologic and staining characteristics and were found to decrease sharply in number along with enzyme activity when the rat tissues were treated with agents as corticosteroids and X-irradiation.

Teir, et. al. (57) find that the bowel wall of most species is abundantly endowed with eosinophils and that the eosinophilia is affected by the histamine content of the area. When the helminths present produce inflammation, histamine is released, eosinophils are attracted to the site and phospholipase B is probably released. The amount of time necessary for this proposed process is unknown, but it is known that the greatest inflammatory response and histamine release occurs soon after trauma is inflicted. Histamine is also a product of immediate hypersensitivity as mediated by humoral antibody. Antibody production, depending upon previous exposure to the same antigen, also elicits a secondary or amnestic response which proceeds quicker, peaks at a higher level and lasts for a longer period of time than the primary response. The fact that an animal had only an initial encounter with a particular inflammatory

causal agent is very significant in showing the relationship of enzyme levels to enducing agents.

Ottolenghi (45, 58) has found significant evidence supporting the theory that phospholipase B is derived from granules of eosinophils. Experiments demonstrate striking correlations between these cells and enzymatic activity in the same tissues and a corresponding decrease in the number of eosinophils and enzymatic activity in tissues of dexamethasone (eosinophil reducer) treated rats (58). It is further hypothesized by Ottolenghi (58) that increased enzymatic activity is due to intestinal inflammation and that the eosinophils undergo morphological changes at the sites of inflammation and release their stores of phospholipase B.

In experiments with Trichinella spiralis, a helminth which produces a striking inflammatory response, Larsh, et. al. (59) noted that greatly elevated levels of phospholipase B found in the intestines of infected rats and mice correlated to the point in time at which inflammation was the greatest. In addition, from data collected from further experiments they hypothesize that eosinophils continually migrate to these inflammed areas and release their stores of phospholipase B until the worms are expelled and the inflammation has run its course (60).

The function of phospholipase B is thought to be one of an anti-inflammatory nature as proposed by several investigators. Rao and Subrahmanyam (19), who found phospholipase B in Culex pipiens fatigens larva have suggested that the

enzyme has a role in preventing the accumulation of lysolipids to cytotoxic levels in insects. That phospholipase B has a similar biologic function in mammalian tissues is supported by Barbanti, et. al. (61) who found the enzyme in Sendai virus. The enzyme was found to alter the hemolytic and fusing ability of the virus due to inactivation of lysolecithin. Modolell and Munder (42) found that phospholipase B acted as an anti-inflammatory agent when used in rats injected with bentonite and $Al(OH)_3$ and further suggest that phospholipase B could be the "natural" therapeutic of the organismal surveillance system.

CHAPTER III

MATERIALS AND METHODS

Procedure for Making Lysophosphatidyl Choline, the Substrate (62)

First Day

Six fresh eggs were placed into a waring blender with 250 ml of acetone, blended and allowed to stand for one hour at room temperature. This preparation was filtered, the residue added to 250 ml of acetone and blended at 10 degrees centigrade. This step was repeated a second time. The filter residue was extracted by stirring for 30 minutes with 150 ml of methanol/chloroform (1:1) at 10 degrees centigrade. The solution was filtered and the residue rewashed twice, the filtrates being preserved. The total amount of filtrate obtained equaled 450 ml. The 450 ml extract was evaporated to dryness using a rotary evaporator at room temperature and the gummy residue was then dissolved in 50 ml of petroleum ether. The sample was then divided into two samples of 25 ml each, placed in 250 ml flasks, each with a magnetic stirrer, while 150 ml of cold acetone was added to each while stirring. The preparation was then refrigerated overnight at 10 degrees centigrade.

Columns were prepared on the first day for the next day's procedures by placing 40 grams of Al_2O_3 into each of three columns along with 100 ml of methanol/chloroform (1:1). The columns were shaken, allowed to stand, and the methanol/chloroform (1:1) added. The procedure of shaking, standing and adding clean methanol/chloroform (1:1) was repeated three more times. The columns then stood overnight in a 10 degree centigrade refrigerator.

Second Day

The 400 ml of solution from the previous day was centrifuged in 12 centrifuge tubes at 120 x g for ten minutes. The supernatant fluid was poured off and the resulting pellet dissolved in 250 ml of methanol/chloroform (1:1). The preparation was then evaporated to dryness in a rotary evaporator and resuspended in 50 ml of methanol/chloroform (1:1) in a 250 ml flask. 15 ml, 15 ml and 20 ml of preparation respectively, was then placed on Al_2O_3 columns and each washed with 90 ml of methanol/chloroform (1:1). When the preparation came from the columns (300 ml), it was filtered twice with #50 filter paper, not changing the filters. The fluid preparation was then evaporated to dryness using a rotary evaporator. The white powder residue was dissolved in 250 ml of ether in a 500 ml flask and set aside for use in the phosphate test.

Phosphate Test. One ml of ether solution was brought

to volume with ether in a 25 ml flask. 48 ten ml test tubes were prepared and numbered one through 48 with the following additives to each: 1, 2, 3, 0.5 ml ether preparation, two glass beads; 4, 5, 6, 1.0 ml ether preparation, two glass beads; 7, 8, 9, 2.0 ml ether preparation, two glass beads; 10, 11, 12, one ml ether; 13, 14, 15, one ml 0.2 M H_3PO_4; 16, 17, 18, one ml 0.5 M H_3PO_4; 19, 20, 21, one ml 0.8 M H_3PO_4; 22, 23, 24, one ml 1.0 M H_3PO_4; 25, 26, 27, one ml 0.1 M H_3PO_4; 28, 29, 30, one ml 0.01 M H_3PO_4; 31, 32, 33, one ml 0.001 H_3PO_4; 34, 35, 36, one ml 0.0001 M H_3PO_4; 37, 38, 39, one ml 0.00001 M H_3PO_4; 40, 41, 42, one ml 0.000001 M H_3PO_4; 43, 44, 45, one ml 0.0000001 M H_3PO_4 and 46, 47, 48, one ml 0.00000001 M H_3PO_4.

