ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1996, p. 2781–2784 Vol. 40, No. 12
0066-4804/96/$04.00+0


# *Cryptosporidium parvum* Metalloaminopeptidase Inhibitors Prevent In Vitro Excystation

PABLO C. OKHUYSEN,[1]* CYNTHIA L. CHAPPELL,[1] CHARLES KETTNER,[2] AND CHARLES R. STERLING[3]

*Division of Infectious Diseases, Department of Internal Medicine, Medical School and Center for Infectious Diseases, School of Public Health, The University of Texas Health Science Center, Houston, Texas 77030[1]; DuPont Merck Pharmaceutical Co., Wilmington, Delaware 19880[2]; and Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721[3]*

Received 26 April 1996/Returned for modification 22 August 1996/Accepted 30 September 1996

***Cryptosporidium parvum* arginine aminopeptidase (RAP) was studied during *in vitro* excystation. Specific RAP inhibitors were identified by using *C. parvum* extracts. Amastatin, a series of α-aminoboronic acids, and the chelating agents EDTA and 1,10-phenanthrolene, but not endoproteinase inhibitors, blocked enzymatic activity. RAP inhibitors found to be effective in soluble enzymatic assays were then studied for their effect on in vitro excystation. 1,10-Phenanthrolene, amastatin, and H-boronorleucine (pinacol) inhibited excystation by 84, 57, and 61%, respectively, compared with solvent-treated control oocysts. Sporozoites remained viable within the oocyst as determined by propidium iodide and fluorescein diacetate dye uptake, suggesting that α-aminoboronic acids were not directly lethal to the parasite.**

*Cryptosporidium parvum*, an intestinal protozoan parasite, can cause diarrhea in human immunodeficiency virus-infected patients (21), children in day care centers (2), and travelers to certain regions of the world (18, 26). *C. parvum* is also responsible for sporadic outbreaks of diarrhea worldwide (6, 20).

In healthy hosts, small numbers of *C. parvum* organisms may cause asymptomatic infection or produce a self-limited diarrheal illness (9). However, in the advanced stages of human immunodeficiency virus infection, *C. parvum* contributes significantly to morbidity (14) and mortality (13). Attempts to treat cryptosporidiosis in human immunodeficiency virus-infected hosts has resulted in failure despite the use of a wide variety of compounds. In AIDS patients with cryptosporidiosis, paromomycin has a parasitologic effect and has been shown to decrease the number of oocysts shed but is ineffective in eradicating infection (27). Agents effective in the treatment of cryptosporidiosis are clearly needed. *C. parvum* proteolytic enzymes essential for parasite survival may provide novel targets for treatment.

During the initial phase of infection, *C. parvum* oocysts undergo excystation in the intestinal lumen, releasing sporozoites that infect the enterocytes of many mammals, including humans (7). After asexual reproduction in a unique intracellular extracytoplasmic vacuole, *C. parvum* meronts are released from the intestinal epithelium, and infection in surrounding enterocytes is established. Meronts may further differentiate and undergo sexual reproduction, yielding new oocysts. Oocysts released into the gut lumen sporulate within the host to become fully infectious and may excyst prior to being excreted into the environment. This process results in autoinfection of the host and may contribute to chronic infection in AIDS patients (11). Since excystation is necessary for initial infection and subsequent amplification, mechanisms that interfere with this process in the intestinal lumen may be of use in the treatment of cryptosporidiosis.

The biological steps involved in *C. parvum* excystation are incompletely understood but require specific environmental conditions, including exposure to reducing agents and specific temperature, time, and pH conditions (10). These criteria suggest that proteolytic enzymes may be involved in the excystation process. These excystation conditions are also important in closely related coccidians, such as *Eimeria* spp. Further, excystation of *Eimeria* oocysts appears to be mediated through metal-binding proteins (8) since 1,10-phenanthrolene and other chelating agents interfere with this process. These agents are also effective aminopeptidase inhibitors, suggesting the possibility of an enzyme-mediated process. In addition to their potential role in excystation in *Eimeria* spp., parasite proteases are important in infection (1, 12). Others have noted that during the initial phases of *C. parvum* excystation, ultrastructural changes occur first within the oocysts (22) and have postulated that specific environmental conditions may trigger enzymes inside the oocyst to initiate excystation (28). A search for endoproteinase activity during *C. parvum* excystation was unsuccessful (28). However, other enzyme classes, such as aminopeptidases, were not addressed.

