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Competitive, Uncompetitive, and Mixed Inhibitors of the Alkaline Phosphatase Activity Associated With the Isolated Brush Border Membrane of the Tapeworm *Hymenolepis diminuta*

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Several compounds were tested as inhibitors of the alkaline phosphatase (AlkPase) activity associated with the isolated brush border membrane of the tapeworm, *Hymenolepis diminuta*. Molybdate, arsenate, arsenite and β-glycerophosphate (BGP) were competitive inhibitors of the hydrolysis of *p*-nitrophenyl phosphate, while levamisole and clorsulon were uncompetitive and mixed inhibitors, respectively. Molybdate was also a competitive inhibitor of the hydrolysis of BGP and 5′-adenosine monophosphate, and levamisole was an uncompetitive inhibitor of BGP hydrolysis. The apparent inhibitor constants (K_i') for molybdate and levamisole were virtually identical regardless of the substrate, and these data support the hypothesis that the AlkPase activity is represented by a single membrane-bound enzyme with low substrate specificity. Quinacrine, Hg^{2+}, and ethylenediaminetetraacetate were also potent inhibitors of the AlkPase activity, but the mechanisms by which these latter three inhibitors function were not clear.

Key words: cestoidea, membrane-bound enzyme, tegument, plasma membrane, enzyme inhibitors, anthelmintic, levamisole, clorsulon, quinacrine

The brush border plasma membrane limiting the external surface of the tapeworm, *Hymenolepis diminuta,* possesses alkaline phosphatase (AlkPase) activity that hydrolyzes and/or is inhibited by a variety of compounds, including nucleotides and hexose mono- and diphosphoesters (reviewed in [1]). The AlkPase activity can be solubilized using ionic or non-ionic detergents [2], but attempts at purifying the enzyme(s) responsible for this activity have been unsuccessful [3]. Kinetic analyses of the AlkPase activity using a variety of substrates and inhibitors indicate that only a single enzyme is involved [1], but this hypothesis has been questioned [4]. Thus, in an attempt to clarify the actual number of enzymes responsible for the AlkPase activity, and to describe more fully the characteristics of this activity, the effects of additional inhibitors on the AlkPase activity have been examined.

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MATERIALS AND METHODS

The "OSU strain" [5] of *H. diminuta* was reared in beetles (*Tenebrio molitor*) and male Sprague-Dawley rats. Rats were infected with 30 cysticercoids and the tapeworms were recovered 12 days post-infection. Tapeworms from several rats were pooled and a brush border membrane fraction was prepared as described previously [3,6]. The protein content of this fraction was determined [7] using bovine serum albumin as a standard.

AlkPase activity was assayed at 37°C in 200 mM Tris buffer, pH 8.8, containing 5 mM $MgCl_2$. Hydrolysis of p-nitrophenyl phosphate (PNPP, Sigma 104 Phosphatase Substrate) was monitored continuously at 405 nm and specific activities (reaction velocities) were calculated in units of μmol PNPP hydrolyzed · mg^{-1} protein · min^{-1} using a millimolar extinction coefficient of 17.5 [8]. Hydrolysis rates of β-glycerophosphate (BGP, Sigma) and 5'-adenosine monophosphate (AMP, Sigma) were determined using 30 and 10 min assay periods, respectively. These assays were terminated and the amount of inorganic phosphate liberated was quantified by the addition of a Malachite Green-ammonium molybdate reagent and measuring the absorbance at 660 nm [3,9].

Ammonium molybdate, sodium arsenate, sodium arsenite, D- and L-phenylalanine, L-homoarginine, $HgCl_2$, ethylenediaminetetraacetate (EDTA) and levamisole (Levisole, Pitman-Moore) were dissolved directly in buffer. A stock solution of each inhibitor was prepared gravimetrically, and the pH was adjusted to 8.8 (when necessary) by the addition of HCl or NaOH.

Stock solutions (50 or 100 mM) of bunamidine (Burroughs Wellcome), clorsulon (Merck Sharp & Dohme), praziquantel (Mobay Corporation) and mebendazole (Pitman-Moore) were prepared gravimetrically in dimethyl sulfoxide (DMSO), while quinacrine HCl (Sterling-Winthrop Research Institute) was dissolved in distilled water. Ivermectin (Merck Sharp & Dohme) was dissolved in absolute methanol and its concentration was determined using a molar extinction coefficient of 30,100 at 245 nm (*The Merck Index*, 9th ed.). Each of these anthelmintics was tested for inhibitory activity by adding an aliquot of the stock solution to the assay buffer (<100 ul/ml assay buffer) and comparing the rate of PNPP hydrolysis with control assays containing an equal amount of DMSO, distilled water or methanol.