The ether was dried in tubes one through nine with nitrogen for 30 seconds followed by addition of one ml of distilled water and one ml of 70 percent perchloric acid. Tubes one through nine were then placed into a hot sand bath under a hood and tapped lightly until ready to boil. When the solution in the tubes reached the boiling point, they were buried in the sand and boiled for 20 minutes. At the end of 20 minutes the tubes were removed from the sand bath and allowed to stand under a hood for five minutes. Three ml of distilled water was then added to each tube and they were then put into a hot water bath for 12 minutes. At the end of 12 minutes, the tubes were put into a beaker of cold water and cooled to room temperature.

To all 48 tubes one ml of ammonium molybdate solution

and 0.4 ml of phosphate reagent was added. The tubes were brought to the ten ml mark with distilled water, mixed and allowed to stand for ten minutes. The tubes were then read at 600 Å on a spectrophotometer after spinning at 140 x g for five minutes.

Phospholipase A Treatment of Phosphatidyl Choline. The phosphatidyl choline-ether solution was brought to a final volume of 2000 ml in a 4000 ml flask with ether. 25 mg of rattlesnake venom and 22 mg of $CaCl_2$ was added to the solution while stirring with a magnetic stirrer in a four liter flask. 1000 ml of the preparation was poured into each of two separatory funnels and allowed to stand overnight in a 10 degree centigrade refrigerator.

Third Day

The lysophosphatidyl choline was washed three times with 2000 ml ether in a 4000 ml flask allowing 30 minutes for settling between washes. The ether was siphoned off on the last wash and the precipitate put into two centrifuge tubes and spun at 120 x g for ten minutes. The precipitate was washed twice in 240 ml cold acetone and hand stirred with a glass rod. The solution was then centrifuged in six large centrifuge tubes at 120 x g for ten minutes. The precepitate was dried with nitrogen while setting the centrifuge tubes in a beaker of 45 degree centigrade water. The lysophosphatidyl choline was then washed in 100 ml of hot

absolute ethyl alcohol by setting four large test tubes containing 25 ml each of the alcohol into a beaker of boiling water on an electric hot plate. The precipitate was collected, dissolved in 60 ml of ethyl alcohol, divided equally and placed into two large centrifuge tubes and spun at 120 x g for ten minutes in a refrigerated centrifuge. The resulting pellet was discarded while the supernatant fluid was poured into a small evaporating flask and evaporated to dryness with nitrogen. The white powder residue was dissolved in 50 ml of distilled water and dialized overnight in a 10 degree centigrade refrigerator in a four liter flask containing cold distilled water.

Fourth Day

The dialized lysophosphatidyl choline was removed from the refrigerator and brought to the final corrected volume of 20 µm/ml with distilled water. The 1930 ml of substrate was then stored in 0.35 ml aliquots in small paraffin covered test tubes and deposited in a Revco freezer at -76 degrees centigrade.

Procedure for Testing for the Presence of Phospholipase B, the Enzyme

The selected tissue sample was weighed, minced finely with scissors and homogenized in 19 volumes of ice cold 12.5 percent glycerol medium in a Ten-Broeck glass homogenizer. Five 5 ul aliquots of homogenate were each brought to a

final volume of 0.6 ml using the 12.5 percent glycerol medium. The five tubes of homogenate were equilibrated in a 37 degree water bath for four minutes. At the end of this time 0.3 ml of prewarmed (37 degree centigrade) lysophosphatidyl choline solution (20 µm/ml) was added to each of the test tubes and incubation continued at 37 degrees centigrade for 30 minutes or until the first sign of a cloudy precipitate. While stirring, 0.1 ml of 2 N H_2SO_4, one ml of isopropyl alcohol, and 0.4 ml of distilled water was added to each tube. Two ml of heptane was then added to each test tube, after which the heptane containing the free fatty acids was pipetted off into another test tube. While bubbling nitrogen into the test tube, the free fatty acids were titrated with 0.01 N NaOH using a Repipet Dispensor (Lab-industries, Berkeley, Cali.) (63). A solution of palmitic acid in heptane (2 µm/ml) was used as the reference standard for the NaOH, and five samples lacking lysophosphatidyl choline, the substate, served as controls.

Procedure for Obtaining Tissue Sample for the Enzyme Assay

Each dog intestine examined for the presence of phospholipase activity was analyzed within five to ten minutes of death. Death was induced in all animals by intravenous administration of Beuthanasia-D Regular Solution (Chromalloy Pharmaceutical Inc., Oakland, Cali.). The peritoneal cavity was opened and the small intestine removed intact from the

pyloric valve to the cecum. A two inch long sample from the proximal one-third of the duodenum was quantitatively analyzed for the presence of phospholipase B activity and paired selected samples were frozen in a Revco freezer (-76 degrees centigrade) and refrigerator freezer (-3 degrees centigrade) for comparative analysis at later times. The remainder of the intestine was saved for examination for helminths.

