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Acid Phosphatase Activity in the Isolated Brush Border Membrane of the Tapeworm, *Hymenolepis diminuta*: Partial Characterization and Differentiation From the Alkaline Phosphatase Activity

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The isolated brush border membrane of the tapeworm, *Hymenolepis diminuta*, hydrolyzes p-nitrophenyl phosphate over a broad pH range. Acid phosphatase activity (pH optimum at 4.0) is inhibited specifically by sodium dodecyl sulfate (SDS) and NaF, while the alkaline phosphatase activity (pH optimum at 8.8) is inhibited specifically by levamisole, 2-mercaptoethanol, and ethylenediaminetetra-acetate (EDTA). These two phosphatase activities are further differentiated in that (1) there is a rapid decrease in alkaline phosphatase activity when the membrane preparation is incubated at pH 4.0, while there is little loss of acid phosphatase activity, and (2) the alkaline phosphatase activity is solubilized with no loss of activity when the membrane is treated with Triton X-100, while such treatment causes a significant loss of acid phosphatase activity. Both activities are nonspecific and hydrolyze a variety of phosphorylated compounds, but the relative activities of the two phosphatases against these substrates vary significantly.

Key words: Cestoidea, tegument, plasma membrane, membrane-bound enzyme

Several membrane-bound enzymatic activities are demonstrable in the brush border membrane limiting the syncytial epithelium of the tapeworm, *Hymenolepis diminuta*. These activities, including ribonuclease, phosphodiesterase, and alkaline phosphatase activities, have pH optima greater than 7 and likely play an important role in the nutrition of this parasitic helminth, as reviewed previously [1]. Recent cytochemical studies [2,3] indicate that the brush border membranes of tapeworms also contain membrane-bound acid phosphatase activity. This paper reports on the demonstration and partial characterization of this acid phosphatase activity by using a preparation of brush border membrane isolated from *H. diminuta*.

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METHODS

The "OSU strain" of *H. diminuta* [4] was maintained in beetles, *Tenebrio molitor*, and male Sprague-Dawley rats. Rats weighing 100–120 g were infected with 30 cysticercoids, and the tapeworms were removed 12 days postinfection. A preparation of brush border membrane was prepared from the tapeworms pooled from several rats [5]; following dialysis against distilled-deionized water (4 days at 2°C), the membrane was sedimented (5,000g for 30 min at 2°C), resuspended in 0.02% NaN_3, and stored at 2°C. The protein content of this final preparation was determined [6] by using bovine serum albumin as a standard.

In most experiments enzymatic activity was assayed as follows. An aliquot of the membrane preparation (<50 μl) was added to buffer and preincubated for 5 min at 37°C, and the reaction was initiated by the addition of substrate (<50 μl). (The volumes of reactants were adjusted to yield a final volume of 1 ml.) Following incubation at 37°C for a predetermined period of time the reaction was terminated, and the amount of hydrolysis was determined. Reactions containing p-nitrophenyl phosphate (PNPP, Sigma 104 Phosphatase Substrate) as the substrate were terminated by adding 1 ml of 1 N NaOH and heating the reaction mixture to 95°C for 2 min; p-nitrophenol was quantified at 405 nm by using a millimolar extinction coefficient of 17.5 [7]. In those assays containing nonchromogenic phosphorylated substrates (e.g., pyrophosphate), the reaction was terminated and the liberated phosphate was quantified in a single step by the addition of a Malachite Green–ammonium molybdate reagent and measuring the absorbance at 660 nm [8,9]. Hydrolysis of o-carboxyphenyl phosphate (OCPP, 1 mM, Sigma) was determined at 37°C by monitoring continuously the increase in absorbance at 300 nm, and the amount of substrate liberated was quantified by using a millimolar extinction coefficient of 1.94 [10]. Similarly, the maximum alkaline phosphatase activity (i.e., the activity in 200 mM Tris buffer, pH 8.8, 5 mM Mg^{2+} [9]) was determined at 37°C by monitoring continuously the hydrolysis of 1 mM PNPP at 405 nm. Control assays containing enzyme which had been heated at 95°C for 5 min and cooled prior to the addition of substrate were included in each set of experiments to correct for spontaneous hydrolysis of substrate.