The effects of inhibitors on the hydrolysis of PNPP, BGP and AMP were determined initially using a Lineweaver-Burk (double reciprocal) plot, and the apparent inhibitor constant (K_i') for each inhibitor was calculated using an appropriate secondary plot. Unless noted otherwise, all lines were fitted by regression analyses and $r^2 > 0.98$.

RESULTS

Preliminary kinetic analyses indicated that BGP, arsenate, arsenite and molybdate were competitive inhibitors of the hydrolysis of PNPP by the isolated membrane preparation (Fig. 1).The effects of increasing concentrations of each of these inhibitors on the hydrolysis of a single concentration of PNPP were determined. When plotted as $(i)^{-1}$ versus $[I]^{-1}$, where i = fractional inhibition and [I] = inhibitor concentration, the data indicated that each of these inhibitors would inhibit PNPP hydrolysis 100% [e.g., $(i)^{-1} = 1$] at infinitely high inhibitor concentrations (Fig. 2).Similar analyses (data not shown) yielded identical results for the effects of molybdate on the hydrolysis of BGP

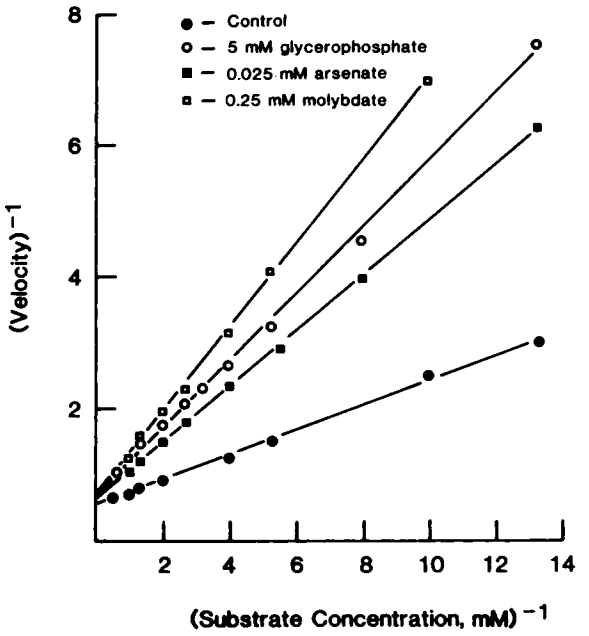

Fig. 1. Lineweaver-Burk plot for the effects of competitive inhibitors on the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean value of six replicate assays. Lines were constructed by use of a minimum of seven substrate concentrations, but some points, especially at higher substrate concentrations, were omitted for clarity. (The data for 30 mM arsenite as an inhibitor were not included because, on this graph, they could not be differentiated from the data for 0.025 mM arsenate as an inhibitor.)

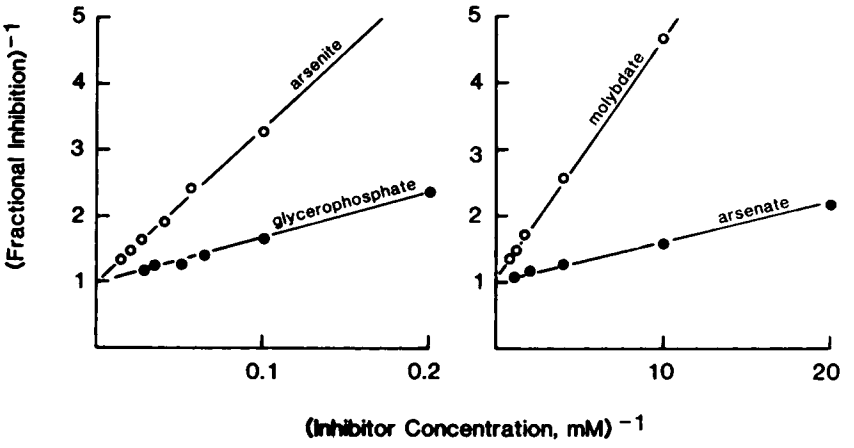

Fig. 2. Plots of the reciprocal of the fractional inhibition versus the reciprocal of the inhibitor concentration for competitive inhibitors of the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean of six replicate assays, and each line was constructed by use of a minimum of seven inhibitor concentrations. Some points, especially at higher inhibitor concentrations, were omitted for clarity. Substrate (PNPP) concentrations for arsenite, β-glycerophosphate, molybdate, and arsenate were 0.1 mM, 0.25 mM, 1 mM, and 1 mM, respectively. These secondary plots were used to calculate K_i' for these competitive inhibitors (Table I).