Source of Experimental Animals

34 dogs were used as experimental animals for assaying for the presence of phospholipase B in the proximal one-third of the duodenum. Most of the dogs were obtained from the pound (16 dogs) while the others were clinical necropsy, student surgery or donated research animals. 12 male and 22 female dogs were examined. Breeds included were collie, poodle, german shepard, german shorthair pointer, brittany spaniel, dachshund, scotty, terrier, hound, schnauzer and mixed. Their age range was from six months to ten years, with most of the animals falling in the six months to two year age group (23 dogs).

Procedure for Determining Helminth Burden of the Small Intestine of the Dogs

Pertinent information including identification number, post mortem findings and clinical history was collected for each dog examined. The small intestine, once removed, was

opened along its entire length, flushed with water, and scraped with a 2 x 2 inch glass slide; care was taken to save all contents. The sediment was then allowed to settle in a plastic mixing bowl and decanted periodically until the supernatant appeared clear. The remaining sediment was poured into a glass pie pan, approximately 25 ml at a time and examined for helminths by gross inspection. All helminths were counted and identified to genus with notation as to state of maturity of the specimens taken.

CHAPTER IV

RESULTS AND DISCUSSION

Presence and Levels of Phospholipase B in the Dog

Phospholipase B Levels

The proximal one-third of the duodenum of the dog has not been previously examined by any other investigators for the presence of the enzyme, phospholipase B. Phospholipase B activity was found in 26 of the 34 dogs examined (76 percent) with a range of 0 µm fatty acid/gm wet tissue/hour to 6283 µm fatty acid/gm wet tissue/hour (Table I). The 34 dogs examined were not a homogeneous group, which may account for the great variability found in this study.

Relationship of Phospholipase B and Helminth Infected Dogs

30 dogs with helminths and four dogs without helminths in the small intestine were examined and compared for levels of phospholipase B in the proximal one-third of the duodenum. Ancylostoma sp. was the most predominant helminth found in parasitized dogs (83 percent). A Student's t test was run and it was shown that the probability of t_{tab} being greater

TABLE I

BREED, SEX, AGE, INTESTINAL HELMINTH BURDEN AND PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM OF 34 EXPERIMENTAL DOGS

Dog	Breed	Sex	Age (Years)	Helminth Genera	*Enzyme Activity
1	Poodle	F	2	0	996
2	Collie	F	1-2	8 Ancylostoma 2 Physaloptera 2 Toxascaris	6283
3	Poodle	F	5	1 Ancylostoma	3691
4	Beagle	F	7	0	0
5	Beagle	F	4½	123 Ancylostoma	651
6	Beagle	F	5½	11 Ancylostoma	423
7	Beagle	F	4½	1 Ancylostoma	0
8	Beagle	F	5	0	0
9	German Shorthair Pointer	M	10	15 Ancylostoma	1646
10	German Shepherd	M	½-1	22 Ancylostoma 5 Dipylidium 3 Physaloptera	576
11	Collie Mix	F	2	5 Taenia	2305
12	Dachshund	F	6	0	1556
13	Scotty	M	1	4 Ancylostoma	760
14	Poodle	F	3	57 Dipylidium	559
15	Brittany Spaniel	F	2-4	14 Ancylostoma 4 Taenia	952
16	Shepherd Mix	F	½	21 Ancylostoma	3922

TABLE I

(CONTINUED)

Dog	Breed	Sex	Age (Years)	Helminth Genera	*Enzyme Activity
17	Shepherd Mix	M	½	24 Ancylostoma 82 Dipylidium 1 Toxocara	3260
18	Hound	M	3	133 Ancylostoma 10 Dipylidium	103
19	Mixed	M	½-1	9 Ancylostoma 2 Taenia	0
20	Schnauzer Mix	M	1-2	7 Dipylidium	2124
21	Schnauzer Mix	F	1-2	4 Ancylostoma	3035
22	Terrier Mix	F	1-2	25 Ancylostoma 1 Taenia	0
23	Terrier Mix	M	1	14 Ancylostoma 5 Taenia	0
24	Terrier Mix	F	1	15 Dipylidium	0
25	Brittany Spaniel	F	½-1	10 Ancylostoma 1 Dipylidium 16 Taenia	423
26	Mixed	F	1-2	2 Ancylostoma	452
27	Mixed	M	1	7 Ancylostoma	413
28	Mixed	M	½	1 Ancylostoma 2 Toxocara	696
29	Mixed	M	½	106 Ancylostoma 1 Toxocara	483
30	Mixed	M	1	2 Dipylidium	0

TABLE I

(CONTINUED)

Dog	Breed	Sex	Age (Years)	Helminth Genera	*Enzyme Activity
31	Mixed	F	½-1	184 Ancylostoma 1 Dipylidium	728
32	Poodle Mix	M	½-1	15 Ancylostoma 3 Dipylidium	2512
33	Beagle Mix	F	½-1	14 Ancylostoma	2261
34	Poodle	F	1-2	40 Ancylostoma 1 Taenia 1 Toxocara	103

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are µm fatty acid/ gm wet tissue/hour.

than t_{calc} was equal to .20; the evidence is strong that the means of the phospholipase B activity of the two groups of dogs are the same (Table II). There is not much difference in phospholipase B activity in the proximal one-third of the duodenum of dogs with helminths and dogs without helminths as exemplified by this test. However, due to the great amount of variability within groups and low number of dogs examined without helminths, these results are inconclusive.

