INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600
Partial characterization of alkaline and acid phosphatases in
cysticercoids and excysted scoleces of the tapeworm, *Hymenolepis
diminuta*

Leiby, David Alan, Ph.D.
The Ohio State University, 1989
PARTIAL CHARACTERIZATION OF ALKALINE AND ACID
PHOSPHATASES IN CYSTICERCoids AND EXCYSTED SCOleCes
OF THE TAPEWORM, HYMENOLEPIS DIMINUTA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

David Alan Leiby, B.S., M.S.

* * * * *

The Ohio State University
1989

Dissertation Committee:        Approved by
P.W. Pappas                        P.W. Pappas
D.W. Garton                      Adviser
D.L. Stetson                  Department of Zoology
ACKNOWLEDGMENTS

I wish to express my sincere thanks to my adviser, Dr. Peter W. Pappas, for his guidance, patience and humor, albeit rather dry, throughout my tenure as his student. As a scientist he demonstrates a level of achievement that I aspire to, but will never enjoy. I also wish to thank Dr. Stetson for his time and critical comments for both my general examination and dissertation. Additional thanks to Dr. Stetson for afternoon forays on the links. Special thanks to Drs. Barriga and Crites for serving on my general examination committee. Extreme indebtedness to Dr. Garton for serving on my dissertation committee under such short notice. Lastly, I wish to thank my wife Vanessa who persevered throughout, providing support, encouragement, understanding, and most of all, not asking too many questions.

These studies were supported in part by a Grant-in-Aid of Research from Sigma Xi and an Alumni Research Award from The Ohio State University, both to DAL.
VITA

April 24, 1959 ............ Born - Easton, Pennsylvania
1981 ..................... B.S., Lafayette College, Easton, Pennsylvania
1981-1984 ................ Research Assistant, Laboratory of Parasitology, University of Pennsylvania, Philadelphia, Pennsylvania
1984 ..................... M.S., Rutgers, The State University of New Jersey, Camden, New Jersey
1984-1988 ................ Graduate Teaching Associate, The Ohio State University, Columbus, Ohio
1986 ..................... M.S., The Ohio State University, Columbus, Ohio
1988-1989 ................ College of Biological Sciences, Dean's Graduate Research Associate, The Ohio State University, Columbus, Ohio

PUBLICATIONS


iii


**FIELDS OF STUDY**

Major Field: Biochemical Parasitology

Studies in Biochemical Parasitology
   Dr. Peter W. Pappas, The Ohio State University

Studies in Helminthology
   Dr. William J. Bacha Jr., Rutgers, The State University of New Jersey
   Dr. K. Darwin Murrell, United States Department of Agriculture
   Dr. Gerhard A. Schad, University of Pennsylvania

Studies in Parasitology
   Dr. Bernard Fried, Lafayette College
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................ ii
VITA ................................................... iii
LIST OF TABLES ........................................ viii
LIST OF FIGURES ....................................... x
LIST OF PLATES ....................................... xi
INTRODUCTION ......................................... 1
MATERIALS AND METHODS .............................. 9
RESULTS ................................................ 17
DISCUSSION ............................................ 51
LITERATURE CITED .................................... 75
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Relative intensities of reaction products localizing alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) at the external surface membrane of cysticercoids, excysted scoleces (ES), 3-day-old (3-DW) and 12-day-old (12-DW) tapeworms of Hymenolepis diminuta</td>
<td>39</td>
</tr>
<tr>
<td>2. Specific activities (nmoles substrate hydrolyzed·mg protein⁻¹·min⁻¹) of alkaline phosphatase from the isolated brush border membrane of Hymenolepis diminuta, measured using Tris buffer (T) or Tris-maleate buffer (TM), and several substrates</td>
<td>40</td>
</tr>
<tr>
<td>3. Specific activities (see Table 2) of acid phosphatase from the isolated brush border membrane of Hymenolepis diminuta, measured using acetate buffer (A) or Tris-maleate buffer (TM) and several substrates at pH 4.0 and 5.0</td>
<td>42</td>
</tr>
<tr>
<td>4. Specific activities (nmoles substrate hydrolyzed·mg protein⁻¹·min⁻¹) of alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) in homogenates of cysticercoids (C) and excysted scoleces (ES) of Hymenolepis diminuta measured using three substrates. Activities of AlkPase and AcPase were determined in Tris buffer (pH 8.8) and acetate buffer (pH 4.0), respectively</td>
<td>45</td>
</tr>
</tbody>
</table>
5. Percentage of alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) activities remaining in the supernates obtained following differential centrifugation of homogenates of cysticercoids and excysted scoleces (ES) of *Hymenolepis diminuta* . . . . 47
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Drawing depicting a longitudinal section of a cysticercoid of <em>Hymenolepis diminuta</em> and showing the relationship of the various tissue layers</td>
<td>5</td>
</tr>
<tr>
<td>PLATE</td>
<td>PAGE</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>I.</td>
<td>Cytochemical localization of alkaline phosphatase in cysticercoids of <em>Hymenolepis diminuta</em> using Tris-maleate buffer (pH 8.0). Electron micrographs of tissues incubated with substrate and without substrate (B) ........................................ 20</td>
</tr>
<tr>
<td>II.</td>
<td>Cytochemical localization of acid phosphatase in cysticercoids of <em>Hymenolepis diminuta</em> using acetate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate and without substrate (B) .................................................. 22</td>
</tr>
<tr>
<td>III.</td>
<td>Cytochemical localization of alkaline phosphatase in excysted scoleces of <em>Hymenolepis diminuta</em> using Tris-maleate buffer (pH 8.0). Electron micrographs of tissues posterior to the neck incubated with substrate (A) and without substrate (B). Also shown is a portion of the scolex proper incubated with substrate (C) ........................................ 23</td>
</tr>
<tr>
<td>IV.</td>
<td>Enlarged view of Plate IIIA which demonstrates alkaline phosphatase localized cytochemically in the excysted scolex of <em>Hymenolepis diminuta</em> using Tris-maleate buffer (pH 8.0) ........................................ 24</td>
</tr>
<tr>
<td>V.</td>
<td>Cytochemical localization of acid phosphatase in excysted scoleces of <em>Hymenolepis diminuta</em> using Tris-maleate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B) ........................................ 27</td>
</tr>
</tbody>
</table>
VI. Cytochemical localization of acid phosphatase in 3-day-old tapeworms of Hymenolepis diminuta using acetate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B) ........................................ 29

VII. Cytochemical localization of acid phosphatase in 3-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 5.0). Electron micrographs of tissues with substrate (A) and without substrate (B) .................. 31

VIII. Cytochemical localization of alkaline phosphatase in 12-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 8.0). Electron micrograph of tissues incubated with substrate (A) and without substrate (B) .............. 34

IX. Cytochemical localization of acid phosphatase in 12-day-old tapeworms of Hymenolepis diminuta using acetate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B) .................. 36

X. Cytochemical localization of acid phosphatase in 12-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B) .............. 38

XI. Polypeptide profiles of homogenates of cysticercoids and excysted scoleces (ES) of Hymenolepis diminuta. Aliquots containing 60 µg of protein were added to wells and separated on a 5-20% acrylamide gradient. Proteins with known molecular masses (in daltons) are shown in the left column ..................... 49
INTRODUCTION

The relationship between a parasite and its host is, by definition, very intimate, and it can be described in terms of physical and biochemical interactions. The region where these physicochemical interactions occur is termed the "host-parasite interface" (Read et al., 1963). Tapeworms (phylum Platyhelminthes, class Cestoidea) represent unique models for the study of interactions at the host-parasite interface because they lack a digestive system in all developmental stages. Indeed, tapeworms must absorb all nutrients and metabolites from their host across their external epithelium, often referred to as the tegument. Absorption of these nutrients and metabolites is mediated by biochemical processes, including numerous tegumental enzymes. Therefore, a better understanding of the complex interactions occurring at the host-parasite interface requires an understanding of these tegumental enzymes.

