The metalloprotease inhibitor 1,10-phenanthroline affects *Schistosoma mansoni* motor activity, egg laying and viability

T. A. DAY* *and* G.-Z. CHEN

Departments of Zoology and Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824, USA

(*Received 11 September 1997; revised 12 November 1997; accepted 12 November 1997*)

SUMMARY

The Zn^{2+}-chelating metalloprotease inhibitor 1,10-phenanthroline (phenanthroline, 5–150 μM) elicited dose-dependent contraction of the longitudinal and circular (transverse) musculature of adult male schistosomes. At the same concentrations, phenanthroline did not cause contraction of dispersed individual muscle fibres. The phenanthroline-induced contractions were reduced by the inclusion of 100 or 300 μM Zn^{2+} in the extracellular medium. Phenanthroline (0·5–150 μM) also inhibited the egg production of adult worm pairs *in vitro*, with a 98% reduction at 50 μM. When worm pairs were exposed to phenanthroline, the males detached from the dish and released the females, resulting in unpaired worms. At the higher concentrations (50 and 150 μM), the worms were killed *in vitro*. Worm burdens were reduced by over 50% in infected mice injected with phenanthroline (20 mg/kg/day for 4 days), but twice the dose resulted in only a 25% reduction. Phenanthroline injections also induced an hepatic shift and an unpairing of adult worms in infected mice, and the female worms appeared degenerate and lacked gut pigmentation. Mice fed a diet containing 0·3% phenanthroline received significant protection from infection when challenged with schistosome cercaria, where phenanthroline-fed mice had 94% fewer adult worms than control mice. The broad range of phenanthroline effects on schistosomes suggests broad and important functions for metalloproteases in these worms.

Key words: platyhelminth, trematode, *Schistosoma mansoni*, metalloprotease, protease, 1,10-phenanthroline.

INTRODUCTION

Proteases play a number of important roles in the biology of schistosomes (for reviews, see McKerrow & Doenhoff, 1988; McKerrow, 1989). Specific proteases have been identified with roles in egg hatching, tissue penetration, transformation, evasion of the host immune system and digestion, among others. Schistosome proteases encompass all of the basic mechanistic types (e.g. serine proteases, aspartic proteases, cysteine proteases and metalloproteases), and include both secreted and membrane-associated proteases.

The importance of proteases in the lives of these parasites led to the idea of protease inhibition as antischistosomal chemotherapy (McKerrow, 1989). For example, an elastinolytic serine protease is important in the process of host penetration (Lewert & Lee, 1956), and serine protease inhibitors hinder cercarial penetration (McKerrow & Doenhoff, 1988). Also, a number of proteases with a role in digestion of haemoglobin have been identified, including a cathepsin B-like cysteine protease responsible for the primary cleavage of ingested haemoglobin (Dresden & Deelder, 1979). Cysteine protease inhibitors interfere with haemoglobin digestion of worms *in vitro* and elicit a decrease in worm burden and liver pathogenesis in schistosome-infected mice (Wasilewski *et al*. 1996).

The only metalloprotease that has been identified from schistosomes is a leucine aminopeptidase, which is present in every lifestage (Xu & Dresden, 1986). The leucine aminopeptidase is prominently expressed in the eggs of schistosomes and appears to play a role in the initiation of the hatching process, since relatively large amounts of the protease are secreted from the egg immediately prior to hatching. Bestatin, which inhibits the protease, inhibits egg hatching (Xu & Dresden, 1986). In addition to bestatin, the leucine aminopeptidase is also inhibited by the Zn^{2+}-chelating metalloprotease inhibitor phenanthroline, an inhibition reversible by Zn^{2+} (Xu, Shawar & Dresden, 1990). A phenanthroline-sensitive aminopeptidase activity plays a role in cleaving host immune molecules from somules (Auriault *et al*. 1981; Cesari, Auriault & Capron, 1983), but it is not clear if this activity is attributable to the same leucine aminopeptidase. Here, the effects of phenanthroline are tested on schistosomes in 2 *in vitro* assays (muscle contraction and egg production) and 2 *in vivo* assays in mice (worm position in

* Corresponding author: Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824-1115, USA. Tel: +517 353 7146. Fax: +517 353 8915. E-mail: dayt@pilot.msu.edu

https://doi.org/10.1017/S0031182097002370 Published online by Cambridge University Press

established infections and host protection from infections upon cercarial challenge).