Because this group of experimental data was statistically difficult to interpret, the animals were grouped by total helminth burden of the small intestine and analyzed as they relate to enzyme activity (Table III). Figure 1 depicts the relationship of numbers of helminths present and phospholipase B activity in seven groups of dogs with interesting results. It appears that there is possibly an "optimum worm burden at which phospholipase B activity peaks and that below or beyond this level activity is lower. The optimum range appears to be ten to 20 helminths present. Although this is by no means conclusive, a general tendency may be drawn from the information collected. It should be noted that Ottolenghi and Rowland (1) found in their studies with Hymenolepis nana and phospholipase B activities in the small intestine of mice that individual variability probably played a major role once a stimulus of sufficient intensity developed. Undoubtedly great individual variability played a major role in the character of the data collected in this study in addition to the influence of varying stages of

TABLE II

STUDENT'S T TEST ON 34 EXPERIMENTAL DOGS WITH AND WITHOUT HELMINTHS IN THE SMALL INTESTINE WITH RELATION TO PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM

Dogs with Helminths		Dogs without Helminths	
Dog	*Enzyme Activity	Dog	*Enzyme Activity
2	6283	1	996
3	3691	4	0
5	651	8	0
6	423	12	1556
7	0		
9	1646		
10	576		
11	2305		
13	760		
14	559		
15	952		
16	3922		
17	3260		
18	103		
19	0		
20	2124		
21	3035		
22	0		
23	0		
24	0		

TABLE II

(CONTINUED)

Dogs with Helminths		Dogs without Helminths	
Dog	*Enzyme Activity	Dog	*Enzyme Activity
28	696		
29	483		
30	0		
31	728		
32	2512		
33	2261		
34	103		

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are μm fatty acid/gm wet tissue/hour.

H_0: Dogs with helminths have phospholipase B activity in the proximal one-third of the duodenum$\neq$phospholipase B activity in the proximal one-third of the duodenum of dogs without helminths.

H_A: The activities of the two groups of dogs are the same.

$t_{calc}=.82$

$P(t_{tab}$ greater than $t_{calc})=.20$ (64)

Since the observed significance level of tabulated t being greater than calculated t is only 20 percent the null hypothesis is not significant and not accepted.

TABLE III

THE RELATIONSHIP OF AVERAGE PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM TO AVERAGE TOTAL NUMBER OF HELMINTHS PRESENT IN 34 EXPERIMENTAL DOGS ARRANGED IN SEVEN GROUPS

Group	Dog	Helminth Genera	Total Number	Mean	*Enzyme Activity	Mean
I	31	184 Ancylostoma 1 Dipylidium	185		729	
	18	133 Ancylostoma 10 Dipylidium	143		103	
	5	123 Ancylostoma	123		651	
	29	106 Ancylostoma 1 Toxocara	107		483	
	17	24 Ancylostoma 82 Dipylidium 1 Toxocara	107		3260	
				133		1045
II	14	57 Dipylidium	57		559	
	34	40 Ancylostoma 1 Taenia 1 Toxocara	42		103	
	10	22 Ancylostoma 5 Dipylidium 3 Physaloptera	30		576	
	25	10 Ancylostoma 1 Dipylidium 16 Taenia	27		423	
	22	25 Ancylostoma 1 Taenia	26		0	
				36		332

TABLE III

(CONTINUED)

Group	Dog	Helminth Genera	Total Number	Mean	*Enzyme Activity	Mean
III	16	21 Ancylostoma	21		3922	
	23	14 Ancylostoma 5 Taenia	19		0	
	32	15 Ancylostoma 3 Dipylidium	18		2512	
	15	14 Ancylostoma 4 Taenia	18		952	
	9	15 Ancylostoma	15		1646	
	24	15 Ancylostoma	15		0	
				18		1505
IV	33	14 Ancylostoma	14		2261	
	2	8 Ancylostoma 2 Physaloptera 2 Toxascaris	12		6283	
	6	11 Ancylostoma	11		423	
	19	9 Ancylostoma 2 Taenia	11		0	
				12		2242
V	27	7 Ancylostoma	7		413	
	20	7 Dipylidium	7		2124	
	11	5 Taenia	5		2305	
				6		1614

TABLE III

(CONTINUED)

Group	Dog	Helminth Genera	Total Number	Mean	*Enzyme Activity	Mean
VI	13	4 Ancylostoma	4		760	
	21	4 Ancylostoma	4		3035	
	28	1 Ancylostoma 2 Toxocara	3		696	
	26	2 Ancylostoma	2		452	
	30	2 Dipylidium	2		0	
	3	1 Ancylostoma	1		3691	
	7	1 Ancylostoma	1		0	
				2		1233
VII	4	0	0		0	
	8	0	0		0	
	12	0	0		1556	
	1	0	0		996	
				0		638

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are μm fatty acid/gm wet tissue/hour.