The stabilities of the acid and alkaline phosphatase activities at pH 4.0 were examined by incubating 1 ml of buffer (200 mM acetate, pH 4.0) containing 6.5 μg of protein at 37°C. At various time intervals a 20 μl aliquot was removed and assayed for alkaline phosphatase activity as above. The remainder of each assay was then assayed for acid phosphatase activity as described above.

The alkaline phosphatase activity of the membrane preparation was solubilized by incubating the preparation in 1% Triton X-100 for 1 hr at 37°C followed by centrifugation at 100,000g for 1 hr at room temperature [11]. Alkaline and acid phosphatase activities of the membrane preparation were determined as above (1) prior to treatment with Triton X-100, (2) following incubation with Triton X-100, but prior to centrifugation, and (3) following centrifugation. In the presence of Triton X-100, protein concentrations were determined following extraction with iso-amyl alcohol [12].

To examine for the presence of cathepsin B activity, tapeworms were removed from the rat host, rinsed briefly in cold homogenization buffer (see below), and homogenized in 10 volumes of cold buffer by using a motor-driven Ten Broeck homogenizer. The homogenate was centrifuged (200g for 15 min at 2°C), and the resulting pellet was again homogenized in 10 volume of cold buffer. Following centrifugation as

above, the 200g supernates were combined and centrifuged at 40,000g (60 min at 2°C). The resulting pellet was resuspended in cold buffer with the aid of a Dounce homogenizer, and the pellet and supernate were dialyzed overnight (at 4°C) against 2 liters of buffer. Protein concentrations of these fractions were determined as noted above [6]. Aliquots of the 40,000g pellet and supernate and the membrane preparation were assayed for cathepsin B activity by using CBZ-L-arginyl-L-arginine 4-methoxy-β-naphthylamide (ZAAMNA, Sigma) as described by Barrett and Kirschke [13].

Solutions with an osmotic pressure of approximately 180 mmol/kg are isosmotic with *H. diminuta* [14]. In order to maintain the integrity of cellular organelles, tapeworms were homogenized in a buffer with a similar osmotic pressure. The buffer used contained 10 mM HEPES, 2 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), 60 mM KCl, 30 mM NaCl, and 2 mM $MgCl_2$, and the pH was adjusted to 7.0 with NaOH (resulting in a final $[Na^+]$ of approximately 39 mM). The osmotic pressure of this solution, as determined by using a vapor pressure osmometer, was 185 mmol/kg.

RESULTS

The 40,000g pellet and supernate separated from homogenized *H. diminuta* contained an enzyme that was active against ZAAMNA. Based on the specificity of this substrate [13,15] and the fact that the activity in both fractions was inhibited by leupeptin, it was assumed that these fractions contained cathepsin B (or B-like) activity. Addition of 0.1% sodium dodecyl sulfate (SDS) or 0.2% Triton X-100 to assays of the pellet fraction resulted in a dramatic increase enzymatic activity (>100% in both cases), and such latency suggested that the cathepsin B activity was sequestered within vesicles. The isolated membrane preparation, on the other hand, contained no activity against ZAAMNA. This lack of activity was apparent even when the amount of protein in these assays was 10 times greater and the length of the assays was 3 times longer than in those assays of the pellet fraction; addition of 0.1% SDS or 0.2% Triton X-100 also had no effect. Thus, while these data indicated the presence of lysosomes in this tapeworm, they also indicated that the isolated membrane preparation was not contaminated with these organelles. (The sole purpose of these experiments was to verify the existence of lysosomes in *H. diminuta* and to then test the isolated membrane preparation for possible contamination. Quantitative data were of no importance in arriving at the above conclusions and, therefore, were omitted.)

In the presence of the isolated brush border membrane preparation, hydrolysis of PNPP occurred over a broad range of pH. Although hydrolysis rates varied dramatically in different buffers, a peak in enzymatic activity was noted at pH 4.0 in both citrate and acetate buffers (Figs. 1, 2), with activity increasing dramatically above pH 6 (Figs. 1–3). It was unclear from these data whether this peak in activity at pH 4.0 represented a distinct enzymatic activity or simply residual alkaline phosphatase activity, so several chemicals were tested as potential inhibitors in hopes of differentiating these two enzymatic activities.