The tapeworm, Hymenolepis diminuta, has been used extensively to study biochemical interactions occurring at the host-parasite interface (for reviews see, Mettrick
and Podesta, 1974; Pappas and Read, 1975; Podesta, 1980; Pappas, 1983). Early studies (Lumsden et al., 1968; Arme and Read, 1970; Dike and Read, 1971a,b; Pappas and Read, 1974) incubated adult tapeworms obtained from the definitive host (a rat) in vitro. These studies demonstrated that the tegument of adult H. diminuta is invested with membrane-bound enzymes, but the incubation of organisms in vitro is of limited use for the characterization of such membrane-bound enzymatic activities. For example, organisms generally are viable only in narrow ranges of pH, thus limiting the measurement of enzymatic activities to these ranges of pH. The biochemical characterization of tegumental enzymes became more practical with the development of techniques for obtaining purified fractions of tegumental membranes (Knowles and Oaks, 1979) and purified subcellular fractions (Siddiqui and Podesta, 1985).

Most studies characterizing tegumental enzymes of H. diminuta have used tapeworms obtained from the definitive host, because large numbers can be recovered easily from experimentally infected rats. There have been, however, few studies examining the biochemical make-up of the metacestode stage (cysticercoid) of H. diminuta (Jeffs and Arme, 1985; Ubelaker, 1980) which develops in
insects. The lack of studies is due largely to the difficulty in culturing, handling and obtaining sufficient quantities of this life-cycle stage. Despite these difficulties, cysticercoids possess several characteristics which make them an attractive model for studies of the host-parasite interface. Unlike adult tapeworms which are found in the small intestine of a vertebrate, cysticercoids grow and develop in the hemocoel of an invertebrate. Furthermore, cysticercoids can be regarded as representing two life-cycle stages; contained within the cysticercoid is a presumptive adult tapeworm (or scolex) which excysts in the small intestine of the definitive host (a rat) (Figure 1). During the development of the cysticercoid and the scolex contained within, biochemical changes likely occur and, thus, represent adaptations for survival in their new environment (i.e., the rat's small intestine). Thus, the cysticercoid and scolex contained within represent unique models for examining the dynamic nature of interactions occurring at the host-parasite interface.

Cysticercoids were thought to be biochemically inactive until Prescott and Voge (1959) demonstrated the synthesis of ribonucleic acid in the peripheral tissue.
Figure 1. Drawing depicting a longitudinal section of a cysticercoid of *Hymenolepis diminuta* and showing the relationship of the various tissue layers (after Voge, 1960). Reprinted with permission from Proceedings of the Helminthological Society of Washington 27: 32-36; copyright 1960, by Allen Press, Lawrence, Kansas.
Figure 1.

- anterior canal
- cysticercoid cavity
- encysted scolex
- cavity lining
- inner cyst
- intermediate cell layer
- fibrous processes
- peripheral cells
- hair-like processes
- acellular deposits
- tail parenchyma
- surface membrane
layers of cysticercoids of *H. diminuta*. Later studies described many enzymes and transport functions in cysticercoids (for review see, Ubelaker, 1980). Among the enzymes that have received the most attention are alkaline and acid phosphatases (AlkPase and AcPase, respectively). These enzymes were first localized histochemically in sectioned cysticercoids of *H. diminuta* by Bogitsh (1967); AlkPase was localized in the tegument of the scolex, and AcPase was localized in the intermediate cell layer and parenchymal cells of the tail. Bogitsh (1967) concluded that AcPase was involved in the excystment of the scolex contained within through tissue degradation, while AlkPase activity localized at the tegument was indicative of membrane transport. In a later study, Bogitsh (1969) localized AcPase cytochemically in the cytoplasm and membrane-bound vesicles of the intermediate cell layer, thereby supporting the earlier histochemical study.

AlkPase and AcPase of *H. diminuta* are non-specific in nature and have been described in greatest detail in preparations of brush border membrane (BBM) from adult *H. diminuta* (Knowles and Oaks, 1979; Pappas, 1988). It is hypothesized that, in adult tapeworms, these enzymes remove phosphate molecules from phosphoesters (e.g.,
glucose 6-phosphate), thereby resulting in substrates that are readily transported across the tapeworm's tegument (Phifer, 1960; Dike and Read, 1971b). However, the functional role and importance of AlkPase and AcPase in cysticercoids remains equivocal, and the present study was undertaken to partially characterize AlkPase and AcPase found in cysticercoids and scoleces of *H. diminuta*. Unlike previous studies, cysticercoids and the scoleces contained within were treated as separate entities in this study (i.e., some cysticercoids were treated chemically, thereby producing excysted scoleces [ES]). The AlkPase and AcPase were characterized in the following manner: 1) enzymatic activities were measured in vitro using intact organisms; 2) enzymatic activities were localized cytochemically using cerium as the capturing agent; 3) enzymatic activities in homogenates of organisms were determined using several substrates; 4) enzymatic activities were determined in fractionated homogenates of organisms; 5) the differences in polypeptide profiles of ES and cysticercoids were determined by polyacrylamide gel electrophoresis; and 6) for comparative purposes, AlkPase and AcPase were localized in 12-day-old tapeworms (12-DW), and AcPase was localized in 3-day-old tapeworms (3-DW). The present
study was designed only to characterize partially these enzymatic activities and, thus, no attempt was made to determine the number of enzymes represented by each activity.
MATERIALS AND METHODS

Maintenance of *Hymenolepis diminuta*

The life-cycle of *Hymenolepis diminuta* ("OSU" strain; Pappas and Leiby, 1986) was maintained in male Sprague-Dawley rats and flour beetles (*Tenebrio molitor*). Eggs of *H. diminuta* were collected from the feces of infected rats by salt flotation, and starved beetles were infected with eggs on two consecutive days. On day 14 post-infection (PI) cysticercoids were flushed from the beetles' hemocoels using Krebs-Ringer saline buffered with 25 mM Tris-maleate (KRT of Read et al., 1963). Rats (125-150 g) were infected by intubation with 30 cysticercoids each, and tapeworms were recovered on day 3 or 12 PI by flushing them from the rats' small intestines using cold (4°C) KRT.

ES were obtained using the methods of Rothman (1959). Briefly, cysticercoids were placed in a 1% pepsin/HCl solution for 15 min (37°C) and then transferred to a 0.5% trypsin/taurocholic acid solution for 20 min (37°C) where the excystment process was completed. ES, cysticercoids, 3-DW and 12-DW were washed
in three changes of cold KRT prior to use in subsequent studies.

**In vitro incubations of ES and cysticercoids**

Reactions measuring AlkPase or AcPase were conducted in tubes containing 100 cysticercoids or 50 ES. Cysticercoids or ES were incubated at 37°C in 1 ml reaction volumes containing p-nitrophenyl phosphate (PNPP) at a final concentration of 1 mM. Buffers used to measure AlkPase and AcPase were 200 mM Tris (pH 8.8) containing 5 mM Mg²⁺ and 200 mM acetate (pH 4.0), respectively. Controls consisted of identical assays containing cysticercoids or ES which had been heated for 5 min at 95°C prior to incubation in substrate (PNPP). Reactions measuring AlkPase and AcPase in cysticercoids were run for 3 and 1 hr, respectively. Reactions measuring AlkPase and AcPase in ES were run for 3 and 4.5 hr, respectively. Assays measuring AcPase were terminated by the addition of 1 ml of 1 N NaOH followed by heating at 95°C for 5 min. Assays measuring AlkPase were terminated by heating alone. The amount of product (p-nitrophenol = PNP) formed was determined by measuring the absorbance of incubation media at 405 nm and calculated based on a millimolar extinction coefficient.
of 17.5. Enzymatic activities were expressed in terms of nmoles of PNP liberated min\(^{-1}\) cysticercoid\(^{-1}\) or ES\(^{-1}\).

**Cytochemistry**

ES, cysticercoids and 3-DW were used intact in cytochemical studies, while 12-DW were placed on Plexiglas, bathed in cold KRT and cut into 1 mm pieces using a razor blade. Individual pieces of 12-DW were washed 3 times in cold KRT prior to use.