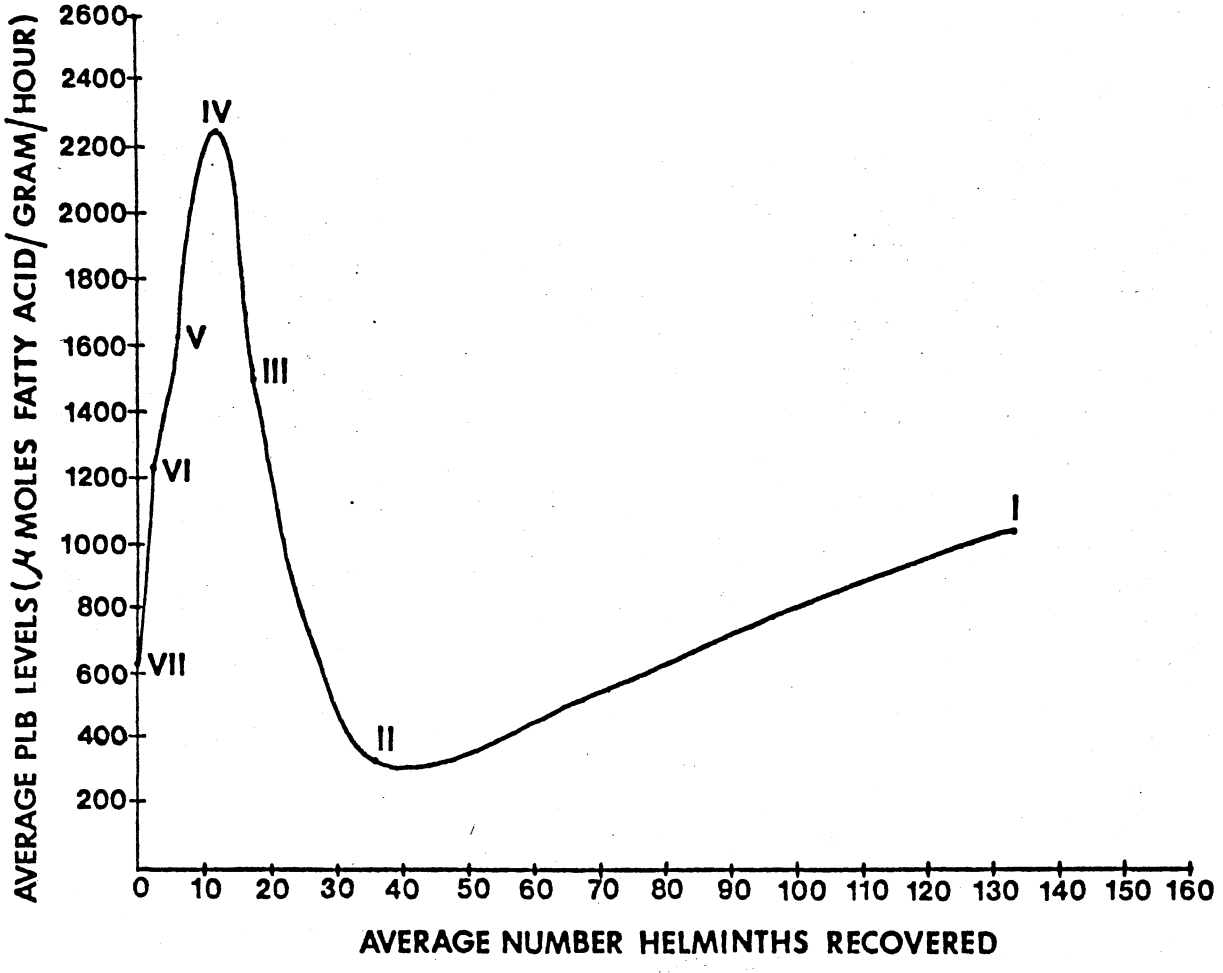

Figure 1. Relationship of Average Phospholipase B Activity in the Proximal One-Third of the Duodenum to Average Numbers of Helminths Present in the Small Intestine.

parasite development and maturity on phospholipase B activity of each animal.

Relationship of Phospholipase B and Nematode Infected Dogs

Phospholipase B activity in the proximal one-third of the duodenum of 14 animals with nematodes only present in the small intestine were compared to those activity levels of four animals with no nematodes in the small intestine. All 14 of the infected group had *Ancylostoma* sp. while dogs 2, 28 and 29 had additional nematodes present, *Toxocara* sp., *Toxascaris* and *Physaloptera* sps. The Student's t test showed that the probability of t_{tab} being greater than t_{calc} was equal to .13; there is not much evidence that phospholipase B activity of the two groups of dogs are different (Table IV). However, those with nematodes did have a relatively higher average of activity; and, more animals were examined with nematodes than without and the possibility of random animal variation exists.

Relationship of Phospholipase B and *Ancylostoma* sp. Infected Dogs

Eleven dogs that were infected with only hookworms in the small intestine were analyzed in simple linear regression for curve fitting to phospholipase B activity (Table V). Four control dogs that had no worms in the small intestine were used in addition to the 11 dogs with one to 123

TABLE IV

STUDENT'S T TEST ON 18 EXPERIMENTAL DOGS WITH AND WITHOUT NEMATODES IN THE SMALL INTESTINE WITH RELATION TO PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM

Dogs with Nematodes		Dogs without Nematodes	
Dog	*Enzyme Activity	Dog	*Enzyme Activity
2	6283	1	996
3	3691	4	0
5	651	8	0
6	423	12	1556
7	0		
9	1646		
13	760		
16	3922		
21	3035		
26	452		
27	413		
28	696		
29	483		
33	2261		

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are μm fatty acid/gm wet tissue/hour.

H_0: Dogs with nematodes have phospholipase B activity in the proximal one-third of the duodenum$\neq$phospholipase B activity in the proximal one-third of the duodenum of dogs without nematodes.

H_A: The activities of the two groups of dogs are the same.

TABLE IV

(CONTINUED)

$t_{calc}=1.17$

$P(t_{tab}$ greater than $t_{calc})=.13$ (64)

Since the observed significance level of tabulated t being greater than calculated t is only 13 percent the null hypothesis is not significant and not accepted.

TABLE V

LACK OF FIT TEST FOR SIMPLE LINEAR REGRESSION CORRELATION ON THE NUMBER OF *ANCYLOSTOMA* SP. IN THE SMALL INTESTINE TO PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM IN 15 EXPERIMENTAL DOGS

Dog	Number *Ancylostoma* sp.	*Enzyme Activity
1	0	996
4	0	0
8	0	0
12	0	1556
3	1	3691
7	1	0
26	2	452
13	4	760
21	4	3035
27	7	413
6	11	423
33	14	2261
9	15	1646
16	21	3922
5	123	651

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are µm fatty acid/gm wet tissue/hour.