TABLE I. A Summary of Inhibitors, Substrates (PNPP Unless Indicated Otherwise), Types of Inhibition, and Apparent Inhibitor Constants (K_i') of the Alkaline Phosphatase (AlkPase) Activity Associated With the Isolated Brush Border Membrane of *Hymenolepis diminuta*

Inhibitor (substrate)	Type of inhibition b	K_i' (mM)
β-glycerophosphate	Competitive	3.8
Molybdate	Competitive	0.090
Molybdate (BGP)	Competitive	0.082
Molybdate (AMP)	Competitive	0.087
Arsenate	Competitive	0.014
Arsenite	Competitive	17.7
Levamisole	Uncompetitive	5.7
Levamisole (BGP)	Uncompetitive	6.0
Hg^{2+}	Unknown[a]	ND[a]
Quinacrine	Unknown[a]	ND[a]
Clorsulon	Mixed	3.1

[a]At low concentrations, these inhibitors appeared to behave as uncompetitive inhibitors, while at higher concentrations they appeared to have concentration-dependent secondary effects on the enzyme such that K_i' varied as a function of [I]. Since secondary plots deviated significantly from linearity, K_i' values were not determined (ND).

and AMP. A K_i' value for each competitive inhibitor was calculated from these data [8] (Table I).

Initial kinetic analyses indicated that levamisole (Fig. 3) was an uncompetitive inhibitor of the AlkPase activity, while clorsulon was a mixed inhibitor (Fig. 4). Lineweaver-Burk plots for the effects of increasing concentrations of quinacrine (Fig. 5) and Hg^{2+} (Fig. 6) on the hydrolysis of PNPP resulted in inconclusive data. At low

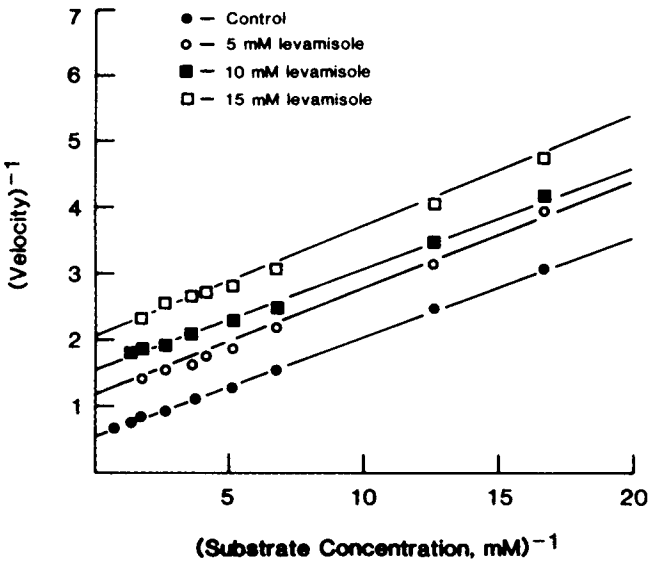

Fig. 3. Lineweaver-Burk plot for the effects of increasing concentrations of levamisole on the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean value of six replicate assays. The slopes of the individual lines were as follows: control = 0.0185; 5 mM levamisole = 0.0208; 10 mM levamisole = 0.0190; 15 mM levamisole = 0.0204.