An arginine aminopeptidase (RAP) of *C. parvum* sporozoites has recently been described. *C. parvum* RAP preferentially cleaves synthetic substrates with arginine or alanine in the amino terminus. This enzyme is present on the surface membrane of freshly excysted sporozoites but not on the exterior of oocyst walls (22). The purpose of this study was to test a series of new synthetic compounds for the ability to inhibit *C. parvum* RAP activity and to examine the effect of RAP inhibition during the in vitro excystation process.

## MATERIALS AND METHODS

**Parasite preparations.** *C. parvum* oocysts (Iowa isolate, originally collected by Harley Moon, Ames, Iowa) were serially passed in neonatal calves and purified as previously described (4). Purified oocysts were stored for 1 to 4 months in 2.5% potassium dichromate at 5°C. Prior to use in each experiment, oocysts used were sequentially washed in 10 mM phosphate-buffered saline to remove dichromate and were counted on a hemacytometer.

In vitro excystation was induced at 5°C with 1.25% sodium hypochlorite for 7

* Corresponding author. Mailing address: Division of Infectious Diseases, The University of Texas Medical School and School of Public Health, JFB 1.728, 6431 Fannin, Houston, TX 77030. Phone: (713) 792-4929. Fax: (713) 792-4937. Electronic mail address: Okhuysen @heart.med.uth.tmc.edu.

min (in the absence of trypsin and bile salts), followed by extensive washing in phosphate-buffered saline and resuspension in Hanks balanced salt solution as previously described (23, 24). Aliquots of $10^5$ oocysts were then placed in microtiter wells (Corning Inc., Corning, N.Y.) previously coated with 2% bovine serum albumin. Excystation was then allowed to proceed by placing plates in a humidified incubator at 37°C and shaking them for 90 min.

Excystation inhibition studies were performed as described above, except that immediately after hypochlorite treatment at 4°C and washing with phosphate-buffered saline, oocysts were placed in microtiter wells with or without inhibitors and preincubated at 5°C for 2 h. After preincubation, oocysts were washed and transferred to microtiter plates at 37°C, and excystation proceeded as already described. At various points during excystation, an aliquot of the mixture was removed and placed on a hemacytometer, and 50 fields were visualized under phase-contrast microscopy. The excystation rate was determined by dividing the number of empty oocysts by the total number of empty and full oocysts multiplied by 100. A minimum of 100 oocysts were counted per well. To evaluate sporozoite viability, preparations were stained with propidium iodide and fluorescein diacetate and visualized as previously described (3). The number of viable sporozoites staining fluorescent green (viable) and those staining red (nonviable) that remained within oocysts were counted, and the percentage of unexcysted oocysts was obtained; a minimum of 100 oocysts were examined. Each inhibitor was tested in triplicate. We investigated the ratio of permeabilized oocysts containing viable sporozoites to nonviable empty oocysts without sporozoites to ensure that the inhibition of excystation was occurring in live oocysts.

*C. parvum* extracts for soluble aminopeptidase assays were prepared by freeze-thawing $10^7$ oocysts in 10 mM phosphate-buffered saline containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma Chemical Co., St. Louis, Mo.). The lysate was then centrifuged at 100,000 × *g* for 1 h at 5°C, and 10-μl aliquots (containing the extracts from approximately $10^5$ oocysts) of the resulting supernatant were used to measure aminopeptidase activity.

**Assay for aminopeptidase activity.** RAP activity was detected by adding aliquots of $10^5$ *C. parvum* oocysts or extracts to 10 mM phosphate-buffered saline, pH 7.2, in a total assay volume of 500 μl. The assay mixture containing the synthetic substrate arginine 7-amino-4-trifluoromethylcoumarin (AFC; Enzyme Systems Products, Inc., Livermore, Calif.) at a final concentration of 20 μM was incubated at 37°C for 15 min. Control tubes contained all of the reagents except oocysts or their extracts. All assays were done in triplicate, and enzymatic activity was read with an FL-750 fluorometer (McPhearson Instruments, Acton, Mass.) as previously described (22). Fluorescence units of unknowns were compared with units generated by using known amounts of the AFC leaving group, and the activity was converted to nanomoles of AFC released per hour per milliliter of test solution. For aminopeptidase assays performed during excystation, aliquots of oocysts were removed from excystation medium at 15-min intervals and kept on ice until assayed. All samples were assayed simultaneously.