The cytochemical techniques described by Robinson and Karnovsky (1983a,b) were used with minor modifications. Tissues (entire organisms or pieces) were fixed in 2% glutaraldehyde-100 mM cacodylate buffer (pH 7.2) for 40 min at 0°C and then washed in three changes of 100 mM cacodylate buffer (this and all subsequent washes were done at 0°C). Tissues used for localization of AlkPase were washed twice in 100 mM Tris-maleate buffer (pH 8.0), while those used for localization of AcPase were washed twice in 100 mM acetate buffer (pH 5.0) or 100 mM Tris-maleate buffer (pH 5.0).

The incubation medium for AlkPase activity consisted of 100 mM Tris-maleate buffer (pH 8.0), 1 mM β-glycerophosphate (βGP) and 2 mM CeCl\(_3\). The incubation medium for AcPase activity consisted of 100 mM acetate or
Tris-maleate buffer (pH 5.0), 1 mM BGP and 2 mM CeCl₃. All media were filtered prior to use (Robinson and Karnovsky, 1983a). Tissues were incubated in the appropriate medium for 1 hr at 37°C. Following incubation, tissues were washed in three changes of the buffer used for the cytochemical reaction. Thereafter, tissues were washed in two changes of 100 mM cacodylate buffer and refixed in 3% glutaraldehyde-100 mM cacodylate buffer for 1 hr at 0°C. Refixed tissues were washed in three changes of 100 mM cacodylate buffer and post-fixed in 1% OsO₄-100 mM cacodylate buffer (pH 7.2) for 1 hr at room temperature. Control incubations consisted of ES, cysticercoids, 3-DW or pieces of 12-DW, incubated in substrate-free (BGP-free) media.

Tissues were washed in three changes of 100 mM cacodylate buffer, dehydrated in acetone and embedded in Epon-Araldite (Anderson and Andre, 1968). Thin sections were cut with a diamond knife, floated onto copper grids and examined unstained in a Philips EM301 at 60 kV.

**In vitro assessment of cytochemical reactions**

A BBM fraction was prepared from 12-DW of *H. diminuta* (Knowles and Oaks, 1979; Pappas, 1988), and the concentration of protein was determined (Markwell et al.,
1978) using bovine serum albumin as a standard. AlkPase and AcPase activities were determined using combinations of several buffers (Tris and Tris-maleate, pH 8.2 and 8.8; acetate and Tris-maleate, pH 4.0 and 5.0) and substrates (βGP, PNPP or 5'-adenosine phosphate [AMP]). Enzymatic activities were quantified in vitro using both colorimetric and turbidimetric assays. Colorimetric assays of AlkPase and AcPase activities were done according to previously published techniques (Pappas, 1982; Pappas, 1988). An aliquot of BBM was added to the appropriate buffer and this mixture (final volume of 0.5 ml) was preincubated at 37°C for 5 min. The reaction was initiated by adding substrate (<20 μl) to a final concentration of 1 mM. Following incubation at 37°C for an appropriate period of time, each reaction was terminated by the addition of 0.8 ml of a Malachite Green-ammonium molybdate solution (Lanzetta et al., 1979). The amount of inorganic phosphate liberated was determined by measuring the absorbance (660 nm) of the resulting solution. Control assays, to correct for spontaneous substrate hydrolysis, were included in each series of assays and contained buffer and substrate only.
To assess the efficacy of cerium as a capturing agent, turbidimetric assays were run using a modification of the techniques of Robinson and Karnovsky (1983a,b). The initial reaction mixture contained an aliquot of BBM and 2 mM CeCl$_3$ in 960 $\mu$l of the appropriate buffer. Following preincubation for 5 min at 37°C, the reaction was initiated by the addition of substrate (40 $\mu$l) to a final concentration of 1 mM. The $\Delta A_{450}$ of each reaction was monitored continuously for 2 min. Appropriate controls lacking BBM were included for each combination of buffer and substrate used.

**Biochemical analyses of ES and cysticercoid homogenates**

Cysticercoids and ES were collected and frozen in a minimal amount of 100 mM Tris buffer (pH 8.0) until sufficient quantities had been collected for enzymatic assays. Cysticercoids or ES were thawed and homogenized in a Dounce tissue grinder (0°C) containing 100 mM Tris buffer (pH 8.0) and 0.1 mM phenylmethyl sulfonyl fluoride. The resulting homogenates were dialyzed for 24 hr at 4°C against 1 mM Tris buffer (pH 7.5) containing 9 mM NaCl. The concentration of protein in these preparations was determined as described above for BBM.
Colorimetric assays of AlkPase and AcPase activities were done in 200 mM Tris buffer (pH 8.8) containing 5 mM Mg$^{2+}$ and 200 mM acetate buffer (pH 4.0), respectively, using AGP, PNPP or AMP. An aliquot of homogenate (<40 μl) was added to the appropriate buffer, and the mixture (final volume of 0.5 ml) was preincubated at 37°C for 5 min. Substrate was added to a final concentration of 1 mM to initiate the reactions which were run for 1-3 hr depending on the enzyme-substrate combination. Termination of the reaction and measurement of inorganic phosphate liberated was done as described for BBM. Control assays, to correct for spontaneous substrate hydrolysis, were run using aliquots of homogenate heated for 5 min at 95°C.

Homogenates of ES and cysticercoids were fractionated by differential centrifugation to determine the activities of AcPase and AlkPase in subcellular fractions. Following sequential centrifugations at 5,000 g, 15,000 g, 30,000 g and 100,000 g (30 min at 4°C), the resulting supernates were assayed for enzymatic activity using PNPP at a final concentration of 1 mM. AcPase activity was measured as described above for the homogenates. The specific activity of AlkPase, however, was determined by measuring the $\Delta A_{405}$ continuously for 15
min at 37°C in 1 ml reaction volumes. For fractionated homogenates of cysticercoids and ES, the specific enzymatic activity in the initial homogenate was also determined and used as a basis to calculate the percentage of enzymatic activity remaining in each supernate.

Polypeptide profiles of homogenized ES and cysticercoids were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the buffer system of Laemmli (1970). Aliquots of the homogenates were lyophilized, redissolved in 1% SDS, and the protein concentration of the reconstituted sample determined as described for BBM. Samples were reduced using 2-mercaptoethanol, loaded onto gels (60 µg protein per well) and separated in a 5-20% acrylamide gradient. Polypeptides were visualized using Coomassie blue.
RESULTS

In vitro incubations of ES and cysticercoids

Estimates of AlkPase and AcPase activities were made based on hydrolysis of PNPP by intact cysticercoids and ES incubated in vitro. Attempts to solubilize cysticercoids in detergents (e.g. SDS) and NaOH were unsuccessful; therefore, activities were expressed in terms of nmoles PNP liberated·min⁻¹·cysticercoid⁻¹ or ES⁻¹. Cysticercoids demonstrated considerably more AcPase activity (6.77 ± 0.42, n=5) than AlkPase activity (0.94 ± 0.07, n=5). For ES, however, the activity of AlkPase (3.83 ± 0.26, n=5) was more than twice that of AcPase (1.80 ± 0.04, n=5). Both cysticercoids and ES were yellow in color following incubation, indicating that they had absorbed some PNP, but no attempt was made to quantify the amount of PNP absorbed by the life-cycle stages. Thus, the values given above must be considered "conservative estimates" of the amount of substrate hydrolyzed.
Cytochemistry

Cytochemical reactions using cysticercoids were difficult to evaluate. The reaction products representing the activities of AlkPase (Plate I) and AcPase (Plate II) were only slightly more intense than those observed in substrate-free controls (Plates I and II). The observed reaction products were limited to the microvillous surface of the cysticercoid.

In ES, AlkPase activity was localized in the tegument, primarily at the microvillous surface posterior to the neck (Plate III). Reaction product was virtually absent at the surface of the scolex proper and in substrate-free controls. The reaction product localized at the microvillous surface was intense, but decreased in intensity posteriad, concomitantly with decreasing thickness of the distal cytoplasm. The distal cytoplasm of the ES tegument contained many vesicles, some of which demonstrated AlkPase activity (Plate IV).