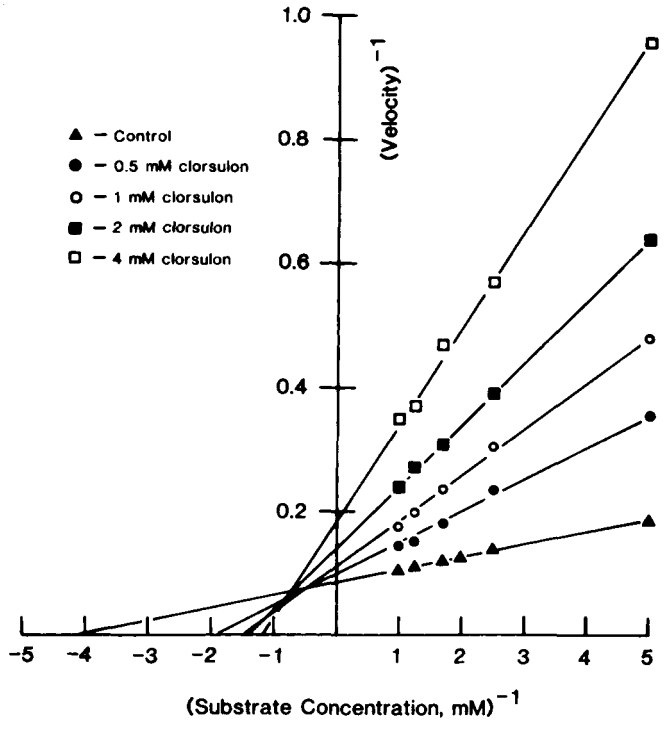

Fig. 4. Lineweaver-Burk plot for the effects of increasing concentrations of clorsulon on the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean value of three replicate assays.

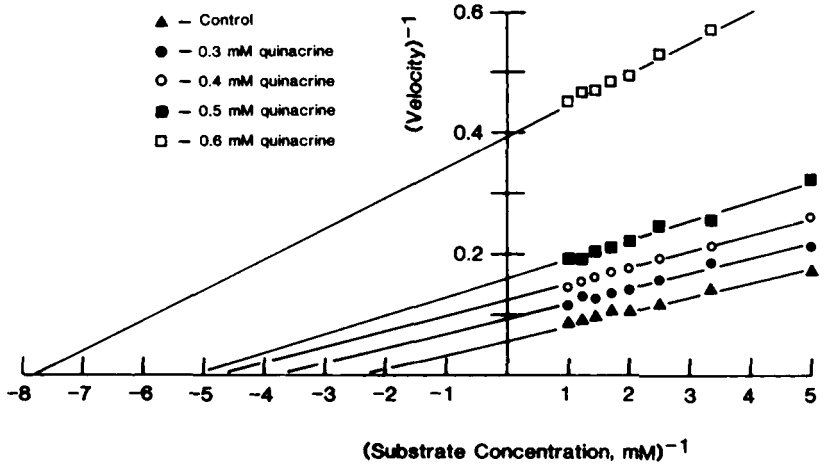

Fig. 5. Lineweaver-Burk plot for the effects of increasing concentrations of quinacrine on the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean of three replicate assays. The slopes of the individual lines were as follows: control = 0.0223; 0.3 mM quinacrine = 0.0247; 0.4 mM quinacrine = 0.0259; 0.5 mM quinacrine = 0.0307; 0.6 mM quinacrine = 0.0517.

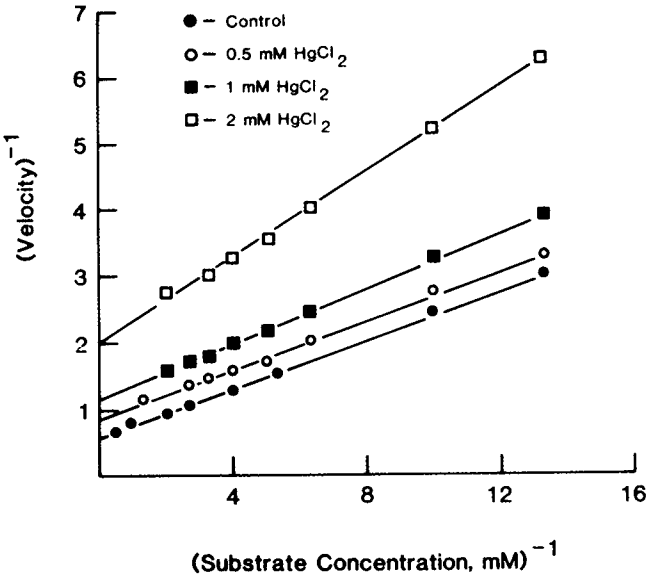

Fig. 6. Lineweaver-Burk plot for the effects of increasing concentrations of Hg^{2+} (as $HgCl_2$) on the hydrolysis of PNPP by the isolated brush border membrane of *Hymenolepis diminuta*. Each point represents the mean value of six replicate assays. The slopes of the individual lines were as follows: control = 0.0185; 0.5 mM Hg^{2+} = 0.0181; 1 mM Hg^{2+} = 0.0206; 2 mM Hg^{2+} = 0.0323.

concentrations, Hg^{2+} and quinacrine appeared to behave as uncompetitive inhibitors of the AlkPase activity. But, at higher inhibitor concentrations regression lines for control assays and assays containing inhibitor were clearly not parallel; as noted below, this concentration-dependent effect was substantiated when secondary plots were constructed.