*C. parvum* RAP inhibition studies were performed with extracts by incubating extracts from $10^5$ oocysts with the following inhibitors at the indicated final assay concentrations (Sigma Chemical Co.): bestatin (10 μM), amastatin (10 μM), phenylmethylsulfonyl fluoride (1 mM), tosyllysyl chloromethyl ketone (TLCK; 100 mM), tosylphenylalanine chloromethyl ketone (TPCK; 100 mM), 1,10-phenanthrolene (1 mM), EDTA (1 mM), leupeptin (1 mM), and *trans*-epoxysuccinyl-1-leucylamido(4-guanidino) butane (10 mM). A number of synthetic α-aminoboronic acid inhibitors (The DuPont/Merck Pharmaceutical Co., Wilmington, Del.) were also tested, including H-boroproline, $NH_2CH(CH_2)_3ClBO_2$, $NH_2CH(CH_2)_3BrBO_2$, H-boroalanine, H-boroleucine, H-borovaline, H-boronorleucine, and H-borophenylalanine, all at a final concentration of 1 nM. Control tubes containing the enzyme with no inhibitors (positive control) but with appropriate solvents (distilled water, 0.05% methanol, or 0.05% dimethyl sulfoxide [DMSO]) and blank tubes containing the substrate alone (negative control) were included in the assays. After incubation for 20 min at 37°C, the fluorescent substrate Arg-AFC (20 μM final concentration) was added and tubes were incubated for an additional 1 h at 37°C. Aminopeptidase activity was determined as described above. The inhibitory activity associated with the solvent alone was deducted from the activity in the presence of the inhibitor. The data are expressed as excystation rates and as the percentage of inhibition observed in the test wells compared with controls.

**Statistical methods.** Differences among excystation rates were compared by analysis of variance. Pairwise comparisons between controls and inhibitors were performed by the Tukey-Kramer test, and $P < 0.05$ was considered significant.

## RESULTS

As an initial step, we investigated the RAP activity of viable *C. parvum* oocysts during the excystation process. RAP activity was undetectable during the initial 15 min of excystation. After this latency phase, a rapid and progressive increase of RAP activity was detected (Fig. 1). In this assay, a minimum of approximately $1.5 \times 10^4$ oocysts were required to have undergone excystation for detection of aminopeptidase activity. After 90 min of incubation, the excystation rate was associated with the highest RAP activity. The number of oocysts containing viable sporozoites as determined by propidium iodide and fluorescein diacetate decreased as excystation progressed (Fig. 1).

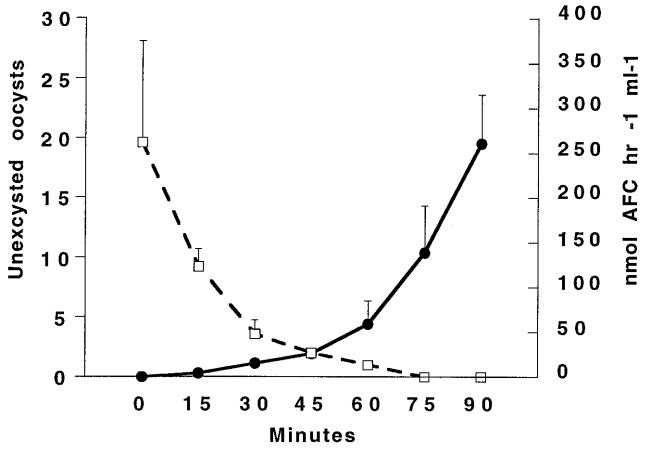


FIG. 1. RAP activity measured during *C. parvum* excystation. Approximately $10^5$ oocysts were used per well; each datum point represents the mean aminopeptidase activity from three experiments, each done in triplicate ($n = 9$). Error bars indicate 1 standard deviation above the mean. Time zero indicates when treated oocysts were transferred from 4°C to 37°C. The number of unexcysted oocysts (□) seen in 50 fields as determined by propidium iodide and fluorescein diacetate staining and RAP activity (●) over the 90-min experiment are shown. Units of measurement are as described in Materials and Methods.