MATERIALS AND METHODS

Phenanthroline

For the *in vitro* assays, phenanthroline (Sigma Chemical Co., St Louis, MO) was dissolved in equal amounts of dimethylsulfoxide (Me_2SO) and distilled water to yield a stock solution of 10^{-1} M phenanthroline. All of the experimental concentrations of phenanthroline were obtained through dilution of this stock. All of the treatments were limited to a final Me_2SO concentration $\leqslant 0{\cdot}1\,\%$, which alone had no effect in these assays.

Muscle contraction

Adult schistosomes were recovered from the mesenteric and portal veins of mice 45–55 days after infection with a Puerto Rican strain of *S. mansoni*. The measurement of longitudinal muscle tension was performed using standard methods (Fetterer, Pax & Bennett, 1977). Briefly, after removal from the host, the adult worms were placed in RPMI–1640 (Gibco, Grand Island, NY) with 1 mg/ml gentamicin and 20 mM HEPES (pH 7·4) and maintained at 37 °C. Individual male worms were then attached, via suction, to 2 thin pipettes in a 1 ml polyethylene dish of the RPMI–1640 maintained at 37 °C. One pipette was fixed and 1 was flexible, and movement of the flexible electrode was transduced by a balance arm system to an optical sensor (Fetterer *et al*. 1978). To relax the worms and provide a more uniform baseline, 10 μM carbachol was added to the bath. Qualitatively similar results were obtained without carbachol but, since some worms were in different contractile states, the results were not as consistent. The length of the worm between the apparatus was then measured and a 1 mg load added. Worms were allowed to equilibrate in this state for 15 min, at which point the baseline was stable. The data shown are changes in tension ($\pm$S.E.M.) induced by addition of phenanthroline to the dish.

Circular muscle shortening was measured in a similar fashion (Pax, Siefker & Bennett, 1984). For these studies, male worms were cut into uniform transverse sections by a dual blade with a fixed 0·5 mm gap. These sections were placed in a polyethylene dish and attached to similar, although slightly smaller, pipette tips in a transverse orientation, perpendicular to the longitudinal axis of the worm. Due to the delicate nature of these thin segments, no load was placed on the segment beyond that supplied inherently by the apparatus. Therefore, only the length of the segment was measured and no attempt was made to calculate the tension change. This apparatus was calibrated daily by physically moving the flexible pipette a given distance, as determined visually with an ocular micrometer, and recording the change induced in the transduction system.

The contraction of individual muscle fibres was measured using methods that have been described previously (Day *et al*. 1994). Briefly, the muscle fibres are dispersed by a combination of enzymatic digestion (papain) and slight mechanical disruption in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St Louis, MO.). The dispersed muscle fibres are plated onto 35 mm plastic Petri dishes in an inorganic version of DMEM (I–DMEM) and stored at 20 °C until use, which was always within 5 h of dispersion. The plates were warmed to 34–36 °C 10 min before use and maintained at this temperature throughout the procedure.

The individual muscle fibre contraction assay was based on observation of responses of individual fibres microperfused with a test agent. The studies here were limited to the muscle type that we have previously described as 'frayed' fibres (Day *et al*. 1993). For each plate, 15–20 individual frayed fibres were microperfused with the test agent dissolved in the I–DMEM culture medium. The data shown are the mean percentages ($\pm$S.E.M.) of fibres that contracted in response to the perfusion, with each n being the percentage observed from the fibres tested in a single plate. Microperfusion of the culture medium alone elicited contraction of $10\pm6\,\%$ of the fibres ($n = 14$), while $86\pm4\,\%$ of the fibres ($n = 14$) contracted in response to 100 nM of the myoactive turbellarian-derived neuropeptide tyrosine-isoleucine-arginine-phenylalanine-amide (YIRFamide, Johnston *et al*. 1996). Each day of experimentation, I–DMEM and 100 nM YIRFamide were tested on 2 plates as negative and positive controls.

In vitro *egg production*

The measurement of *in vitro* egg production of schistosome pairs has been used as an assay of drug activity (Vande Waa *et al*. 1989). Fifteen pairs of adult worms were placed in each flask along with 50 ml of incubation medium, which consisted of sterile RPMI–1640 supplemented with 50 % heat-inactivated horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM mercaptoethanol buffered to pH 7·4 with 20 mM HEPES. The worms were incubated on a gentle shaker table at 37 °C. The general appearance of the adult worms was checked daily and, after 3 days, eggs were counted. To count the eggs, the flasks were manually shaken and 5 ml of the incubation medium was withdrawn. All eggs in the 5 ml sample were counted, the 5 ml sample was replaced and the procedure was repeated. The 2 counts were averaged and multiplied by 10 for an estimate of the number of eggs in the 50 ml flask and that number was divided by 15 to obtain the number

of eggs/worm pair. The egg count from each experimental flask was compared to the average of 2 control flasks from the same experiment, yielding a percentage reduction in egg production due to the experimental treatment.