Secondary plots of (maximum specific activity)$^{-1}$ versus [I] for levamisole and clorsulon resulted in straight lines, thus substantiating that levamisole and clorsulon were uncompetitive and mixed inhibitors, respectively (Fig. 7). Values of K_i' for levamisole and clorsulon were calculated from these secondary plots (Table I). Similar plots of the data for quinacrine and Hg^{2+} deviated from linearity (Fig. 7). Thus, inhibition of the AlkPase activity by these latter two inhibitors did not appear uncompetitive in nature and K_i' values were not calculated (Table I).

Inhibition of PNPP hydrolysis by EDTA was time- and concentration-dependent. AlkPase activity was inhibited 90% in 10 min by the addition of 10 mM EDTA, and the amount of inhibition increased to >99% following a 50 min incubation at 37°C. However, the AlkPase activity was inhibited only 18% in 10 min by the addition of 2.5 mM EDTA, and the amount of inhibition increased to >60% over the next 180 min of incubation at 37°C. The effects of 2.5 mM EDTA on the AlkPase were virtually identical using an intact membrane preparation, or a preparation solubilized in 0.1% SDS (sodium dodecyl sulfate) [2].

Hydrolysis of PNPP (0.1 mM) by the isolated membrane preparation was not inhibited by D- or L-phenylalanine (9 mM), L-homoarginine (10 mM), praziquantel (0.1 mM), mebendazole (0.1 mM), bunamidine (0.1 mM) or ivermectin (0.1 mM).

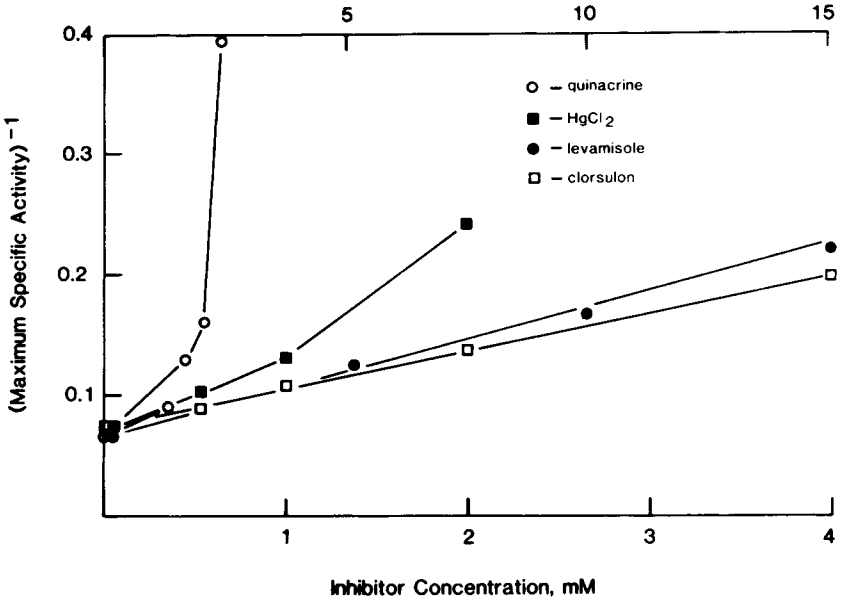

Fig. 7. A plot of (maximum specific activity)$^{-1}$ versus inhibitor concentration for Hg^{2+} (as $HgCl_2$), clorsulon, quinacrine (bottom abscissa), and levamisole (top abscissa) as inhibitors of PNPP hydrolysis by the isolated brush border membrane of *Hymenolepis diminuta*. The lines for quinacrine and Hg^{2+} were drawn by inspection. These secondary plots were used to calculate K_i' for levamisole and clorsulon (Table I).

(Due to the limited solubilities of these latter four compounds, they were not tested at higher concentrations.)