A series of inhibitors were screened for the ability to inhibit RAP activity by using disrupted oocyst extracts. The aminopeptidase-specific inhibitors, amastatin, and the chelating agents EDTA and 1,10-phenanthrolene were effective RAP inhibitors (i.e., they demonstrated >50% inhibition compared with control tubes without the inhibitor), while endopeptidase inhibitors demonstrated little or no activity (Table 1). The α-aminoboronic acids screened demonstrated significant inhibition, except for H-boroproline, which inhibited only 36% of the RAP activity, and the cyclic derivative of H-boroproline $NH_2CH(CH_2)_3ClBO_2$ (5), which also had little effect. While the conventional aminopeptidase inhibitors demonstrated inhibitory activity in the micromolar range, the α-aminoboronic acids tested were effective in the nanomolar range.

To further elucidate the function of RAP, we investigated the ability of RAP inhibitors to arrest excystation in a microwell assay. RAP inhibitors were solubilized in methanol, DMSO, or distilled water. The excystation rates observed in control wells containing solvents alone were 63.4% ± 25.0% for methanol and 49.6% ± 7.1% for DMSO, which were comparable to the excystation rate seen in wells containing Hanks balanced salt solution alone (61.4% ± 14.0%; $P > 0.5$). After 2 h of preincubation with the inhibitors, a significant ($P < 0.05$) decrease in excystation was observed in the wells containing 1,10-phenanthrolene, amastatin, and the α-aminoboronic acid inhibitor H-boronorleucine (19 versus 49.6%; $P < 0.05$) (Table 2) compared with the respective controls. None of the other α-aminoboronic acids tested showed a statistically significant decrease in excystation. In the wells containing RAP inhibitors, free sporozoites remained viable but demonstrated decreased motility. The inhibition of excystation seen with 2 h of preincubation was not observed when the time of exposure to the inhibitors was reduced to 5 min (data not shown).

A dose-dependent effect on excystation was observed for H-boronorleucine (Fig. 2). In this experiment, the excystation rate in the absence of inhibitors was 58.2%. No significant

TABLE 1. Effect of protease inhibitors on aminopeptidase activity from disrupted *C. parvum* oocysts[a]

| Compound | Final assay concn | Mean enzymatic activity ± SD | % Inhibition |
|---|---|---|---|
| Aminopeptidase inhibitors | | | |
| Amastatin | 10 μM | 5.3 ± 2 | 93 |
| Methanol control | | 74.3 ± 68 | |
| Bestatin | 10 μM | 75.0 ± 23 | 29 |
| Methanol control | | 98.6 ± 29 | |
| 1,10-Phenanthrolene | 1 mM | 2.3 ± 4.5 | 95 |
| Methanol control | | 48.2 ± 81 | |
| EDTA | 1 mM | 22.4 ± 4.4 | 67 |
| Water control | | 67.5 ± 42 | |
| Endopeptidase inhibitors | | | |
| PMSF[b] | 1 mM | 20.4 ± 79 | 18 |
| Methanol control | | 24.8 ± 2.6 | |
| TPCK | 100 mM | 33.4 ± 79 | 0 |
| Methanol control | | 24.8 ± 45 | |
| TLCK | 100 mM | 19.5 ± 10 | 21 |
| Methanol control | | 27.1 ± 30 | |
| Leupeptin | 1 mM | 19.0 ± 10 | 28 |
| Methanol control | | 27.4 ± 13 | |
| E-64[c] | 10 mM | 39.8 ± 26 | 0 |
| Methanol control | | 27.3 ± 10 | |
| Experimental compounds | | | |
| H-boroalanine | 1 nM | 5.3 ± 0.78 | 88 |
| H-boronorleucine | 1 nM | 1.9 ± 1.6 | 96 |
| H-borovaline | 1 nM | 8.3 ± 2.6 | 81 |
| H-boroleucine | 1 nM | 9.8 ± 0 | 78 |
| H-borophenylalanine | 1 nM | 9.0 ± 0.6 | 79 |
| $NH_2CH(CH_2)_3ClBO_2$ | 1 nM | 22.5 ± 9 | 49 |
| H-boroproline | 1 nM | 28.4 ± 14 | 36 |
| $NH_2CH(CH_2)_3BrBO_2$ | 1 nM | 9.8 ± 0.10 | 78 |
| DMSO control | | 44.2 ± 4 | |

[a] Results shown are from a representative experiment carried out in duplicate on at least three occasions. Enzymatic activity is expressed as nanomoles of AFC released per hour per microliter for aliquots containing extracts from $10^5$ oocysts.
[b] PMSF, phenylmethylsulfonyl fluoride.
[c] E-64, *trans*-epoxysuccinyl-l-leucylamido(4-guanidino) butane.