TABLE V

(CONTINUED)

Analysis of Variance Table

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F Ratio
Between Distinct X's	13,670,157	9		
Linear	35,621	1	35,621	.02
Remainder	13,634,536	8	1,704,317	.76
Within Distinct X's	11,184,612	5	2,236,922	

Since the linear tabulated F=6.61 and the linear calculated F=.02 is less than 6.61, the null hypothesis in not accepted at the five percent significance level and no linear relationship exists. Since the remainder tabulated F=4.82 and the remainder calculated F=.76 is less than 4.82, the null hypothesis is not accepted at the five percent significance level and no higher degree curve fits the data (65).

Ancylostoma sp. present. The remainder F test at the five percent level equaled .76 which indicated that a higher degree curve was not needed to fit the data and a straight line might fit. The linear F test at the five percent level was equal to .02 indicating that there was not even a linear relationship among the data.

In an alternate method of examining the data the 15 dogs were arranged in three groups according to numbers of Ancylostoma sp. present, averaged and plotted as to phospholipase B activity (Table VI). Figure 2 indicates that the Ancylostoma sp. and phospholipase B relationship is one of an upward right sloping nature. This relationship may be more valid than the previous statistical work indicates because of the grouping of animals. There is likely to be some point at which the phospholipase B activity will rise no higher with step-wise gradations and minimum levels below. With the data provided only conjecture is applicable.

Effects of Storage and Temperature on Phospholipase B

The activity of phospholipase B in canine small intestine after a period of storage or freezing has not been examined by any previous researchers. Most enzymes are known to decompose rather quickly in vitro due to their biochemical make-up and the assumption would be that this enzyme reacts in much the same way. Since heat denatures the protein of all enzymes, cold was used in this study as a means

TABLE VI

THE RELATIONSHIP OF AVERAGE PHOSPHOLIPASE B ACTIVITY IN THE PROXIMAL ONE-THIRD OF THE DUODENUM TO AVERAGE TOTAL NUMBER OF *ANCYLOSTOMA* SP. PRESENT IN 15 EXPERIMENTAL DOGS ARRANGED IN THREE GROUPS

Group	Dog	Number *Ancylostoma* sp.	Mean	*Enzyme Activity	Mean
I	1	0		996	
	4	0		0	
	8	0		0	
	12	0		1556	
			0		638
II	3	1		3691	
	7	1		0	
	26	2		452	
	13	4		760	
	21	4		3035	
	27	7		413	
			3		1392
III	6	11		423	
	33	14		2261	
	9	15		1646	
	16	21		3922	
	5	123		651	
			37		1781

TABLE VI

(CONTINUED)

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are μm fatty acid/gm wet tissue/hour.