Preliminary experiments tested the effects of ammonium molybdate, 2-mercaptoethanol (2ME), ethylenediaminetetra-acetate (EDTA), and SDS on phosphatase activity at pH 4.0 and 8.8. Ammonium molybdate inhibited hydrolysis at both pH values (data not shown), but the latter three inhibitors appeared to have distinctly different effects. Thus, the effects of these inhibitors were examined over a broader range of pH values.

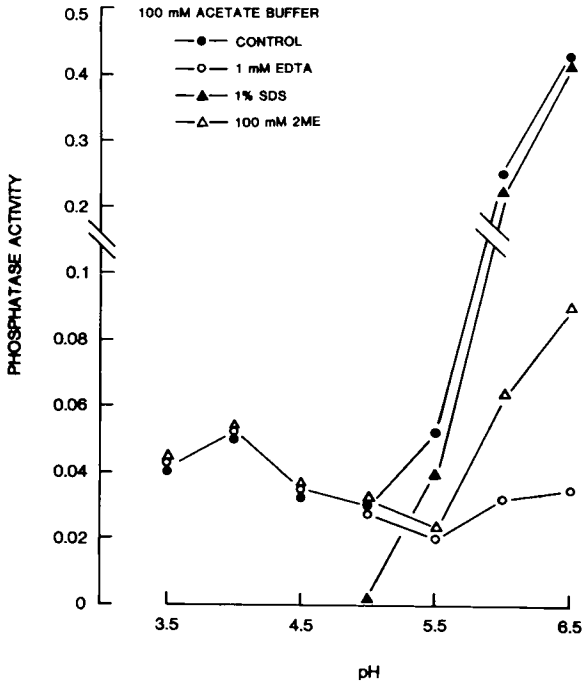

Fig. 1. Hydrolysis of PNPP (1 mM) by the isolated brush border membrane of *Hymenolepis diminuta* as a function of pH in 100 mM acetate buffer; "phosphatase activity" = μmol p-nitrophenol liberated·mg^{-1} protein·min^{-1}. Each assay contained 6.5 μg of membrane protein. Assays were incubated at 37°C for 60 min and terminated. Hydrolysis rates of PNPP in the presence of 1 mM EDTA, 1% SDS, and 100 mM 2ME are also indicated. Each point represents the mean of triplicate assays. (Hydrolysis rates of PNPP in 50 mM or 200 mM acetate buffer were identical to those in 100 mM acetate buffer.)

When these pH profiles were examined, and the effects of the inhibitors were determined, it was readily apparent that EDTA and 2ME inhibited only the alkaline phosphatase activity, while SDS inhibited only the acid phosphatase activity (Figs. 1–4).

NaF and levamisole (Levisole, Pitman Moore, Inc.) were also tested as inhibitors, but only at pH 4.0 and 8.8. (Levamisole is an uncompetitive inhibitor of some vertebrate alkaline phosphatases [see 16, for example].) NaF inhibited phosphatase activity at pH 4.0, and when a series of 2-fold serial dilutions of NaF (beginning with 10 mM) was tested, it was determined that the minimal concentration of NaF required to inhibit completely the acid phosphatase activity was 1.25 mM (i.e., a 1:8 dilution). However, at pH 8.8, 2 mM NaF had no effect on the alkaline phosphatase activity. On the contrary, 10 mM levamisole had no effect on phosphatase activity at pH 4.0, but activity at pH 8.8 was inhibited 58%.

The distinct nature of these two enzymatic activities was demonstrated further when their stabilities at pH 4.0 and solubilities in Triton X-100 were determined. Incubation of the membrane preparation at pH 4.0 resulted in a rapid and significant loss of alkaline phosphatase activity, while acid phosphatase activity decreased only slightly (Fig. 5).

Treatment of the isolated membrane preparation with 1% Triton X-100 resulted in a significant loss of acid phosphatase activity (i.e., the "relative activity," or activity on a per volume basis, decreased 54% during the incubation period), and the remaining