TABLE 2. Percent inhibition of *C. parvum* excystation after exposure to aminopeptidase-specific inhibitors and chelating agents[a]

| Compound | Concn (μM) | Excystation rate (mean ± SD)(%) | % Inhibition |
|---|---|---|---|
| Hanks balanced salt solution control | | 61.4 ± 14.0 | 0 |
| 1,10-Phenanthrolene | 100 | 10.0 ± 7.3 | 84[b] |
| Methanol control | | 63.4 ± 25.2 | 0 |
| Amastatin | 100 | 27.0 ± 13.6 | 57[c] |
| DMSO control | | 49.6 ± 7.1 | 0 |
| H-boronorleucine | 10 | 19.0 ± 6.9 | 61[c] |
| H-boroalanine | 10 | 29.1 ± 24.0 | 41 |
| H-borophenylalanine | 10 | 22.0 ± 9.5 | 55 |
| H-boroleucine | 10 | 34.0 ± 11.0 | 31 |
| H-boroproline | 10 | 48.0 ± 18.1 | 3 |

[a] Inhibition of excystation for each of the aminopeptidase inhibitors was compared to control wells without the aminopeptidase inhibitor. Results represent mean excystation rates from three experiments, each done in duplicate.
[b] $P < 0.01$ by analysis of variance.
[c] $P < 0.05$ by analysis of variance.

differences in excystation rates were observed in oocysts preincubated with 1 μM H-boronorleucine compared with control oocysts (56.9% ± 13% versus 58.2% ± 13%). However, the excystation rates decreased with increasing concentrations of the inhibitor. A 36% reduction in excystation was seen with 10 μM inhibitor (37.1% ± 16% versus 58.2% ± 13%; $P < 0.05$), and a 60% reduction was observed with 100 μM H-boronorleucine (23.6% ± 12% versus 58.2% ± 13%; $P < 0.01$). Wells in which decreases in excystation rates were observed also had a higher number of oocysts containing unreleased sporozoites as determined by fluorescein diacetate-propidium iodide staining (Fig. 2).

## DISCUSSION

In prior studies, *C. parvum* RAP activity was associated with the membrane of the sporozoite after excystation but not with intact oocysts (22), indicating a lack of RAP on oocyst walls. This observation prompted us to investigate if aminopeptidase activity is present and plays a role during the excystation process.

A series of specific aminopeptidase inhibitors were identified in soluble assays using crude parasite extracts. The inhibitory profile observed was characteristic of an enzyme belonging to the metalloaminopeptidase class. In this study, amastatin, an inhibitor of alanine and leucine aminopeptidases, was an effective inhibitor of *C. parvum* aminopeptidase, while bestatin, an inhibitor of soluble RAPs, demonstrated only partial RAP inhibition. This apparent preferential inhibition yielded by amastatin suggests that *C. parvum* aminopeptidase is an alanine-arginine aminopeptidase. It is unlikely that the aminopeptidase activity observed in our study is due to a cathepsin H-like enzyme, since no cleavage of conventional cathepsin H substrates (19) was observed (data not shown) and *trans*-epoxysuccinyl-l-leucylamido(4-guanidino) butane was noninhibitory.

In addition to the conventional inhibitors, several experimental α-aminoboronic acid inhibitors were also found to be effective inhibitors of *C. parvum* RAP. Agents belonging to this class are potent reversible inhibitors (in the nanomolar and

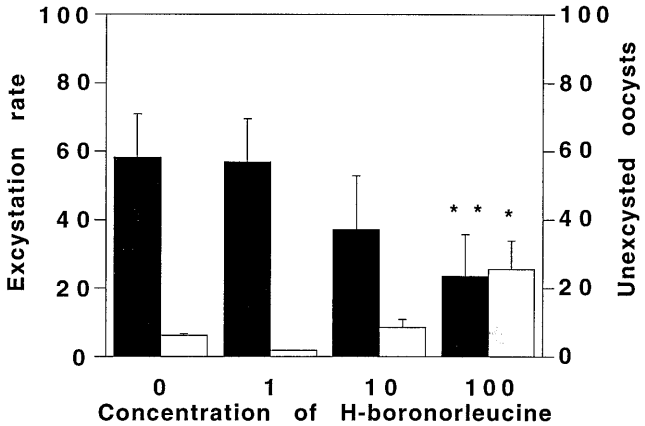


FIG. 2. Inhibition of *C. parvum* excystation by various concentrations of the α-aminoboronic acid H-boronorleucine. Results are the mean values from three experiments, each performed in triplicate as described in Materials and Methods. The final inhibitor concentration (micromolar) in the assay is shown. Dark columns represent the excystation rate, and light columns represent the number of unexcysted oocysts seen in 50 fields. The error bars indicate standard deviations. *, $P < 0.05$; **, $P < 0.01$ (compared to control wells with no inhibitor as determined by analysis of variance with the Tukey-Kramer test for comparisons between groups).