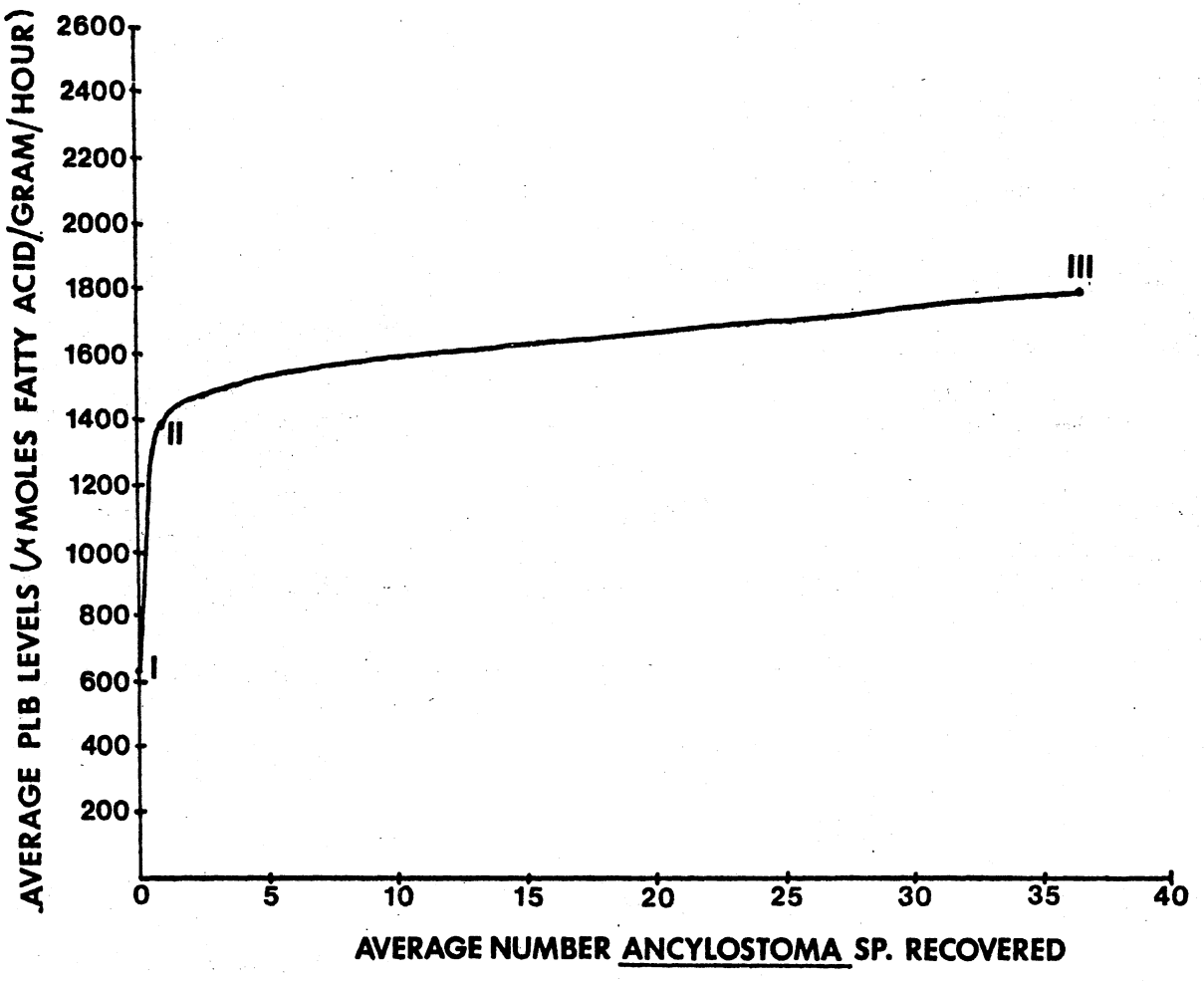

Figure 2. Relationship of Average Phospholipase B Activity in the Proximal One-Third of the Duodenum to Average Numbers of Ancylostoma sp. Present in the Small Intestine.

of assaying storability of test tissue.

Tissue samples from the ten dogs examined had an initial activity range of 728 µm fatty acid/gm wet tissue/hour to 3922 µm fatty acid/gm wet tissue/hour. Each tissue was divided into six pieces and stored in a Revco at -76 degrees centigrade and in a refrigerator freezer at -3 degrees centigrade and examined at 24, 48 and 168 hours after storage. Five assays were run on each tissue sample for statistical homogeniety and an average taken for the standard value.

After 48 and 168 hours of storage, no enzyme activity could be found in the tissue samples at either -76 degrees centigrade or -3 degrees centigrade (Tables VII and VIII). At 24 hours those samples that did show activity exhibited markedly reduced values. Undoubtedly the life of phospholipase B is quite short, as most enzymes, and reliable activity measurements can be made only within a very short time after removal of the tissue from the living host animal.

The statistical tests examining interaction and contrasts of temperature and storage of the enzyme in intestinal tissue show no significance except at the treatment level (Table IX). Treatment significance is shown at the 95 percent level; this is merely an indication that the six treatments examined were indeed different. Since none of the individual treatment comparisons were significant it can be assumed that phospholipase B activity is not influenced by storage at temperatures of -76 degrees centigrade and -3 degrees centigrade; it will lose activity within a

TABLE VII

THE EFFECTS OF -3 DEGREE CENTIGRADE STORAGE OVER 24, 48 AND 168 HOURS ON THE PHOSPHOLIPASE B ACTIVITY OF THE PROXIMAL ONE-THIRD OF THE DUODENUM OF TEN EXPERIMENTAL DOGS

		0 Hours	24 Hours	48 Hours	168 Hours	
	9	1646	481	0	0	
	11	2305	0	0	0	
	13	760	0	0	0	
	14	559	0	0	0	
Dog	15	952	901	0	0	*Enzyme Activity
	16	3922	1575	0	0	
	17	3260	300	0	0	
	31	728	0	0	0	
	32	2512	0	0	0	
	33	2261	0	0	0	

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are μm fatty acid/gm wet tissue/hour.

TABLE VIII

THE EFFECTS OF -76 DEGREE CENTIGRADE STORAGE OVER 24, 48 168 HOURS ON THE PHOSPHOLIPASE B ACTIVITY OF THE PROXIMAL ONE-THIRD OF THE DUODENUM OF TEN EXPERIMENTAL DOGS

		0 Hours	24 Hours	48 Hours	168 Hours	
	9	1646	237	0	0	
	11	2305	0	0	0	
	13	760	0	0	0	
	14	559	442	0	0	
Dog	15	952	0	0	0	*Enzyme Activity
	16	3922	0	0	0	
	17	3260	0	0	0	
	31	728	0	0	0	
	32	2512	728	0	0	
	33	2261	579	0	0	

*Each reading is based on five observations which were then averaged. Phospholipase B activity units are µm fatty acid/gm wet tissue/hour.

TABLE IX

ANALYSIS OF VARIANCE TABLE-CONTRASTS AND INTERACTION OF TIME AND TEMPERATURE ON PHOSPHOLIPASE B ACTIVITY OF THE PROXIMAL ONE-THIRD OF THE DUODENUM OF THE DOG

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F Ratio
Treatments	28,864,756	6	4,810,793	20.6
C_1	278,679	1	278,679	1.2
C_2	269	1	269	.001
C_3	269	1	269	.001
C_4	43	1	43	.0001
C_5	808	1	808	.003
C_6	0	1	0	0
Error	12,518,088	54	231,816	

Since the treatment tabulated F=1.63 and the treatment calculated F=20.6 is greater than 1.63, the null hypothesis is accepted at the five percent significance level and the treatments are indeed different. Since the individual treatment tabulated F=2.02 and the individual treatment calculated F=1.2, .001, .0001, .003, 0 are less than 2.02, the null hypothesis is not accepted at the five percent significance level and the individual treatments have no effect on enzyme activity (65).

C_1=Initial vs. others

C_2=Temperature effect averaged over time

C_3=Time effect averaged over temperature

C_4=Interaction of time and temperature

C_5=Temperature effect at 24 hours

C_6=Temperature effect at 48 hours

few hours after the host animal dies and readings should be taken immediately.