AcPase was initially localized in ES using acetate buffer (pH 5.0), however the microvillous surface was observed to detach from the distal cytoplasm when this buffer was used. (Cysticercoids incubated in acetate buffer demonstrated a similar lack of cellular integrity.) Therefore, Tris-maleate buffer (pH 5.0) was
Plate I. Cytochemical localization of alkaline phosphatase in cysticercoids of *Hymenolepis diminuta* using Tris-maleate buffer (pH 8.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). Tissues examined unstained. MV, microvilli; DC, distal cytoplasm; BL, basal lamina.
Plate I.
Plate II. Cytochemical localization of acid phosphatase in cysticercoids of *Hymenolepis diminuta* using acetate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). Only minimal reaction product is present, and these tissues demonstrate clearly the degradative effects of the acidic buffer. The cellular integrity is poorly maintained, and cells generally lack cytoplasm and organelles (compare to Plate I). Tissues examined unstained.
Plate II.
Plate III. Cytochemical localization of alkaline phosphatase in excysted scoleces of *Hymenolepis diminuta* using Tris-maleate buffer (pH 8.0). Electron micrographs of tissues posterior to the neck incubated with substrate (A) and without substrate (B). Also shown is a portion of the scolex proper incubated with substrate (C). Tissues examined unstained. V, vesicle; LD, lipid droplet; M, mitochondria; CM, circular muscle; LM, longitudinal muscle; TC, tegumental cyton; N, nucleus.
Plate IV. Enlarged view of Plate IIIA which demonstrates alkaline phosphatase localized cytochemically in the excysted scolex of Hymenolepis diminuta using Tris-maleate buffer (pH 8.0). Enzymatic activity is localized in tegumental vesicles, as well as, at the surface membrane. Tissue examined unstained.
used to localize AcPase in ES because this buffer maintained cellular integrity. Cytochemical reactions localizing AcPase in ES were negative, as were substrate-free controls (Plate V).

AcPase activity, however, was localized cytochemically in 3-DW. Unlike ES, 3-DW were unaffected by acetate buffer; therefore, both acetate and Tris-maleate buffers were used to localize AcPase activity in 3-DW. AcPase activity localized in 3-DW using Tris-maleate buffer (Plate VI) was less intense than that observed using acetate buffer (Plate VII). However, AcPase localized in 3-DW using Tris-maleate buffer was much more intense than AcPase observed in ES using the same buffer and length of incubation (40 min). Regardless of the buffer, the patterns of localization in 3-DW were similar; activity was virtually absent from the scolex proper, and occurred primarily in association with the microvilli posterior to the neck. As was observed in ES, activity in 3-DW decreased posteriad, while substrate-free controls demonstrated minimal reaction product.

AlkPase and AcPase activities also were demonstrable cytochemically in the tegument of 12-DW. In the case of AlkPase activity, the reaction product was intense and
Plate V. Cytochemical localization of acid phosphatase in excysted scoleces of *Hymenolepis diminuta* using Tris-maleate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). The tissues are negative for enzymatic activity. Tissues examined unstained.
Plate VI. Cytochemical localization of acid phosphatase in 3-day-old tapeworms of Hymenolepis diminuta using acetate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). Tissues examined unstained.
Plate VI.
Plate VII. Cytochemical localization of acid phosphatase in 3-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 5.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). Tissues examined unstained. DG, discoidal granules; IP, internuncial process; MW, membranous whorls.
limited exclusively to the brush border (Plate VIII). Similar results were obtained when AcPase activity was localized using acetate buffer at pH 5.0 (Plate IX). However, when Tris-maleate buffer (pH 5.0) was used, the reaction product was less intense and of finer texture, thereby providing better resolution of enzymatic activity (Plate X). Regardless of the buffer used, substrate free controls demonstrated minimal reaction product (Plates VIII-X). For comparative purposes, the relative intensities of reaction products localizing AlkPase and AcPase activities in cysticercoids, ES, 3-DW and 12-DW of *H. diminuta* are summarized in Table 1.

**In vitro assessment of cytochemical reactions**

The AlkPase activity of the BBM was affected significantly by the composition of the incubation medium. As summarized in Table 2, these data demonstrated the following: (1) AlkPase activity was greatest in Tris buffer and in the presence of Mg$^{2+}$; (2) βGP was hydrolyzed faster at pH 8.2 than at 8.8, while the opposite was true for PNPP and AMP; and (3) PNPP was hydrolyzed faster than βGP under all experimental conditions, and faster than AMP under most experimental conditions.
Plate VIII. Cytochemical localization of alkaline phosphatase in 12-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 8.0). Electron micrographs of tissues incubated with substrate (A) and without substrate (B). Tissues examined unstained. G, glycogen.
Plate VIII.
Plate IX. Cytochemical localization of acid phosphatase in 12-day-old tapeworms of *Hymenolepis diminuta* using acetate buffer (pH 5.0). Electron micrographs of tissue incubated with substrate (A) and without substrate (B). Tissues examined unstained.
Plate IX.
Plate X. Cytochemical localization of acid phosphatase in 12-day-old tapeworms of Hymenolepis diminuta using Tris-maleate buffer (pH 5.0). Electron micrographs of tissue incubated with substrate (A) and without substrate (B). The reaction product demonstrated in these tissues is finer grained compared to the reaction product in Plate IX. Tissues examined unstained.
Plate X.
Table 1. Relative intensities of reaction products localizing alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) at the external surface membrane of cysticercoids, excysted scoleces (ES), 3-day-old (3-DW) and 12-day-old (12-DW) tapeworms of Hymenolepis diminuta. The relative intensities of reaction products varied from most intense (++++) to least intense/questionable (+/-).

<table>
<thead>
<tr>
<th></th>
<th>AlkPase</th>
<th>AcPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris-maleate</td>
<td>Tris-maleate</td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>buffer</td>
</tr>
<tr>
<td>Cysticercoids</td>
<td>+/-</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>ES</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>3-DW</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>12-DW</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

\(^a\)ND = not done
Table 2. Specific activities (nmoles substrate hydrolyzed·mg protein\(^{-1}\)·min\(^{-1}\)) of alkaline phosphatase from the isolated brush border membrane of *Hymenolepis diminuta*, measured using Tris buffer (T) or Tris-maleate buffer (TM), and several substrates. Specific activities were measured at pH 8.2 and 8.8, with (Mg\(^{2+}\)) or without (-Mg\(^{2+}\)) the addition of 5 mM Mg\(^{2+}\). Values represent the means of triplicate assays using a single membrane preparation prepared from a pooled sample of tapeworms.

<table>
<thead>
<tr>
<th>Substrate/ (buffer)</th>
<th>pH 8.2</th>
<th>pH 8.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg(^{2+})</td>
<td>-Mg(^{2+})</td>
</tr>
<tr>
<td>BGP/(T)</td>
<td>4140</td>
<td>1915</td>
</tr>
<tr>
<td>BGP/(TM)</td>
<td>1457</td>
<td>213</td>
</tr>
<tr>
<td>PNPP/(T)</td>
<td>20479</td>
<td>10896</td>
</tr>
<tr>
<td>PNPP/(TM)</td>
<td>7015</td>
<td>1742</td>
</tr>
<tr>
<td>AMP/(T)</td>
<td>13371</td>
<td>5984</td>
</tr>
<tr>
<td>AMP/(TM)</td>
<td>6378</td>
<td>1792</td>
</tr>
</tbody>
</table>
The AcPase activity of the BBM was dramatically lower than the AlkPase activity, regardless of the assay conditions, and was also affected by the pH of the incubation medium, the substrate used, and composition of the buffer. As summarized in Table 3, these data demonstrated the following: (1) AcPase activity was lower in Tris-maleate buffer than in acetate buffer; (2) hydrolysis of PNPP was greater at pH 4.0 than at pH 5.0, while just the opposite was true for BGP and AMP; and (3) the rate of substrate hydrolysis was highest for PNPP, and lowest for AMP, under all experimental conditions.

Turbidimetric assays of the AlkPase activity were attempted using each of the buffer and substrate combinations listed in Table 2, but many of these combinations were found to be incompatible with such assays. For example, any combination of AMP and Ce³⁺ resulted in rapid precipitation of the reaction medium. Similarly, any combination containing Tris buffer and Ce³⁺ precipitated. PNPP was not tested as a substrate in turbidimetric assays because the hydrolysis product (PNP) absorbs light at 450 nm. Thus, turbidimetric assays were run using BGP in combination with Tris-maleate buffer. The results, expressed as the Δ\(A_{450}\)·mg protein\(^{-1}\)·min\(^{-1}\), represent the means of five assays using a single
Table 3. Specific activities (see Table 2) of acid phosphatase from the isolated brush border membrane of Hymenolepis diminuta, measured using acetate buffer (A) or Tris-maleate buffer (TM) and several substrates at pH 4.0 and 5.0. Values represent the means of triplicate assays using a single membrane preparation prepared from a pooled sample of tapeworms.