micromolar ranges) of a variety of microsomal and cytosolic aminopeptidases (25). The use of α-aminoboronic acid inhibitors as a pharmacologic adjuvant to the nasal delivery of peptide drugs has been investigated. In these studies, no adverse effects were observed in animal models when the compounds were used in this manner (16, 17).

The reduction in *Cryptosporidium* excystation observed in our study with the use of 1,10-phenanthrolene is in agreement with prior reports regarding the ability of this and other chelating agents to block the excystation of oocysts of *Eimeria tenella*, a closely related coccidian parasite (8). Taken together, these observations suggest that *E. tenella*, *C. parvum*, and perhaps other coccidians require zinc-binding aminopeptidases to complete excystation. Since a brief incubation with inhibitors was ineffective in preventing excystation, it is likely that inhibitors were required to penetrate hypochlorite-permeabilized oocysts rather than interacting with sporozoites as they exited. While nanomolar concentrations of α-aminoboronic acid inhibitors were effective in inhibiting aminopeptidase activity in *C. parvum* extracts, significantly higher concentrations (>10 μM) were required to demonstrate significant impact on excystation. This is in agreement with prior observations in which H-borophenylalanine inhibited the aminopeptidase of the filariid parasite *Brugia pahangi* at nanomolar concentrations in solution but was effective in arresting larval molting at micromolar concentrations (15).

Although the increase in RAP activity closely paralleled excystation, this fact alone does not establish causality. However, the decrease in excystation observed with 1,10-phenanthrolene, amastatin, and H-boronorleucine suggests that a metalloaminopeptidase, such as RAP, is important at some point in *C. parvum* excystation. Further studies are needed to determined if these inhibitors of *C. parvum* RAP can be effective in decreasing the infectivity of the parasite.

## ACKNOWLEDGMENTS

This work was supported by General Clinical Research Center (GCRC) NIH MCAP supplemental award M01-RR-02558. The research described here has been funded in part by the U.S. Environmental Protection Agency under assistance agreement CR-81984.

We thank Marilyn Marshall for technical assistance.