Established schistosome infections in mice

Female ICR mice were infected with schistosomes by i.p. injection of 250–300 cercariae. On days 45–48 post-infection, mice were injected i.p. with 40 mg/kg/day phenanthroline, 20 mg/kg/day phenanthroline, or the sterile carboxy-methyl cellulose vehicle. Repeated i.p. phenanthroline has been used in rodents at 20 mg/kg/day without increased mortality and little toxicity (Chang, Mann & Gautieri, 1977; Chvapil *et al*. 1974*a*). On day 48, the mice were autopsied and worms were collected.

Establishment of infections in mice

Female ICR mice were offered mouse chow that contained 0·3% phenanthroline, a dose which was found to have no overt effect on the mice. The mouse chow was prepared by grinding the pellets to a powder and 300 mg of phenanthroline was added to 100 g of the powdered mouse chow. The control group received the powdered mouse chow without phenanthroline, and both groups were allowed to eat *ad libitum*. Both groups of mice were injected intraperitoneally with 250–300 cercariae 2 days after initiation of the diet. Mice were maintained on the diet for 30 days after the challenge, then both groups were given powdered chow without phenanthroline for 15 days (days 31–45 post-infection). At day 45, the mice were autopsied and the worms were tallied from the mesenteric veins, the portal vein and the liver. Each group contained 5 mice and the experiment was repeated twice.

RESULTS

Phenanthroline-induced contractions

Phenanthroline elicited contraction of the longitudinal somatic musculature of whole male schistosomes in a concentration-dependent fashion at concentrations between 5 and 150 μM (Fig. 1). At the same concentrations, phenanthroline also induced contraction of the circular muscle of worm segments (Fig. 1). The contractile response of both the longitudinal and the circular muscle was still escalating in response to increasing concentrations up to 150 μM, the maximum concentration allowed by the limitation of phenanthroline solubility in the protocol. At the highest concentrations, contractures of both the longitudinal and circular muscles were immediate and sustained. Generally, maximum contractions were achieved within a few sec and maintained at a constant level for > 10 min. At lower concentrations, there was often a short delay before the initiation of contraction, especially in the circular muscle. The longitudinal musculature of the female worms was similarly contracted by phenanthroline, but it was not possible to test the circular muscle of the females. Individual muscle fibres did not contract even in 150 μM phenanthroline (Fig. 1).

The phenanthroline-induced contractions of the whole male worms were diminished by the addition of sufficient Zn^{2+} to the medium (Fig. 2). The contractions induced by 50 μM phenanthroline were unaltered by the addition of 30 μM Zn^{2+} to the culture medium. However, 100 μM Zn^{2+} reduced the 50 μM phenanthroline response by 62%; at 300 μM Zn^{2+}, the response was reduced by 72%.

Phenanthroline effects on in vitro *egg production*

Phenanthroline reduced egg production by worm pairs *in vitro* in a concentration-dependent fashion at concentrations between 0·5 and 150 μM (Fig. 3). Egg production was reduced by 81% by the inclusion of 5 μM phenanthroline in the incubation medium. Although the adult worms survived the 3-day *in vitro* incubation at this concentration, both the males and the females were clearly affected. For example, none of the males was attached to the flask after 24 h, whereas more than 75% of the males in the control flasks remained attached. Less than 10% of the worms remained paired; in controls, over 50% of the worms remained paired. All worms examined from the 5 μM groups (> 40) were alive at the end of the incubation, as judged by their responsiveness to prodding and/or exposure to elevated (25 mM) K^+. However, only a few of these worms displayed spontaneous motility, while almost all the control worms were independently active.

Egg production was almost completely inhibited by higher concentrations of phenanthroline. No eggs were found in 150 μM phenanthroline, compared to 1326 ± 230 eggs in the control flasks ($n = 8$, 15 pairs/flask). After 24 h, none of the worms was attached to the flasks and none was found paired in either the 50 or 150 μM groups. At 150 μM, most of the worms appeared dead by the end of the first day, as judged by unresponsiveness to prodding and to 25 mM K^+. The males were stiff, and the females were often knotted. At 50 μM, over half the worms appeared dead after the first 24 h, and over 75% were dead at the end of the 3-day incubation.