<table>
<thead>
<tr>
<th>Substrate/ (buffer)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>βGP/(A)</td>
<td>11.4</td>
</tr>
<tr>
<td>βGP/(TM)</td>
<td>5.9</td>
</tr>
<tr>
<td>PNPP/(A)</td>
<td>98.6</td>
</tr>
<tr>
<td>PNPP/(TM)</td>
<td>76.1</td>
</tr>
<tr>
<td>AMP/(A)</td>
<td>8.5</td>
</tr>
<tr>
<td>AMP/(TM)</td>
<td>3.8</td>
</tr>
</tbody>
</table>
membrane preparation prepared from a pooled tapeworm sample. At pH 8.2, AlkPase activity was higher with Mg$^{2+}$ (2.274) than without Mg$^{2+}$ (0.272). Lower activities were observed at pH 8.8, where AlkPase activity with Mg$^{2+}$ (0.897) again was higher than without Mg$^{2+}$ (0.168). Indeed, these results were quantitatively similar to those for the colorimetric assays measuring AlkPase in Tris-maleate buffer (Table 2).

When compared with colorimetric assays, the turbidimetric assays were found to be relatively insensitive in detecting AlkPase activity. For example, when AlkPase assays were run in Tris-maleate buffer using βGP, only 0.1 μg of protein (BBM) was required for colorimetric assays (Table 2). To detect AlkPase activity in turbidimetric assays under the same conditions, it was necessary to add 9.9 μg of protein to obtain a measurable $\Delta A_{450}$.

Colorimetric assays containing βGP and acetate buffer easily detected AcPase activity when as little as 9.9 μg of protein was assayed for 60 min (Table 3). However, no increase in turbidity was detectable when turbidimetric assays containing an identical reaction mixture were monitored continuously at $A_{450}$ for 5 hr.
Biochemical analyses of ES and cysticercoid homogenates

Unfractionated homogenates of ES had considerably higher specific activities of AlkPase compared to AcPase, regardless of the substrate (Table 4). However, homogenates of cysticercoids had higher specific activities of AcPase, except when AMP was used as substrate, when AlkPase activity was slightly higher. In all instances, the greatest enzymatic activities were observed when PNPP was used as substrate. Enzymatic activities in reactions with βGP were generally lowest, except for assays measuring AcPase activity in cysticercoids, when AMP resulted in a slightly lower specific activity. When comparing enzymatic activities between homogenates, AlkPase was always greater in ES than cysticercoids, while AcPase activity was always greater for ES than cysticercoids, regardless of the substrate.

As an added note, ES were observed to be homogenized completely by the Dounce tissue grinder. When cysticercoids were homogenized, however, the capsule surrounding the scolex was not affected. Other cellular elements, including the scolex, were completely homogenized as verified by microscopic examination.
Table 4. Specific activities (nmoles substrate hydrolyzed·mg protein⁻¹·min⁻¹) of alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) in homogenates of cysticercoids (C) and excysted scoleces (ES) of Hymenolepis diminuta measured using three substrates. Activities of AlkPase and AcPase were determined in Tris buffer (pH 8.8) and acetate buffer (pH 4.0), respectively. Values represent the means of triplicate assays using a single homogenate prepared from samples of pooled cysticercoids or excysted scoleces.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate/(homogenate)</th>
<th>AlkPase</th>
<th>AcPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BGP/(C)</td>
<td>2.34</td>
<td>6.29</td>
</tr>
<tr>
<td></td>
<td>BGP/(ES)</td>
<td>4.77</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>PNPP/(C)</td>
<td>11.08</td>
<td>66.87</td>
</tr>
<tr>
<td></td>
<td>PNPP/(ES)</td>
<td>47.55</td>
<td>10.85</td>
</tr>
<tr>
<td></td>
<td>AMP/(C)</td>
<td>7.25</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>AMP/(ES)</td>
<td>35.13</td>
<td>1.73</td>
</tr>
</tbody>
</table>
Analyses of fractionated homogenates of cysticercoids revealed that a large percentage of AlkPase and AcPase activities were present in solubilized form (Table 5). The activities of AlkPase and AcPase in the 5,000 g supernate of homogenized cysticercoids were 65% and 64%, respectively, of the enzymatic activities in the unfractionated homogenate, and the percentage of activity remaining in the 100,000 g supernate was not appreciably lower. The results of similar experiments using ES were quite different. AlkPase activity was greatly reduced following centrifugation at 5,000 g, with only 21% of the original activity remaining in the supernate (Table 5). Further centrifugation removed most AlkPase activity and only 6% of the original activity remained following centrifugation at 100,000 g. AcPase activity in ES was also reduced by centrifugation, but not to the same degree as for AlkPase. Following centrifugation at 5,000 g, 48% of the AcPase activity remained. Further centrifugation resulted in only 26% of AcPase activity remaining after fractionation at 100,000 g.

The polypeptide profiles of homogenized cysticercoids and ES, as determined by SDS-PAGE, were different, particularly for polypeptides of high molecular mass (Plate XI). Numerous high molecular mass
Table 5. Percentage of alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) activities remaining in the supernates obtained following differential centrifugation of homogenates of cysticercoids and excysted scoleces (ES) of Hymenolepis diminuta. Values represent the means of triplicate assays using homogenates prepared from pooled samples of cysticercoids or excysted scoleces.

<table>
<thead>
<tr>
<th>Supernate</th>
<th>Cysticercoids</th>
<th>ES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AlkPase AcPase</td>
<td>AlkPase AcPase</td>
</tr>
<tr>
<td>5,000 g</td>
<td>65 64</td>
<td>21 48</td>
</tr>
<tr>
<td>15,000 g</td>
<td>76 50</td>
<td>11 32</td>
</tr>
<tr>
<td>30,000 g</td>
<td>69 50</td>
<td>10 32</td>
</tr>
<tr>
<td>100,000 g</td>
<td>63 61</td>
<td>6 26</td>
</tr>
</tbody>
</table>
Plate XI. Polypeptide profiles of homogenates of cysticercoids and excysted scoleces (ES) of Hymenolepis diminuta. Aliquots containing 60 μg of protein were added to wells and separated on a 5-20% acrylamide gradient. Proteins with known molecular masses (in daltons) are shown in the left column.
Plate XI.
polypeptides (> 116 Kd) were present in the homogenate of
cysticercoids, but few were detected in the homogenate of
ES. When compared to the polypeptide profile of the
homogenized cysticercoids, the profile for the homogenate
of ES demonstrated few polypeptides. In general, few low
molecular mass polypeptides were detected in either
homogenate.
DISCUSSION

Parasitic organisms demonstrate very complex life-cycles which often include several larval or developmental stages, each of which is found in a different host or "environment." Particularly interesting are those life-cycle stages which are introduced into different environments or hosts after release from a protective "cyst" or the previous life-cycle stage. Thus, each life-cycle stage must possess morphological and physicochemical adaptations which enable it to survive and perpetuate the parasite's life-cycle in the new environment or host. An understanding of these adaptations is important for developing a better appreciation of the complex interactions occurring at the host-parasite interface.

Many parasitic organisms demonstrate patterns of transmission similar to the one described above, but to my knowledge, only two studies have examined this phenomenon from the standpoint of the host-parasite interface. Uglem et al. (1973) compared leucine transport and aminopeptidase activity in encysted and
excysted cystacanths of Moniliformis dubius (phylum Acanthocephala). More recently, Rosen and Uglem (1988) compared glucose transport in cysticercoids and ES of H. diminuta. Thus, the present study, which describes the activities of AlkPase and AcPase in ES and cysticercoids of H. diminuta, represents the third such study.