## REFERENCES

1. **Adams, J., and G. Bushell.** 1988. The effect of protease inhibitors on *Eimeria vermiformis* invasion of cultured cells. Int. J. Parasitol. **18:**683–685.
2. **Alpert, G., L. M. Bell, C. E. Kirkpatrick, L. D. Budnick, J. M. Campos, H. M. Friedman, and S. A. Poltkin.** 1984. Cryptosporidiosis in a day care center. N. Engl. J. Med. **311:**860–861.
3. **Arrowood, M. J., J. M. Jaynes, and M. C. Healey.** 1991. In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*. Antimicrob. Agents Chemother. **95:**224–227.
4. **Arrowood, M. J., and C. R. Sterling.** 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J. Parasitol. **73:**314–319.
5. **Bachovchin, W. W., A. G. Plaut, G. R. Flentke, M. Lynch, and C. A. Kettner.** 1990. Inhibition of IgA1 proteinases from *Neisseria gonorrhoeae* and *Haemophilus influenzae* by peptide prolyl boronic acids. J. Biol. Chem. **265:**3738–3743.
6. **Casemore, D. P., E. G. Jessop, D. Douce, and F. B. Jackson.** 1986. *Cryptosporidium* plus *Campylobacter*, an outbreak in a semi-rural population. Trans. R. Soc. Trop. Med. Hyg. **96:**95–105.
7. **Current, W.** 1986. *Cryptosporidium*: its biology and potential for environmental transmission. Crit. Rev. Environ. Control **17:**21–51.
8. **Dulski, P.** 1990. *Eimeria tenella* incomplete excystation in the presence of EDTA in a taurodeoxycholate-based medium. J. Protozool. **37:**524–528.
9. **DuPont, H., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose, and W. Jakubowski.** 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. **332:**855–859.
10. **Fayer, R., and R. G. Leek.** 1984. The effects of reducing conditions, medium, pH, temperature, and time on in vitro excystation of *Cryptosporidium*. J. Protozool. **31:**567–569.
11. **Fayer, R., C. A. Speer, and J. P. Dubey.** 1990. General biology of *Cryptosporidium*, p. 1–29. *In* J. P. Dubey, C. A. Speer, and R. Fayer (ed.), Cryptosporidiosis of man and animals. CRC Press, Inc., Boca Raton, Fla.
12. **Fuller, A. L., and L. R. McDougald.** 1990. Reduction in cell entry of *Eimeria tenella* (coccidia) sporozoites by protease inhibitors, and partial characterization of proteolytic activity associated with intact sporozoites and merozoites. J. Parasitol. **76:**464–467.
13. **Goldstein, S. T., D. D. Juraneck, O. Ravenholt, A. W. Hightower, D. G. Martin, J. L. Mesnick, S. D. Griffiths, A. J. Bryant, R. R. Reich, and B. L. Herwaldt.** 1996. Cryptosporidiosis: an outbreak associated with drinking water despite state-of-the-art water treatment. Ann. Intern. Med. **124:**459–468.
14. **Goodgame, R. W., K. Kimball, C. N. Ou, A. C. White, R. M. Genta, C. H. Lifschitz, and C. L. Chappell.** 1995. Intestinal function and injury in acquired immunodeficiency syndrome-related cryptosporidiosis. Gastroenterology **108:**1075–1082.
15. **Hong, X., J. Bouvier, M. M. Wong, G. Y. Yamagata, and J. H. McKerrow.** 1993. *Brugia pahangi*: identification and characterization of an aminopeptidase associated with larval molting. Exp. Parasitol. **76:**127–133.
16. **Hussain, M. R., C. Koval, A. Shevni, and B. Aungst.** 1990. Recovery of rat nasal mucosa from the effects of aminopeptidase inhibitors. J. Pharm. Sci. **79:**398–400.
17. **Hussain, M. R., A. Shenvi, S. Rowe, and E. Shefter.** 1989. The use of alpha-aminoboronic acid derivatives to stabilize peptide drugs during their intranasal absorption. Pharm. Res. **6:**186–189.
18. **Jokipii, L., S. S. Pohjola, and A. M. Jokipii.** 1985. Cryptosporidiosis associated with traveling and giardiasis. Gastroenterology **89:**838–842.
19. **Kirschke, H., J. Langner, B. Wiederanders, S. Ansorge, P. Bohley, and H. Hanson.** 1977. Cathepsin H: an endoaminopeptidase from rat liver lysosomes. Acta Biol. Med. Ger. **36**(2)**:**185–199.
20. **Mac Kenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, and J. Davis.** 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. **331:**161–167.
21. **Navin, T. R., and A. M. Hardy.** 1987. Cryptosporidiosis in patients with AIDS. J. Infect. Dis. **155:**150–155.
22. **Okhuysen, P. C., C. L. Chappell, H. L. DuPont, and C. R. Sterling.** 1994. Arginine aminopeptidase, an integral membrane protein of the *Cryptosporidium parvum* sporozoite. Infect. Immun. **62:**4667–4670.
23. **Reducker, D. W., and C. A. Speer.** 1985. Factors influencing excystation in *Cryptosporidium* oocysts from cattle. J. Parasitol. **71:**112–115.
24. **Riggs, M. E., and L. E. Perryman.** 1987. Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect. Immun. **55:**2081–2087.
25. **Shevni, A.** 1986. Alpha-aminoboronic acid derivatives: effective inhibitors of aminopeptidases. Biochemistry **25:**1286–1291.
26. **Soave, R., and P. Ma.** 1985. Cryptosporidiosis: travelers' diarrhea in two families. Arch. Intern. Med. **145:**70–72.
27. **White, A. C., C. L. Chappell, C. S. Hayat, K. T. Kimball, T. P. Flanigan, and R. W. Goodgame.** 1994. Paromomycin treatment for cryptosporidiosis in AIDS: a prospective, double blind trial. J. Infect. Dis. **170:**419–424.
28. **Woodmansee, D. B.** 1987. Studies of *in vitro* excystation of *Cryptosporidium parvum* from calves. J. Protozool. **34:**398–402.