Phenanthroline effects on established infections in mice

When mice with mature schistosome infections were given intraperitoneal injections of phenanthroline for 4 days, there was a slight reduction in the worm burden and a significant shift of the worms to the

https://doi.org/10.1017/S0031182097002370 Published online by Cambridge University Press

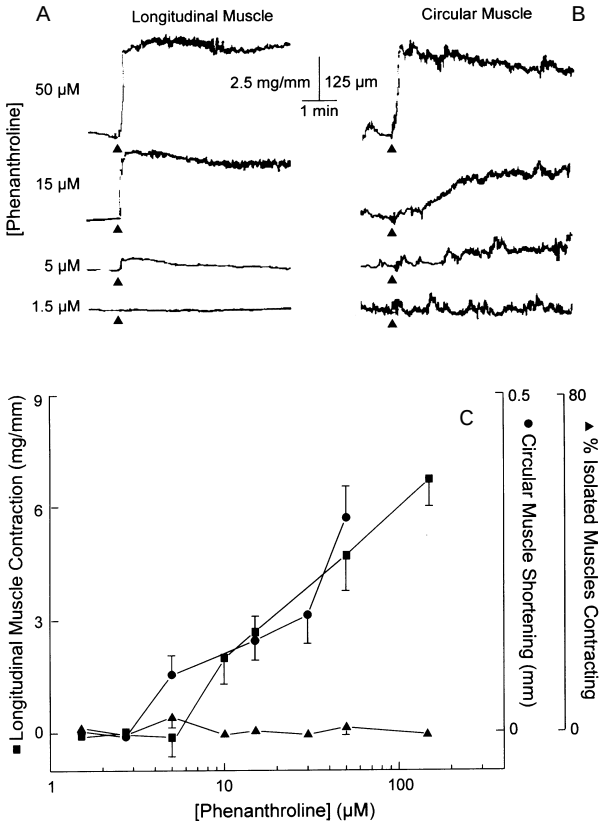

Fig. 1. Phenanthroline effects on muscle contractility. Examples of individual recordings of longitudinal muscle tension (A) and circular muscle length (B), with phenanthroline added at the arrow head. (C) Concentration–response relationship for longitudinal muscle (■), circular muscle (●) and isolated muscle fibres (▲). For the longitudinal and circular muscle data, $n \geqslant 6$ for each point. For the isolated muscle, at least 4 plates were sampled with $\geqslant 20$ individual fibres tested in each plate.

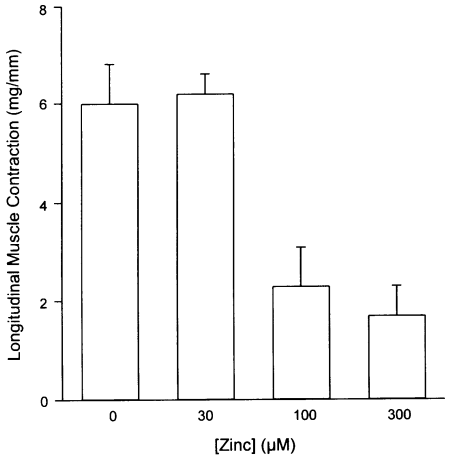

Fig. 2. Zn^{2+} effects on phenanthroline-induced contractions. The contractions compared are all longitudinal muscle contractions in response to 50 μM phenanthroline ($n \geqslant 5$). The various amounts of Zn^{2+} added in the form of $ZnCl_2$, and the parasites were pre-incubated in the Zn^{2+} for 15 min. Identical studies using $ZnSO_4$ produced indistinguishable results.

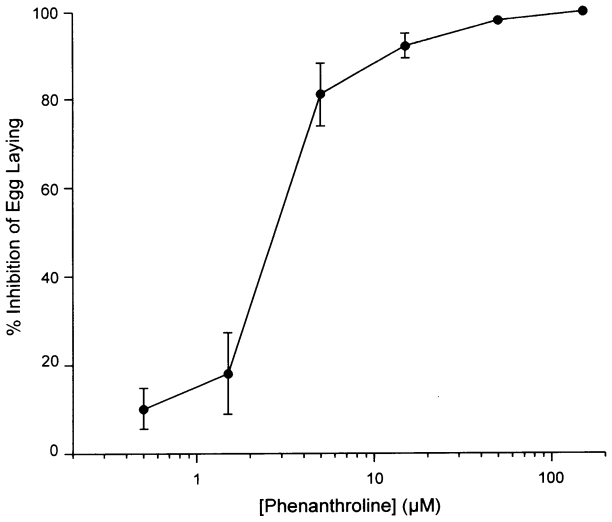

Fig. 3. Phenanthroline effects on *in vitro* egg production. Percentage inhibition for each trial was determined by comparing the experimental value to the average of 2 parallel controls. The controls averaged 88 ± 15 eggs/worm pair ($n = 8$). For each dose, $n = 4$.