DISCUSSION

AcPase and AlkPase activities are demonstrable in the external brush border membrane of *H. diminuta* [10,11], and enzymatic analyses provide convincing evidence that these activities represent distinct enzymes [12]. The AcPase and AlkPase activities lack substrate specificity [3,8,12–14], and, although it is currently unknown whether the AcPase activity represents more than one enzyme, the data of the current and previous studies suggest strongly that the AlkPase activity is represented by a single enzyme with low substrate specificity. The fact that the hydrolysis of nucleotides, hexose mono- and diphosphates and PNPP is inhibited by identical compounds, and that each of these substrates is an inhibitor of the hydrolysis of the others [3,8,13,14], supports this conclusion. Further evidence is provided by the observations that nucleoside mono- and triphosphates, hexose mono- and diphosphates, BGP, and pyrophosphate are competitive inhibitors of the hydrolysis of PNPP, and that each of these substrates will potentially inhibit hydrolysis of PNPP 100% at high inhibitor to substrate ratios [3,8,13]. And lastly, the K_i' values for competitive and uncompetitive inhibitors (molybdate and levamisole, respectively) are virtually identical when PNPP, BGP or AMP is used as the substrate.

As demonstrated cytochemically, levamisole inhibits the hydrolysis of BGP by membrane-bound AlkPases of rats and guinea pigs, but it has no effect on the hydrolysis

of adenosine triphosphate, AMP, thiamine pyrophosphate or glucose-1-phosphate [15,16]. Thus, in these cases hydrolysis of BGP is believed to involve a non-specific AlkPase that is inhibited specifically by levamisole. On the other hand, levamisole inhibits the hydrolysis of BGP, PNPP and AMP by the brush border membrane of *H. diminuta*. Thus, these data do not support the assumption [4] that the brush border membrane of *H. diminuta* contains more than one AlkPase enzyme.

Levamisole is a potent uncompetitive inhibitor of AlkPases from various tissues of vertebrates, but varies in its affinity for enzymes from different tissues. For example, levamisole has a high affinity (K_i of about 1 μM) for AlkPases from canine liver, kidney, bone and placenta, while its affinity for canine intestinal AlkPase is significantly lower (K_i of about 1 mM). AlkPase of bacterial origin (*Escherichia coli*), on the other hand, is unaffected by this anthelmintic [17]. Levamisole also behaves as an uncompetitive inhibitor of the membrane-bound AlkPase activity of *H. diminuta*, but, compared to enzymes from vertebrate sources, the affinity of this anthelmintic for the tapeworm's enzyme is low (K_i = 5.7 mM).

Mercurials are potent inhibitors of many classes of enzymes, and may display characteristics of competitive, non-competitive, uncompetitive or mixed inhibitors [18]. At low concentrations, Hg^{2+} appears to inhibit the tapeworm's AlkPase activity in an uncompetitive fashion, while at higher concentrations the effects of Hg^{2+} are not consistent with those of an uncompetitive inhibitor. The reason for this disparity is not clear, but it likely represents some type of concentration-dependent secondary effect of Hg^{2+} on the enzyme. The nature of this effect is discussed in greater detail below.

Inhibition of the AlkPase activity by EDTA is both time- and concentration-dependent, and inhibition of bone, intestinal and placental AlkPases of vertebrates by EDTA displays similar characteristics [19,20]. It is assumed that inhibition of these enzymes is due to the removal of an essential cation [13,14,20], but the time-and concentration-dependent effects of EDTA on these enzymes do not allow one to determine whether EDTA binds to the cations and causes a conformational change in the enzymes, or whether EDTA removes the cations immediately, thereby resulting in an unstable enzyme which undergoes denaturation [20]. Regardless of the mechanism involved, the membrane-bound and SDS-solubilized AlkPase activities of *H. diminuta* are affected in a similar manner, and this observation seemingly rules out the possibility that the time-dependent effect is due to the fact that the cations are not accessible to the inhibitor.

Clorsulon and quinacrine inhibit the AlkPase activity, but the mechanisms by which these inhibitors affect the activity differ. Kinetic data demonstrate that clorsulon behaves as a mixed inhibitor of the membrane-bound AlkPase of *H. diminuta*, but these data indicate little more than the binding of substrate (PNPP) and inhibitor to the enzyme are not independent events. At low concentrations, quinacrine appears to inhibit the AlkPase activity in an uncompetitive fashion, but at higher concentrations the kinetics are not consistent with uncompetitive inhibition. Although the effects of quinacrine and Hg^{2+} on the AlkPase activity are similar superficially, these inhibitors probably affect the enzyme in quite different ways, as discussed below.