The present study demonstrates clearly that AlkPase and AcPase are present in cysticercoids and ES of H. diminuta; when these life-cycle stages are incubated in PNPP, PNP is liberated. Based on in vitro incubations, cysticercoids demonstrate considerably greater AcPase activity than AlkPase, while AlkPase predominates in ES. In vitro incubations are of limited use in demonstrating enzymatic activities, since they fail to establish the cellular location of these activities. For example, the results discussed above do not indicate whether PNPP is hydrolyzed at the surface of the cysticercoids or the ES, or whether the substrate is absorbed and then hydrolyzed. Therefore, alternative methods were chosen for the localization of AlkPase and AcPase. Initially, enzymatic activities were localized cytochemically in cysticercoids and ES. Additional information regarding the specific activities and cellular locations of AlkPase and AcPase
was obtained from assays of unfractionated and fractionated homogenates of cysticercoids and ES.

Previous studies localizing AlkPase and AcPase in cysticercoids relied upon histochemical (Bogitsh, 1967; Moczon, 1973) and cytochemical (Bogitsh, 1969) techniques which used lead as the capturing agent. Cytochemical methods generally are preferable to histochemical methods because they provide more accurate localization and better resolution of enzymatic activity. Most cytochemical methods, however, use lead as the capturing agent to localize enzymatic activity. The use of lead may pose several problems (Barka and Anderson, 1962; Essner, 1973; Robinson and Karnovsky, 1983a), including non-specific adsorption of lead to cellular structures, diffusion of the reaction product from the actual site of the reaction, and a coarse reaction product that often obscures the actual site of enzymatic activity. In fact, attempts to use lead as the capturing agent in the present study resulted in many of these undesirable side effects. Recently, Robinson and Karnovsky (1983a,b) described a cytochemical technique that uses cerium in place of lead, and which reportedly reduces or eliminates many of the problems of lead-based cytochemical reactions. Therefore, cerium was used as
the capturing agent for the cytochemical localization of AlkPase and AcPase in ES and cysticercoids of *H. diminuta*.

Initially, the validity of cerium-based cytochemical reactions was examined using pieces of 12-DW. Previous studies have demonstrated that the BBM fraction of 12-DW contains AlkPase and AcPase (Pappas, 1982, 1988), and these enzymes are demonstrable cytochemically in the brush border membrane of 12-DW using cerium. The observed reaction product is fine grained, demonstrates minimal non-specific adsorption to cellular structures, and it apparently does not diffuse from the site of reaction. Thus, cerium appears to be a suitable alternative to lead for the localization of AcPase and AlkPase in 12-DW, and presumably, cysticercoids and ES of *H. diminuta*.

To better understand those factors which might affect cerium-based cytochemical reactions, *in vitro* assays were conducted using a BBM preparation obtained from 12-DW. The BBM preparation was used instead of homogenized ES or cysticercoids, because sufficient quantities of BBM are obtained easily from 12-DW. Indeed, colorimetric assays demonstrate that the composition of the reaction media affects the activities
of AcPase and AlkPase in the BBM; in particular, the inclusion of Mg$^{2+}$ increases greatly AlkPase activity. AlkPase in adult *H. diminuta* is reported to be a metalloenzyme in which cations are functionally important cofactors; AlkPase is inhibited in living adult tapeworms when they are incubated in chelating agents (Lumsden and Berger, 1974), and Mg$^{2+}$ is required to demonstrate maximal AlkPase activity in the BBM (Pappas, 1980).

However, Mg$^{2+}$ does not appear to be an essential component of media used to localize AlkPase cytochemically in adult tapeworms. Rothman (1966) reported that the deletion of Mg$^{2+}$ from incubation media had no effect on the localization of AlkPase in *H. diminuta*, and AlkPase is demonstrable in ES and 12-DW without using Mg$^{2+}$. These data suggest that sufficient quantities of Mg$^{2+}$ are present in the tapeworm's tegument to maintain enzymatic activity. In fact, previous studies suggest that Mg$^{2+}$ is bound very tightly to the tapeworm's external membrane (and membrane-bound enzymes), and that complete removal of this cation results in irreversible denaturation of the AlkPase activity (Gamble and Pappas, 1981; Pappas, 1982). Thus, it is not surprising that, in living tapeworms, AlkPase is active even in the absence of exogenous Mg$^{2+}$. 
As noted above, cytochemical reactions do not necessarily have to be run under the same conditions that give optimal enzymatic activities during in vitro assays. In fact, cytochemical reactions are intended only for the localization of enzymatic activity, while in vitro assays are designed to measure enzymatic activity. Thus, optimal conditions observed in vitro may not be desirable or comparable with the results obtained in cytochemical reactions. For example, cytochemical and in vitro studies demonstrate that the activity of AcPase in adult *H. diminuta* is greater in acetate than Tris-maleate buffer. However, in Tris-maleate buffer the reaction product is finer grained and provides better resolution of enzymatic activity. In addition, when using the BBM fraction, the activity of AlkPase measured in Tris-maleate buffer is considerably greater than the activity of AcPase measured in acetate buffer. However, when these same buffers are used for cytochemical localization of the two enzymatic activities, the resulting reaction products appear nearly equal in intensity. Furthermore, in vitro assays do not indicate how tissues will be affected by buffers, as demonstrated by the disruptive effects of acidic buffers on cysticercoids and ES. Indeed, conditions which may appear optimal for in vitro
assays may prove to be otherwise in cytochemical reactions, and vice versa.

The use of cerium as a capturing agent in cytochemical reactions was assessed using turbidimetric assays similar to those described by Robinson and Karnovsky (1983a,b). In our hands, however, turbidimetric assays were not particularly useful, perhaps due to the source of our enzymes. Robinson and Karnovsky (1983a,b) used purified enzymes purchased from commercial sources, while the enzymes in the BBM are unpurified. In the present study, turbidimetric assays were unable to measure AcPase activity, even when reactions were monitored for 5 hr, while colorimetric and cytochemical reactions readily detected activity. This suggests that turbidimetric assays are not sufficiently sensitive to detect very low enzymatic activities. However, when used in conjunction with colorimetric assays, turbidimetric assays are potentially useful for selecting components of media for cytological studies. In turbidimetric assays, for example, neither AMP nor PNPP is a suitable substrate, since any combination of AMP and cerium precipitates, and since PNP absorbs at 450 nm. Thus, despite demonstrating lower specific activities than PNPP in colorimetric assays, BGP was
selected as substrate. The use of 8GP as substrate may result in a less intense reaction product, but it assures better resolution of enzymatic activity; it is more stable and, thus, less susceptible to non-enzymatic hydrolysis than PNPP (Robinson and Karnovsky, 1983a).

Similarly, turbidimetric assays demonstrate that any combination of cerium and Tris buffer without maleic acid precipitates. Previously, Barka and Anderson (1962) suggested that the inclusion of maleic acid in Tris buffer helps to stabilize lead cations. Apparently, cerium cations are also stabilized by the inclusion of maleic acid, because cerium in combination with Tris-maleate buffer does not precipitate. As discussed previously, cytochemical reactions run in Tris-maleate are also preferable because they provide better resolution and localization of enzymatic activity.

Cytochemical studies indicate that phosphatase activities are present only at the surface membrane, and occasionally in underlying vesicles, of those life-cycle stages examined. However, when adults of H. diminuta are homogenized and fractionated by differential centrifugation, AlkPase and AcPase activities are demonstrable in all of the subcellular fractions (Siddiqui and Podesta, 1985). Similarly, differential
centrifugation of homogenized cysticercoids and ES indicates that AlkPase and AcPase are not limited to the surface membrane. Thus, one would expect that AlkPase and AcPase would be localized in areas of cysticercoids, ES, 3-DW and 12-DW other than the surface membrane. There are only two possible explanations for this discrepancy. One is that the enzymes are denatured during fixation, but this seems unlikely since the phosphatases associated with the brush border membrane remain active following fixation.