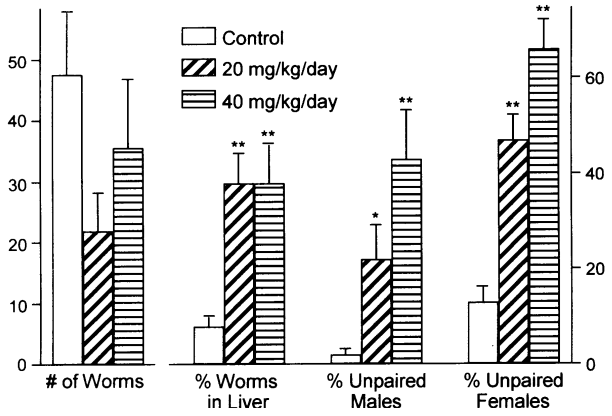

Fig. 4. Phenanthroline effects on established schistosome infections in mice. Each group contained 4 mice and the experiment was performed twice. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, experimental *vs.* control Student's *t*-test.

liver and unpairing of the worms *in vivo* (Fig. 4). There were no deaths among the phenanthroline-treated mice. By the third day, some of the mice in the higher dosage group behaved sluggishly and had some tremors for the 1–2 h following the injection, but were not otherwise noticeably different from the vehicle-injected controls.

Control mice, injected only with the vehicle, averaged 48 ± 10 worms ($n = 9$), compared to 22 ± 6 ($n = 8$) worms in the mice injected with phenanthroline at 20 mg/kg/day and 36 ± 11 worms ($n = 8$) in the mice injected with 40 mg/kg/day. Both dosage groups showed equal shifting of the worms to the liver, with 38% of the worms being found in the liver as opposed to $< 10\%$ in controls.

The most striking and dose-dependent effect on the established infections was the unpairing of worms in the phenanthroline-treated mice. Whereas

https://doi.org/10.1017/S0031182097002370 Published online by Cambridge University Press

only 2 % of females and 13 % of males were unpaired in control mice, 22 % of females and 48 % of males were unpaired in the mice that received the lower dose of phenanthroline. At the higher dose, the unpairing was even more dramatic, with 44 % of females and 65 % of males being found unpaired. Female schistosomes from the mice receiving phenanthroline were visibly different from those of control mice. Firstly, they lacked dark gut pigmentation. They were also undersized, ranging from slightly smaller to almost threadlike in appearance. Like the females exposed to phenanthroline *in vitro*, these females were often knotted.

Phenanthroline effects on the establishment of infections in mice

Mice on a diet containing 0·3 % phenanthroline were protected against schistosomiasis induced by cercarial challenge. The phenanthroline-containing diet was begun 2 days before cercarial challenge and maintained for 32 days. The phenanthroline-fed mice ate and gained weight in a manner indistinguishable from the controls and no adverse effects were apparent. Mice on a phenanthroline-containing diet averaged $3{\cdot}3 \pm 1{\cdot}2$ ($n = 10$) adult parasites/mouse, compared to $59{\cdot}4 \pm 16{\cdot}1$ ($n = 10$)/mouse in the control group. Importantly, there was an apparent absence of liver pathology in the phenanthroline-fed group, as opposed to the typical pathology observed in the infected control mouse livers. Upon gross inspection, the livers of the phenanthroline-fed mice were indistinguishable from those of unchallenged controls, in contrast to the numerous granulomas and enlargement of the infected mouse livers.

DISCUSSION

The wide range of effects of phenanthroline on schistosomes suggests broad functions for the metalloprotease(s) in this animal. *In vitro*, this metalloprotease inhibitor elicits paralytic contraction of the somatic musculature of both male and female worms, and decreases egg production of worm pairs. *In vivo*, dosing with phenanthroline elicits a reduction in worm burden, and changes in the position and pairing of worms in established infections and provides mice with protection from cercarial challenge.

Previous studies involving repeated i.p. phenanthroline doses of 20 mg/kg/day and higher have produced little apparent toxicity in mice (Change *et al*. 1977) and rats (Chvapil *et al*. 1947*a*; Chvapil & Ryan, 1972). The most detailed study in rats involved doses of 20 mg/kg every other day for 3 weeks, with no measurable effects on mortality, weight gain, liver weight or serum protein, among other markers (Chvapil *et al*. 1974*a*). Doses of 30 mg/kg for 4 days produced no overt adverse maternal effects in pregnant mice (Chang *et al*. 1977). Tremors are the most apparent and commonly reported adverse effect with phenanthroline administration (Rothman, Yang & Webb, 1962; Chvapil *et al*. 1974*b*). In the studies reported here, 20–40 mg/kg dosing for 4 days produced little evidence of toxicity in the mice. In some mice, there was tremor and sluggishness immediately after injection of the higher doses. Also, the inclusion of 0·3 % phenanthroline in the diet was without discernible effect on the health of the mice.