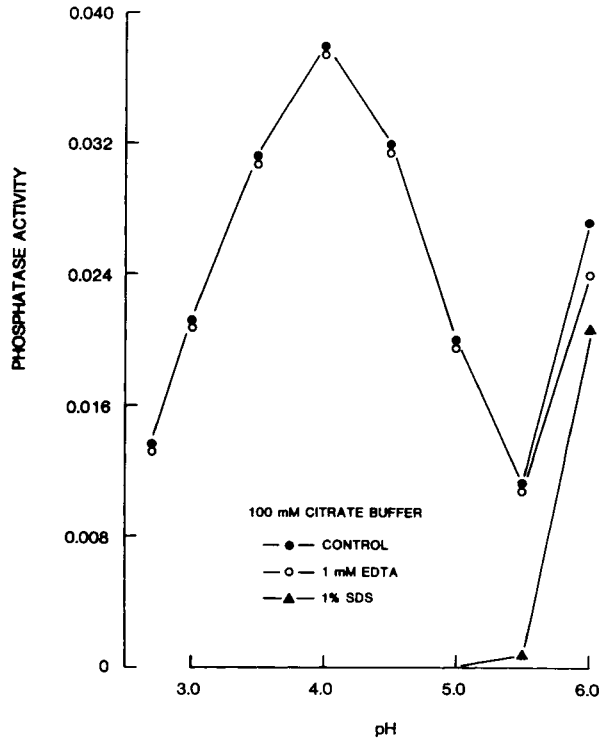

Fig. 2. Hydrolysis of PNPP (1 mM) by the isolated brush border membrane of *H. diminuta* as a function of pH in 100 mM citrate buffer, and the effects of 1 mM EDTA and 1% SDS on hydrolysis. These assays were run under the same conditions as those described in Figure 1, except they were terminated after 90 min. Each point represents the mean of triplicate assays.

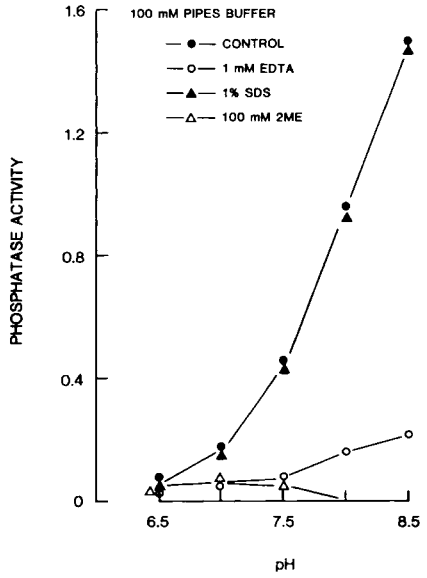

Fig. 3. Hydrolysis of PNPP (1 mM) by the isolated brush border membrane of *H. diminuta* as a function of pH in 100 mM PIPES buffer, and the effects of 1 mM EDTA, 1% SDS, and 100 mM 2ME on hydrolysis. These assays were run under the same conditions as those described in Figure 1, except they were terminated after 10 min. Each point represents the mean of triplicate assays.

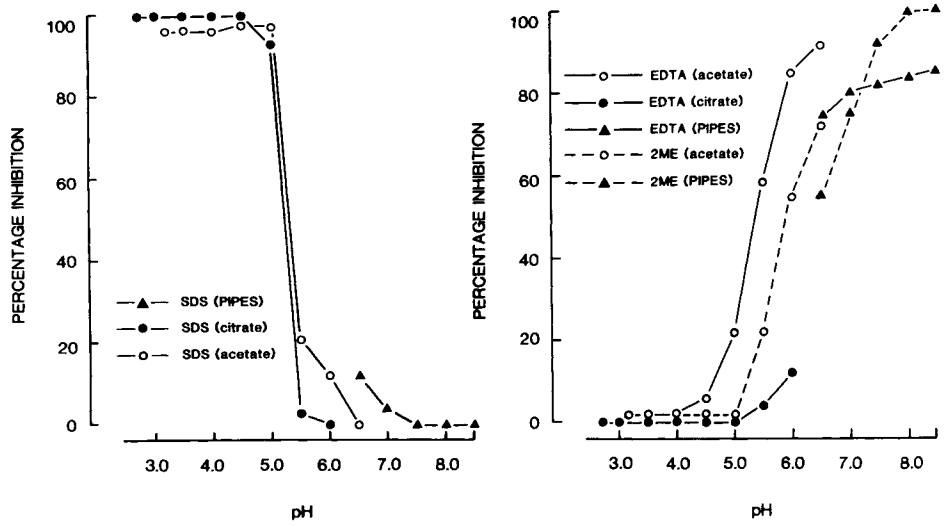

Fig. 4. Inhibition of phosphatase activity by 1% SDS (**left graph**), 10 mM EDTA, and 100 mM 2ME (**right graph**) as a function of pH in different buffers (indicated in parentheses on graphs). The data (calculated from Figs. 1–3) are presented as a "percentage inhibition" compared to control assays containing no inhibitor. (The effects of 1.0% and 0.1% SDS were identical regardless of pH or buffer.)