Significance and Variability of Phospholipase B in Canine Small Intestine

The examination of data gathered in this study demonstrates that phospholipase B has a range of activity in the proximal one-third of the duodenum of dogs. Since a heterogeneous collection of animals was examined it may be assumed that dogs do have various duodenal phospholipase B levels. However, the heterogenisity itself may mask the true relationship of altered enzyme activity to a specific causal agent, as helminth induced inflammation. Several very important factors may be considered responsible for the great variability of activity demonstrated. Some of these factors would include age of animals, length of infections, number of previous or challenge exposures to the same helminth, sex of animal, nutritional state, general physiological state and stage of estrus cycle of the females. All of these items have been shown by various investigators to alter eosinophil levels which would in turn affect phospholipase B activity levels.

Archer (55) states that eosinophilia of the tissues is classically associated with invasion by nematode parasites but that tissue eosinophilia is subject to rapid changes especially during shock of the animal. Biggart (66), Heidenhain (67), Opie (68), Tausk (69) and Godlawski (70,71)

have all reported that dogs fed a protein rich meal incurr a marked eosinophilia in the bowel wall soon afterwards. Both Heindenhain (67) and Godlawski (72) found that starved animals (protein deficient) have relatively few eosinophils in the intestinal submucosa. Pound dogs are usually maintained at a minimal nutritional level and are often captured in a state of semi or complete shock and/or starvation. Thus, if the eosinophil content of the small intestine is altered due to one or more of these secondary factors, phospholipase B activity levels may also be altered to the extent that assays may not reflect a true condition.

The great variability and randomness of some of the phospholipase B activity levels of the dogs in this study was of great value since it provided some information on almost every breed, age or sex of dog available. The purpose of the investigation was achieved, the identification and quantitation of phospholipase B in the proximal one-third of the duodenum of the dog, in addition to providing material on the relation of helminths, nematodes, hookworms, storage and freezing to phospholipase B activity.

CHAPTER V

SUMMARY

The lysophosphatidyl splitting enzyme, phospholipase B, is probably a universal moiety which is just now being characterized and related to biological mechanisms. With its widespread prevalence in bacteria, insects, plants and animals as an isoenzyme it undoubtedly has a basic function essential for normal biological activity of these organisms. Although its role is not known, the presence of phospholipase B in the duodenum of dogs is unsurprising but not without significance. The levels of enzyme demonstrated in canines in this study were low in comparison to work done with rodent tissues but no comparable values are available for domestic animals at this time.

No direct statistical relationship was found between enzyme tissue levels and species or incidence of helminths in parasitized dogs. However, a general tendency for elevated enzyme activity in dogs with over five Ancylostoma sp. or ten to 20 helminths was noted. Due to the great heterogeneity of test animals exact correlations were not found, but further studies using homogeneous groups of animals will probably establish baseline information.

Statistical tests revealed that phospholipase B

activity cannot be accurately quantitated and compared unless the tissue is analyzed immediately upon the death of the experimental animal. After storage at -3 degrees and -76 degrees centigrade for 24, 48 and 168 hours, tissue samples with high initial enzyme activity were markedly reduced or negligible. Both time and freezing temperatures were found to adversely affect the activity of phospholipase B and it is probable that the half-life is a few hours.

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APPENDIX

REAGENTS

Preparation of 12.5 percent Glycerol Medium

Combine 25 ml of 50 percent glycerol, 14.7 ml of .5 M KH_2PO_4, 5.1 ml of .5 M K_2HPO_4, 10 ml of 1.5 M $HgSO_4$ and 1 ml of .2 M EDTA. Bring mixture to 100 ml by using distilled water. Just before using add 20 ml of cystamine.

Preparation of .2 M EDTA

Place 14.4 g of ethylenediamine tetraacetic acid in a 200 ml beaker containing 100 ml of distilled water. While stirring with a magnetic stirrer, slowly add 3 N KOH to the solution until all the EDTA is dissolved. At this point about 50 to 55 ml of KOH should have been used and the pH should read 5.5 to 6.0. Now add the KOH dropwise until the pH reads 6.5 continuously. Quantitatively transfer the solution to a 200 ml flask and bring to volume with distilled water.

Preparation of Phosphate Reagent

Combine 0.5 g of 1-amino-2-napthol-4-sulfuric acid, 200 ml of 15 percent sodium bisulfite, anhydrous and 1 g of

sodium sulfite, anhydrous.

Preparation of Ammonium Molybdate Solution

Add 1 g of ammonium molybdate to 20 ml of distilled water and boil until the preparation goes into solution.

VITA

Katherine Elizabeth Sartain

Candidate for the Degree of

Master of Science

Thesis: THE INFLUENCE OF HELMINTH INFECTION ON THE PRESENCE AND LEVELS OF PHOSPHOLIPASE B ACTIVITY IN THE DUODENUM OF THE DOG

Major Field: Veterinary Parasitology and Public Health

Biographical:

Personal Data: Born in Tulsa, Oklahoma, August 29, 1953, the daughter of Mr. and Mrs. R. R. Phillips

Education: Graduated from Memorial High School, Tulsa, Oklahoma in May, 1971: received Bachelor of Science degree in Zoology from Oklahoma State University in 1975; completed requirements for the Master of Science degree at Oklahoma State University in May, 1977.

Professional Experience: Parasitology Laboratory Technologist and Teaching Assistant at Oklahoma State University, Department of Parasitology, Microbiology and Public Health, 1975-1977

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