Similar to the effects of this metalloprotease inhibitor, irreversible cysteine protease inhibitors decrease the worm burden and liver pathology of infected mice (Wasilewski *et al*. 1996). These inhibitors also block schistosome haemoglobin digestion *in vitro* (Wasilewski *et al*. 1996), attributable to an inhibition of the cysteine 'haemoglobinase' that performs the initial digestion of haemoglobin in the worm gut (Grant & Senft, 1971). The most obvious explanation is that the inhibition of digestion causes the *in vivo* effects, which is supported by the temporal relationship between the inhibition of digestion *in vitro* and the anthelmintic effects *in vivo*.

The only phenanthroline-sensitive enzyme thus far identified in schistosomes is a leucine aminopeptidase. This protease activity has been demonstrated to play a role in egg-hatching (Xu & Dresden, 1986) and in the cleavage of host immune molecules from the parasite surface (Auriault *et al*. 1981), although it is not clear if these activities are mediated by the same enzyme. Phenanthroline inhibition of the aminopeptidase mediating host immune molecule cleavage may play a role in the *in vivo* results presented in this study. Inhibition of mechanisms for contesting the immune system could compromise the parasite's ability to maintain its position in the host, as well as elicit an unpairing of the male and female worms. Since the female relies on the male for some nutritional needs (Cornford & Huot, 1981), unpairing could lead to the visibly poor condition of the females in phenanthroline-treated mice. Similarly, if the developing somule depends on this leucine aminopeptidase-mediated cleavage of host molecules to evade the immune system, inhibition of this protease could explain the protection afforded to mice fed phenanthroline.

However, the *in vitro* effects reported here – the inhibition of egg-laying and the contraction of the somatic musculature – cannot be accounted for by the currently established functions of the leucine aminopeptidase activity in these parasites. Therefore, either the leucine aminopeptidase activity has functions that have not yet been identified, or there are other metalloproteases in the schistosome that are yet to be identified. Since the characterized leucine aminopeptidase is present in relative abundance throughout the life-cycle of schistosomes (Xu

& Dresden, 1986) and the demonstrated functions are quite focused and stage specific, it seems likely that the protease serves functions not yet elucidated. Also, it is likely that more than 1 metalloprotease is present in the schistosome, based on inference from other animals.

The contraction of somatic musculature by phenanthroline stands out from the other effects in immediacy of onset. It is difficult to attribute this rapid motor response to the inhibition of any nutritional or metabolic function of metalloproteases. Another potential role for proteases in the biology of flatworms is in the degradation of peptide neurotransmitters. Peptide neurotransmitter signal termination in other systems occurs by protease-mediated degradation (Kenny & Hooper, 1991), and almost all the proteases involved are metalloproteases (Turner, 1987). Over the past few years, it has become clear that neuropeptides play a central role in the biology of parasitic worms (Shaw, Maule & Halton, 1996). Flatworm-derived peptides potently contract the somatic musculature of flatworm parasites (Marks *et al*. 1996; Moneypenny *et al*. 1997), including non-innervated muscle (Day *et al*. 1994; Johnston *et al*. 1996), suggesting that these peptides could be excitatory transmitters on the muscle.

If metalloproteases terminate peptide transmitter action in schistosomes, the inhibition of excitatory peptide degradation could explain the rapid contraction of worm musculature elicited by phenanthroline. More specifically, if there is a tonic release of an excitatory peptide transmitter onto the muscle, phenanthroline-mediated inhibition of a transmitter-degrading protease would result in a persistent excitation of the somatic musculature. If such is the case, preparations containing neurons should respond, and those without neurons should not. In these studies, whole worms and neuron-containing worm segments were excited by phenanthroline, but individual muscle fibres isolated from neural influence were not. These same isolated muscle fibres contracted in response to the platyhelminth-derived neuropeptide YIRFamide. All the contraction data presented here could be explained if schistosome motorneurons tonically release an excitatory peptide whose action is terminated by metalloprotease-mediated degradation.

The loss of muscular control resulting from paralytic contraction could contribute to the wide range of effects observed with phenanthroline. For example, a loss of muscle control could contribute to the displacement and unpairing of the worms *in vivo*. Also, in addition to the demonstrated effects of peptides on flatworm somatic musculature, many specialized muscle groups have peptidergic innervation, including the reproductive system (Halton *et al*. 1991) and the suckers (Magee *et al*. 1989; Skuce *et al*. 1990; Marks *et al*. 1995). If indeed peptidergic transmission is important in the control of these systems, phenanthroline interference with signal termination could lead to effects on egg production and maintenance of position.