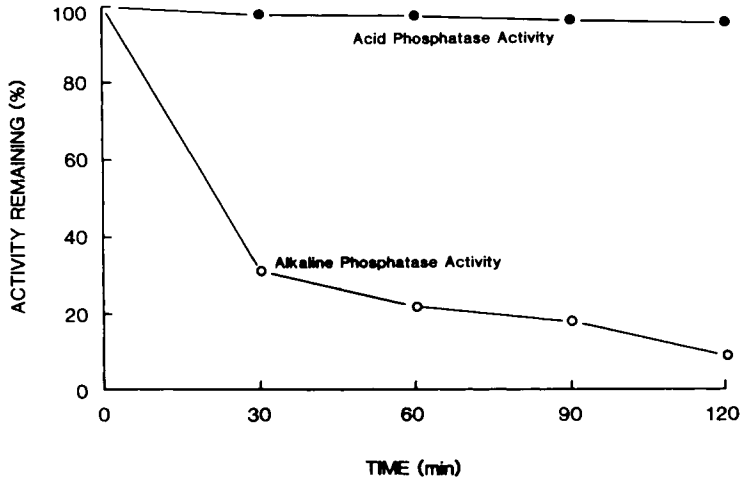

Fig. 5. Stability of acid and alkaline phosphatase activities of the brush border membrane of *H. diminuta* at pH 4.0. The membrane preparation was incubated at 37°C in 200 mM acetate buffer, pH 4.0, for the indicated time periods and then assayed for acid and alkaline phosphatase activities (see Methods). Data were calculated as "percentage of activity remaining" compared to time zero. Each point represents the mean of triplicate assays.

acid phosphatase activity was not soluble (i.e., the "specific activity," or activity based on the amount of protein, did not increase following centrifugation). On the other hand, treatment of the membrane with Triton X-100 resulted in no loss and virtually complete solubilization of the alkaline phosphatase activity (Table I). (The "relative activity" did not change, but the "specific activity" increased dramatically following centrifugation.)

The specificities of the two phosphatase activities were assessed by measuring the hydrolysis of several substrates at pH 4.0 and 8.8. (The latter value is the pH optimum for hydrolysis of many phosphorylated substrates by the membrane preparation [7,9].) Additional assays containing SDS, EDTA, or 2ME were also run to insure that the hydrolysis of each substrate at each pH value was due to only a single enzymatic activity (i.e., acid or alkaline phosphatase). These data (Table II) indicated that both phosphatase activities hydrolyzed a variety of substrates and that their relative activities against the various substrates differed dramatically.

DISCUSSION

Acid phosphatase activity is demonstrable in a wide variety of organisms, including plants, animals, bacteria, and fungi [17], and parasitic platyhelminths (trematodes and tapeworms) are no exception. While many previous studies have demonstrated acid phosphatase activity in homogenates of various species of flatworms [18], or localized this activity histochemically [19], such studies are of limited value in determining in which cellular organelles such activity is localized. More recent studies [2,3] have, however, demonstrated cytochemically that the external brush border membrane of several species of tapeworms contains acid phosphatase activity.

Acid phosphatase activity is also demonstrable within membranous vesicles in the syncytial epithelium of several tapeworms [3,20], and such data, although not conclusive, suggest the presence of lysosomes. This assumption is supported by the presence of latent cathepsin B (or B-like) activity in *H. diminuta*, since such activity is indicative of lysosomes (at least in vertebrates) [13,15,21]. However, lysosomal contamination

TABLE I. The Solubilities of Acid and Alkaline Phosphatase Activities of the Isolated Brush Border Membrane of *Hymenolepis diminuta* in Triton X-100*

Parameter	Prior to addition of Triton	Following addition of Triton	Following centrifugation
Protein concentration	460 μg/ml	460 μg/ml	118 μg/ml
AlkPase Act.			
"Relative"	12.4	12.4	11.5
"Specific"	27.0	27.0	97.8
AcPase Act.			
"Relative"	0.027	0.012	0.0028
"Specific"	0.055	0.026	0.024

*The concentrations of protein and alkaline and acid phosphatase activities (AlkPase and AcPase, respectively) of the membrane preparation were determined (1) prior to the addition of Triton X-100, (2) following the addition of Triton X-100 and a 60-min incubation at 37°C, and (3) following centrifugation at 100,000g for 60 min. Units for "relative enzymatic activities" and "specific enzymatic activities" = mmol substrate hydrolyzed·ml^{-1}·min^{-1} and μmol substrate hydrolyzed·mg^{-1} protein·min^{-1}, respectively.