An analysis of the system used in this study suggests that a second explanation is more likely. That is, substrate and/or capturing agent (Ce$^{3+}$) do not penetrate the brush border plasma membrane, and any reaction in the underlying tissues is thereby precluded. Moczón (1973) proposed a similar explanation for the lack enzymatic activity in underlying tissues of cysticercoids of *H. diminuta*. As noted previously, *H. diminuta* appears to be impermeable to phosphoesters; that is, living tapeworms do not absorb detectable amounts of hexose phosphates, BGP or nucleotides. Rather, these compounds are hydrolyzed at the tapeworm's surface and only the dephosphorylated form is transported across the tegument (Dike and Read, 1971b). In addition, the surface
membrane of *H. diminuta* is covered with a glycocalyx that bears a net negative charge (Lumsden, 1972) and adsorbs cations (Lumsden et al., 1970; Lumsden, 1973; Lumsden and Berger, 1974). This suggests that the trivalent cation Ce³⁺ adsorbs to the glycocalyx, thus preventing its penetration of the brush border. Therefore, it is conceivable that neither Ce³⁺ nor βGP penetrates the surface of *H. diminuta*, thereby limiting reaction product to the surface membrane.

It is also conceivable that the non-specific reaction product present in substrate-free controls is the result of electrostatic interactions between electronegative moieties of the glycocalyx and Ce³⁺, and the observed reaction product may represent the binding of free Ce³⁺ cations to the anionic surface of the tapeworm. Lumsden et al. (1968) proposed a similar explanation for the binding of free lead ions to the surface of *H. diminuta* during cytochemical reactions. Studies by Lumsden (1973) indicate that the glycocalyx of *H. diminuta* contains binding sites for Ca²⁺, a metabolically important divalent cation. These Ca²⁺ binding sites are competitively inhibited by the binding of lanthanum ions (La³⁺). In addition to having identical valences, Ce³⁺ and La³⁺ have similar atomic
weights (138.91 and 140.12, respectively) and atomic radii (1.14 and 1.07, respectively). Considering the similarities between Ce$^{3+}$ and La$^{3+}$, it seems reasonable that Ce$^{3+}$ cations would also bind to the anionic surface of *H. diminuta*.

In substrate-free controls, less Ce$^{3+}$ is deposited on the external membrane of tapeworms incubated in acidic media than alkaline media. This suggests that pH affects the binding characteristics of the external membrane; as the pH decreases, the net electronegative charges on the external membrane/glycocalyx would decrease and less Ce$^{3+}$ ions would bind. This assumption also supports the hypothesis that non-specific binding between Ce$^{3+}$ and anionic sites of the glycocalyx occurs.

Cytochemical and biochemical studies indicate that AlkPase and AcPase are distributed differentially in cysticercoids and ES of *H. diminuta*; AlkPase predominates in ES, while AcPase predominates in cysticercoids. A similar distribution of AlkPase and AcPase was reported by Bogitsh (1967) in sectioned cysticercoids of *H. diminuta*. He localized AcPase in the intermediate cell layer below pH 6.0. Above pH 6.0, reaction product indicative of AlkPase appeared in the tegument of the scolex and persisted through pH 9.0. In a related study,
Varma et al. (1985) determined the activities of AlkPase and AcPase in homogenates of cysticerci and adult tapeworms of *Taenia hydatigena* and *T. solium*. In both species of tapeworms, AlkPase predominates in adult tapeworms, while AcPase predominates in cysticerci. Therefore, the differential distribution of these enzymes in tapeworms and their immature stages (i.e.; cysticercoids, cysticerci) suggests that AlkPase and AcPase may have different functional roles in these life-cycle stages.

Bogitsh (1967) hypothesized that, because AcPase was localized throughout the intermediate cell layer of cysticercoids, this tissue layer ultimately is dissolved during excystation. Bogitsh (1969) provided further evidence for this hypothesis by localizing cytochemically AcPase in the cytoplasm and smooth membrane-bound vesicles of the intermediate cell layer. Several lines of evidence from the present study support this hypothesis.

Cysticercoids incubated in media for the localization of AcPase demonstrated a loss of cellular integrity, while those incubated in media for the localization of AlkPase were intact. This difference is not due to improper fixation, since no loss of
cytoplasmic integrity was observed in other life-cycle stages incubated in the same acidic media. Thus, the responses of cysticercoids to incubation in alkaline and acidic media are quite different. Presumably, AlkPase present in the cells would not be functional at an acidic pH as has been demonstrated in BBM fractions (Pappas, 1988). This suggests that cellular degradation may be due to AcPase.

Similar losses of cellular integrity have been reported for H. diminuta in vivo. In a series of experiments (Goodchild and Harrison, 1961), rats were infected with cysticercoids and killed at different intervals to determine the location of the cysticercoids within the rat. After 3 hr, cysticercoids were found in the stomach and were observed to consist only of the scolex and the surrounding inner cyst. These results suggest that AcPase rapidly degrades cysticercoids in the rat's stomach, however, pepsin also may have a synergistic effect.

The localization of AcPase in cysticercoids has been suggested by Bogitsh (1969) to be indicative of lysosomal activity. The present study, however, indicates that a majority of AcPase activity in cysticercoids is solubilized and, thus, is not likely to be associated
with lysosomes. Recent studies of AcPase in 12-DW of *H. diminuta* (Pappas, 1988) have established reasonably well the presence of lysosomes in these tapeworms. Therefore, it seems likely that cysticercoids also would possess lysosomes, but their presence remains equivocal.

Unlike the intermediate cell layer, the inner cyst tissue of cysticercoids is unaffected by homogenization or incubation in an acidic medium. Attempts to homogenize cysticercoids using a Dounce tissue grinder resulted in incomplete homogenization. Microscopic examination revealed that the inner cyst remains intact, but the scolex is disrupted. In a similar study using cysticercoids of *H. microstoma*, Caley (1974) reported that the inner cyst protects the encysted scolex from acid (pH 2.0) for 30 min at 37°C. He also reported that the inner cyst and encysted scolex of *H. microstoma* remains intact after 5 min of grinding with glass beads.

The resilience of the inner cyst may be attributable to the fibrous nature of this tissue layer. Indeed, it has been suggested that the inner cyst layer of *H. diminuta* is produced by fibroblasts (Krasnoshchekov et al., 1979; Richards and Arme, 1984b), thus implying that the main structural component of the inner cyst is collagen. However, these fibrils do not present the same
appearance as vertebrate collagen; they demonstrate differences in size, a lack of periodicity and a general a hollow appearance (Richards and Arme, 1984b). Despite a somewhat different appearance, substantial evidence exists to suggest that the fibrils of the inner cyst are indeed made of collagen. Caley (1974) demonstrated that the inner cyst of H. microstoma is susceptible to collagenase. Also, the fibrils observed in H. diminuta (Richards and Arme, 1984b) are similar to collagen fibers described in cysticerci of T. solium (Torre-Blanco, 1982). The presence of collagen in these cysticerci is confirmed by the demonstration of the following similarities with vertebrate collagen: amino acid composition, intrinsic viscosities, and susceptibilities to bacterial collagenase. Whether or not collagen associated with the inner cyst plays a protective role, as the scolex passes through the stomach of the definitive host, remains to be determined empirically.

Richards and Arme (1983), however, discounted a protective role for the inner cyst based on several observations. They reported that 11-day-old cysticercoids are infective, despite incomplete fibrogenesis of the inner cyst. Thus, they discount a protective role for the inner cyst based on the lack of
complete fibrogenesis. However, this line of reasoning does not preclude a protective function, because even at 11 days the inner cyst may be developed sufficiently to provide some protection from the acidic environment encountered in the stomach of the rat. The mere fact that the scolex survives passage through the stomach suggests that it is protected in some way prior to excystment.

Richards and Arme (1983) also suggest that the inner cyst limits the growth of the scolex and, thus, serves as a preadaptive advantage. Presumably the fibrogenesis of the cyst wall reduces the flow of nutrients to the presumptive scolex, thereby limiting its growth. In contrast, Arme and Coates (1973) reported that the anterior canal of the cysticercoid is continuous with the inner cyst throughout development of the cysticercoid, and that this would serve as a route for nutrient uptake. Clearly, the functional importance of the inner cyst is equivocal at present, but perhaps it both protects and limits the growth of the encysted scolex.