In the presence of an ideal uncompetitive inhibitor, the K_m and V_{max} of an enzyme decrease by an equal factor $[1 + ([I]/K_i)]$ as demonstrated by the identical slopes of lines (on Lineweaver-Burk plots) for control assays and assays in the presence of inhibitor (for example, see Fig. 3). Quinacrine and Hg^{2+} display such ideal behavior at low concentra-

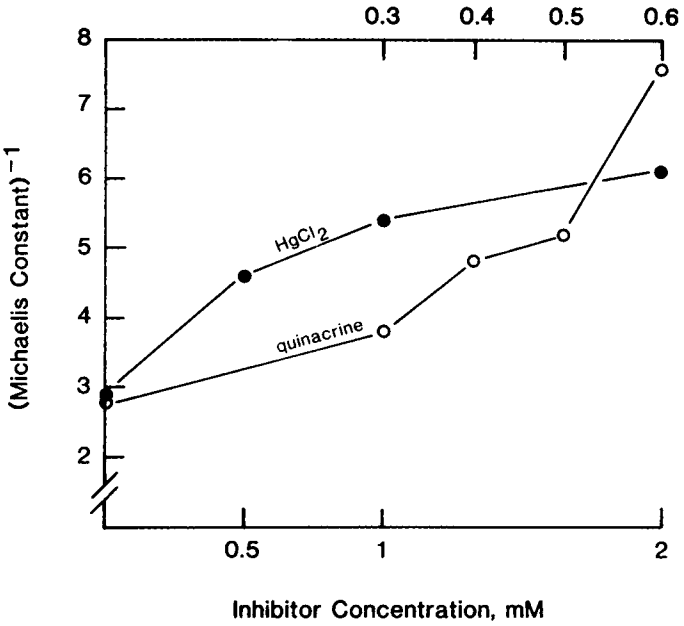

Fig. 8. A plot of (Michaelis constant)$^{-1}$ versus inhibitor concentration for Hg^{2+} (bottom abscissa) and quinacrine (top abscissa) as inhibitors of the AlkPase activity of the isolated brush border membrane of *Hymenolepis diminuta*. The lines were drawn by inspection.

tions. However, at higher concentrations both inhibitors display kinetics which are inconsistent with uncompetitive inhibition. In one respect, Hg^{2+} and quinacrine have a similar effect on the AlkPase activity; they both have a greater than expected effect on the V_{max} (maximum specific activity) of the AlkPase activity as demonstrated by the fact that secondary plots of (maximum specific activity)$^{-1}$ versus [I] for these inhibitors are similar in shape and not linear. However, the effects of these inhibitors on the K_m of the enzyme are quite different, as is clear when the relationship of $(K_m)^{-1}$ versus [I] is plotted (Fig. 8). Although these data do not permit one to identify the mechanisms by which quinacrine and Hg^{2+} inhibit the AlkPase activityof *H. diminuta,* they indicate (1) that the K_i' of each inhibitor in concentration-dependent and (2) that the mechanisms by which these inhibitors inhibit AlkPase activity are likely different.

Levamisole, quinacrine and clorsulon are effective anthelmintics for the treatment of cestode infections, but it is unknown whether the pharmacological effects of these drugs are due to their effects on the tapeworm's membrane-bound enzymes. On the one hand, the low affinities of these drugs for the AlkPase activity suggests that inhibition of the AlkPase activity is not their primary mode of action. On the other hand, the effects of these anthelmintics on the membrane-bound AlkPase activity of *intact* (i.e., living) tapeworms may be quite different than their effects on the isolated membrane preparation used in this study. Regardless of the pharmacological effects of these drugs, one of them (levamisole) has already been shown to useful in cytochemical studies [15,16] and in comparing the AlkPases from disparate groups of animals. It would be interesting to know if clorsulon and quinacrine might find similar uses.

L-homoarginine inhibits specifically the AlkPases from vertebrate bone and liver [21,22], while L-phenylalanine inhibits specifically the AlkPases from vertebrate intestine and placenta [22,23]. Neither of these amino acids inhibits the membrane-bound AlkPase of *H. diminuta*. Thus, although the AlkPases of vertebrates and the brush border membrane of *H. diminuta* share some characteristics, as noted above, each of these enzymes in unique unto itself.

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