TABLE II. The Hydrolysis of Phosphorylated Substrates by the Isolated Brush Border Membrane of *H. diminuta

Substrate	Hydrolysis rate pH 8.8	Hydrolysis rate pH 4.0	Ratio[a]
5′-AMP	3.93[b]	0.0010[c]	3,930
5′-ATP	1.05[b]	0.016[c]	65
Glucose-1-PO_4	0.50[b]	0.0017[c]	295
Glucose-6-PO_4	0.84[b]	0.0017[c]	494
Glycerophosphate	2.22[b]	0.013[c]	171
Pyrophosphate	2.42[b]	0.077[c]	31
PNPP	20.0[d]	0.055[e]	363
OCPP	5.0[d]	0.096[e]	52

*Substrate concentrations were 1 mM (except glycerophosphate, which was 2 mM), and hydrolysis rates were calculated in terms of the amount of reaction product (inorganic phosphate or p-nitrophenol) liberated (i.e., $\mu mol \cdot mg^{-1}$ protein$\cdot min^{-1}$). Each rate represents the mean of triplicate assays.

[a][rate at pH 8.8]/[rate at pH 4.0].

[b]Assay conditions: protein = 0.16 μg/assay; 60 min assay period; 200 mM Tris buffer containing 5 mM Mg^{2+}. Addition of 10 mM EDTA inhibited hydrolysis 99+%.

[c]Assay conditions: protein = 6.5 μg/assay; 60 min assay period; 200 mM acetate buffer. Addition of 10 mM EDTA had no effect on hydrolysis.

[d]Assay conditions: protein = 0.16 μg/assay; substrate hydrolysis monitored continuously at 405 nm; buffer as in b above. Hydrolysis was inhibited by 10 mM 2ME (93+%) and 10 mM EDTA (99%+) but was unaffected by 0.1% SDS.

[e]Assay conditions: protein = 6.5 μg/assay; substrate hydrolysis monitored continuously at 300 nm; buffer as in c above. Hydrolysis was inhibited by 0.1% SDS (100%) but was unaffected by 10 mM 2ME and 10 mM EDTA.

does not appear to be the source of the acid phosphatase activity associated with the isolated brush border membrane used in this study.

Acid and alkaline phosphatases have, historically, been differentiated simply on the basis of their pH optima, but these enzymes also differ with respect to their substrate specificities and the effects of various inhibitors (reviewed in 17). The alkaline and acid phosphatase activities present in the isolated brush border membrane of *H. diminuta* can also be differentiated clearly by using these same criteria. For example: (1) the relative rates at which different substrates are hydrolyzed at pH 4.0 and 8.8 differ dramatically; (2) phosphatase activity at pH 4.0 is inhibited when the membrane is solubilized in SDS or Triton X-100, while similar treatment has no effect on activity at pH 8.8; and (3) EDTA, 2ME, and levamisole inhibit alkaline but not acid phosphatase activity, while NaF has just the opposite effect.

Tapeworms lack any remnant of a functional digestive tract, and, therefore, their brush border surfaces have both absorptive and digestive capabilities. The several membrane-bound enzymatic activities demonstrable in the membrane limiting this surface appear to play an essential role in the production of low molecular weight substrates which are subsequently absorbed by the tapeworms [1]. One might assume a priori that the acid phosphatase would play an insignificant role in this regard since it would be relatively inactive in the pH range of 6.5–7.5 (as found in the rat's small intestine). However, *H. diminuta* excretes significant amounts of acidic metabolic end products, resulting in acidification of the external environment [22,23]. Thus, it is possible that the pH of the environment in the tapeworm's immediate vicinity (i.e., its microenvi-

ronment) could be low enough to result in significant acid phosphatase activity, and, therefore, that this membrane-bound enzymatic activity plays an important role in the nutrition of this parasitic helminth.

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