Rothman (1959) conducted extensive studies to determine the factors involved in the excystment of scolecites from cysticercoids. Pretreatment in acid, particularly below pH 2.0, increases the rate of
subsequent excystation by dissolving the cyst wall. Rothman (1959) suggests, however, that the mammalian stomach-intestine rarely demonstrates a pH below 2.0, usually demonstrating a pH of approximately 3.5. Since considerable AcPase activity is demonstrable in homogenates of cysticercoids at pH 4.0, the AcPase in cysticercoids would appear to be functional in the mammalian stomach-intestine. This hypothesis warrants further examination.

The above discussion suggests the following series of events occurs during the excystation of the scolex from the cysticercoid. Upon introduction to the rat's stomach, AcPase activity in the intermediate cell layer of the cysticercoid is activated in response to the acidic pH. AcPase, in conjunction with other lytic enzymes reported in the cysticercoid (i.e., aryl sulfatase; Bogitsh, 1969), dissolves all tissue layers surrounding the inner cyst, leaving only the inner cyst and scolex intact. The inner cyst presumably provides the scolex a degree of protection from enzymatic degradation during its passage through the stomach. Once in the intestine, the scolex, activated by trypsin and/or bile salts (Lackie, 1975), excysts by muscular action through the inner cyst which presumably has been weakened
by acid/pepsin treatment. Thus, this series of events suggests the functional importance of AcPase in the excystment process.

AlkPase, unlike AcPase, appears to have a minor functional role in cysticercoids of *H. diminuta*. Previous histochemical studies (Bogitsh, 1967; Moczon, 1973) localized AlkPase activity only at the tegument of the encysted scolex and the lining of the scolex cavity. In the present study, AlkPase activity was demonstrated in homogenates of cysticercoids and by using *in vitro* incubations. However, the percentage of AlkPase activity contributed by the encysted scolex is not readily discernable. The fact that a considerable percentage of AlkPase remains in solubilized form in cysticercoids (67%), but not in ES (6%), argues that AlkPase is present in regions of the cysticercoid other than the encysted scolex. A role for AlkPase in the excystment process is unlikely due to the acidic nature of the rodent stomach. Therefore, the functional role of AlkPase in cysticercoids, beyond a possible nutritional role is unclear.

The localization of AlkPase in the tegument of ES, however, suggests that this enzyme has a nutritional role in the developing tapeworm. Various phosphoesters found
in the rat's small intestine represent an important nutritional resource for tapeworms, but the attached phosphate moiety prevents their direct absorption. The tegument of *H. diminuta* demonstrates phosphohydrolase activity which hydrolyzes these esters, and the resulting dephosphorylated compounds are absorbed (Phifer, 1960; Dike and Read, 1971b). Phosphohydrolases are recognized as a ubiquitous biochemical feature of cell surfaces engaging in transport functions (Lumsden, 1975). Furthermore, the geometric growth demonstrated by developing tapeworms necessitates the rapid assimilation of nutritional metabolites. Thus, phosphohydrolase activity would be expected to occur at the surface of the brush border membrane of *H. diminuta*. Indeed, this study localized AlkPase at the brush border of ES, supporting a possible nutritional role for this enzyme.

Localized AlkPase was observed to be associated with the brush border and underlying vesicles, posterior to the scolex proper. In a similar study using adult *H. diminuta*, Rothman (1966) also described the lack of AlkPase activity in the scolex. Bogitsh (1967), however, reported that AlkPase is present in the scolex proper of unexcysted scoleces. Therefore, he suggested that AlkPase activity and related transport capabilities are
lost by the scolex during maturation. By using ES, it is clear that the scolex proper is devoid of AlkPase activity, thus suggesting that the scolex lacks appreciable AlkPase activity throughout its development. Therefore, despite appearing morphologically similar to other portions of the ES, the scolex proper appears to function primarily as an attachment organ. Why the scolex lacks AlkPase, or perhaps lacks the capability to produce it, is not clear and warrants further investigation.

As described above, vesicles are a common tegumental component of ES, but are greatly reduced in 3-DW and absent in 12-DW of *H. diminuta*. Richards and Arme (1984a) reported that vesicles increase in number during the development of the encysted scolex. In addition, they observed the fusion of these vesicles, as well as the release of internal contents at the surface membrane, which they suggest represents glycocalyx formation. The localization of AlkPase in these vesicles also suggests that they may be important for insertion of enzymes at the surface membrane. Presumably enzymes would be produced in the Golgi, packaged in vesicles and transported to the surface where they would be incorporated into the surface membrane.
The synthesis of AlkPase in ES, however, appears to be a dynamic process, dependent upon the development and maturation of the tegument and underlying cytons. The distal cytoplasm of the tegument in ES decreases in thickness and the degree of development as it proceeds posteriad. Concomitantly, the microvilli are less developed (Richards and Arme, 1984a) and virtually absent at the extreme posterior end of ES. Indeed, the posterior end of ES often contain a hollow cavity surrounded by poorly defined cells. The intensity of localized AlkPase activity in ES is observed to be directly proportional to the degree of cellular and tegumental development. This suggests that the cellular organelles necessary for the production of enzymes (i.e., Golgi apparatus) may not be completely developed in immature portions of the ES.

Richards and Arme (1984a) reported that the tegumental syncytium of the encysted scolex is adapted for existence in the definitive host in at least two ways. First, the presumptive scolex demonstrates adult-type microvilli; and secondly, the presence of vesicles in the cytoplasm suggests the ability to rapidly form a glycocalyx. In addition, the present study suggests that the scoleces also may be adapted biochemically based on
the presence of AlkPase. Indeed, the ability to transport nutrients and metabolites immediately upon excystation in the rat's small intestine would augment growth and development. In fact, Richards and Arme (1984a) infer that the survival of the ES in the vertebrate host is dependent upon the prior development of microvilli on the encysted scolex. Perhaps survival of the ES in the small intestine of the rat is ensured only when sufficient quantities of AlkPase are present in the tegument to conduct necessary nutritional, digestive and metabolic functions. Therefore, ES are adapted not only morphologically, but also biochemically for survival in the definitive host.

Cytochemical assays measuring AcPase in 12-DW indicate that considerable enzymatic activity is present. The lack of localized AcPase in ES suggests that the synthesis of AcPase in H. diminuta is related to the maturation of the tapeworm. This hypothesis is supported by the localization of AcPase in 3-DW using Tris-maleate buffer; 3-DW demonstrated greater AcPase activity than ES, but less than 12-DW. Thus, maturation of H. diminuta and synthesis of AcPase appear to be directly related.

Erasmus (1957) reported similar results for the localization of AcPase in adults of T. pisiformis. In
tapeworms which are approximately 50 days old, reactions localizing AcPase demonstrate only questionable amounts of activity in anterior portions of the tapeworm. However, in older tapeworms, AcPase is present in the tegument of mature proglottids. Therefore, unlike AlkPase, AcPase does not appear to be necessary in ES at excystation, however, its functional role remains unclear.

Considerable amounts of AlkPase and AcPase were demonstrable in 12-DW. The functional role of AlkPase in the tegument was discussed previously for ES, and applies to 12-DW as well. However, the functional role of AcPase in 12-DW is less clear. Pappas (1988) reported AcPase activity in a BBM fraction from *H. diminuta* which suggests a nutritional role. However, AcPase functions optimally at a pH well below those found in the rat intestine, making a nutritional role unlikely. Recent studies (Uglem and Just, 1983; Zavras and Roberts, 1984) have demonstrated that *H. diminuta* releases several acidic metabolites into its surrounding environment, thus suggesting that the "microenvironment" in the vicinity of the tapeworm may be acidic (Uglem and Just, 1983; Pappas, 1980); conditions under which AcPase could play a
nutritional role. Cytochemical localization of AcPase at the brush border supports this hypothesis.

In conclusion, this study suggests that AcPase and AlkPase have different functional roles at the host-parasite interface in ES and cysticercoids of *H. diminuta*: (1) AcPase is apparently important for the degradation of tissues in cysticercoids prior to excystation of the scolex; (2) AcPase has a limited role in ES, but a nutritive/digestive role is suggested in 3-DW and 12-DW; (3) the functional importance of AlkPase in cysticercoids may be nutritional in nature, but is unclear at this time; and (4) localization of AlkPase at the external membranes of ES and 12-DW indicates clearly a nutritional/digestive role in these tapeworms.
LITERATURE CITED