Some of the effects of phenanthroline may be attributable to general zinc chelation. There is no clearly defined role for zinc in these helminths. However, growing worms contain more Zn^{2+} than older worms, and Zn^{2+} is concentrated in multiplying and differentiating tissue (Chowdhury & Singh, 1989). Since Zn^{2+} is known to play a role in DNA replication in other organisms (Chesters, 1974), it seems reasonable to infer such a role in these worms. As such, phenanthroline chelation of Zn^{2+} could be involved in the inhibition of egg production or the inhibition of the establishment of infection. In apparent contradiction to this interpretation, intraperitoneal injection of Zn^{2+} in schistosome-infected hamsters caused a reduction in total worm counts and hepatic pathology (Mansour, Mikhail & Gurirgis, 1983) and a reduction in cercarial viability (Asch & Dresden, 1977).

The authors thank Dr James McKerrow for his input in this project and Ms Mary Thomas for technical assistance.

REFERENCES

ASCH, H. L. & DRESDEN, M. H. (1977). *Schistosoma mansoni*: effects of zinc on cercarial and schistosomule viability. *Journal of Parasitology* **63**, 80–86.

AURIAULT, C., OUASSI, G., TORPIER, H., EISEN, H. & CAPRON, A. (1981). Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomule. *Parasite Immunology* **3**, 33–44.

CESARI, I. M., AURIAULT, C. & CAPRON, A. (1983). Aminopeptidase activity in adult *Schistosoma mansoni*. *Journal of Parasitology* **69**, 280–284.

CHANG, C. H., MANN, D. E. & GAUTIERI, R. F. (1977). Teratogenicity of zinc chloride, 1,10-phenanthroline, and a zinc-1,10-phenanthroline complex in mice. *Journal of Pharmaceutical Sciences* **66**, 1755–1758.

CHESTERS, J. K. (1974). Biochemical functions of zinc with emphasis on nucleic acid metabolism and cell division. In *Trace Element Metabolism in Animals* (ed. Hoekstra, W. G., Suttie, J. W., Ganther, H. E. & Mertz, W.), pp. 39–50. University Park Press, Baltimore, MD., USA.

CHOWDHURY, N. & SINGH, R. (1989). Distribution of zinc in parasitic helminths. *Journal of Helminthology* **63**, 149–152.

CHVAPIL, M. & RYAN, J. N. (1972). Diverse effect of 1,10-phenanthroline and 2,2′-dipyridyl on the synthesis of collagen and noncollagenous proteins in *in vivo* and *in vitro* systems. *Biochimica et Biophysica Acta* **273**, 208–211.

CHVAPIL, M., CARLSON, E. C., MADDEN, J. W. & PEACOCK, E. E. (1974*a*). Different effects of 1,10-phenanthroline on chronic liver injury induced by dimethylnitrosamine or ethionine in rats. *Life Science* **14**, 1991–2002.

CHVAPIL, M., MCCARTHY, D., MADDEN, J. W. & PEACOCK, E. E. (1974*b*). Effect of 1,10-phenanthroline and

desferrioxamine *in vivo* on prolyl hydroxylase and hydroxylation of collagen in various tissues of rats. *Biochemical Pharmacology* **23**, 2165–2173.

CORNFORD, E. M. & HUOT, M. E. (1981). Glucose transfer from male to female schistosomes. *Science* **213**, 1269–1271.

DAY, T. A., ORR, N., BENNETT, J. L. & PAX, R. A. (1993). Voltage-gated currents in muscle cells of *Schistosoma mansoni*. *Parasitology* **106**, 471–477.

DAY, T. A., MAULE, A. G., SHAW, C., HALTON, D. W., MOORE, S., BENNETT, J. L. & PAX, R. A. (1994). Platyhelminth FMRFamide-related peptides (FaRPs) contract *Schistosoma mansoni* (Trematoda: Digenea) muscle fibres *in vitro*. *Parasitology* **109**, 455–459.

DRESDEN, M. H. & DEELDER, A. M. (1979). *Schistosoma mansoni*: thiol proteinase properties of adult worm 'hemoglobinase'. *Experimental Parasitology* **48**, 190–197.

FETTERER, R. H., PAX, R. A. & BENNETT, J. L. (1977). *Schistosoma mansoni*: direct method for simultaneous recording of electrical and motor activity. *Experimental Parasitology* **43**, 286–294.

FETTERER, R. H., PAX, R. A., STRAND, S. & BENNETT, J. L. (1978). *Schistosoma mansoni*: physical and chemical factors affecting the mechanical properties of the adult male musculature. *Experimental Parasitology* **46**, 59–71.

GRANT, C. T. & SENFT, A. W. (1971). Schistosome proteolytic enzyme. *Comparative Biochemistry and Physiology* **38B**, 663–678.

HALTON, D. W., BRENNAN, G. P., MAULE, A. G., SHAW, C., JOHNSTON, C. F. & FAIRWEATHER, I. (1991). The ultrastructure and immunogold labeling of pancreatic polypeptide-immunoreactive cells associated with the egg-forming apparatus of a monogenean parasite, *Diclidophora merlangi*. *Parasitology* **102**, 429–436.

JOHNSTON, R. N., SHAW, C., HALTON, D. W., VERHAERT, P., BLAIR, K. L., BRENNAN, G. P., PRICE, D. A. & ANDERSON, P. A. V. (1996). Isolation, localization, and bioactivity of the FMRFamide-related neuropeptides GYIRFamide and YIRFamide from the marine turbellarian *Bdelloura candida*. *Journal of Neurochemistry* **67**, 814–821.

KENNY, A. J. & HOOPER, N. M. (1991). Peptidases involved in the metabolism of bioactive peptides. In *Degradation of Bioactive Substances : Physiology and Pathophysiology* (ed. Henriken, J. H.), pp. 47–79. CRC Press, Boca Raton, Fl., USA.

LEWERT, R. M. & LEE, C. (1956). Quantitative studies of the collagen-like enzymes of cercariae of *S. mansoni* and the larvae of *Strongyloides ratti*. *Journal of Infectious Diseases* **99**, 1–14.

MAGEE, R. M., FAIRWEATHER, I., JOHNSTON, C. F., HALTON, D. W. & SHAW, C. (1989). Immunocytochemical demonstration of neuropeptides in the nervous system of the fluke, *Fasciola hepatica* (Trematoda: Digenea). *Parasitology* **98**, 227–238.

MANSOUR, M. M., MIKHAIL, M. M. & GURIRGIS, N. I. (1983). Effect of zinc supplementation on *S. mansoni*-infected hamsters. *Annals of Tropical Medicine and Parasitology* **77**, 517–521.

MARKS, N. J., HALTON, D. W., MAULE, A. G., BRENNAN, G. P., SHAW, C., SOUTHGATE, V. R. & JOHNSTON, C. F. (1995). Comparative analyses of the neuropeptide F (NPF)- and FMRFamide-related peptide (FaRP)-immunoreactivities in *Fasciola hepatica* and *Schistosoma* spp. *Parasitology* **110**, 371–381.

MARKS, N. J., JOHNSON, S., MAULE, A. G., HALTON, D. W., SHAW, C., GLARY, T. G., MOORE, S. & THOMPSON, D. P. (1996). Physiological effects of platyhelminth RFamide peptides on muscle-strip preparations of *Fasciola hepatica* (Trematoda: Digenea). *Parasitology* **113**, 393–401.

McKERROW, J. H. (1989). Parasite proteases. *Experimental Parasitology* **68**, 111–115.

McKERROW, J. H. & DOENHOFF, M. F. (1988). Schistosome proteases. *Parasitology Today* **4**, 334–337.

MONEYPENNY, C. G., MAULE, A. G., SHAW, C., DAY, T. A., PAX, R. A. & HALTON, D. W. (1997). Physiological effects of platyhelminth FMRFamide-related peptides (FaRPs) on the motility of the monogenean *Diclidophora merlangi*. *Parasitology* **115**, 281–288.

PAX, R. A., SIEFKER, C. & BENNETT, J. L. (1984). *Schistosoma mansoni*: differences in acetylcholine, dopamine and serotonin control of circular and longitudinal parasite muscle. *Experimental Parasitology* **58**, 314–324.

ROTHMAN, S., YANG, W. C. & WEBB, J. L. (1962). Observations on the antifibrillatory activity of 1,10-phenanthroline on the rabbit heart. *Journal of Experimental Therapeutics* **137**, 1–4.

SHAW, C., MAULE, A. G. & HALTON, D. W. (1996). Platyhelminth FMRFamide-related peptides. *International Journal for Parasitology* **26**, 335–345.

SKUCE, P. J., JOHNSTON, C. F., FAIRWEATHER, I., HALTON, D. W., SHAW, C. & BUCHANAN, K. D. (1990). The immunoreactivity to the pancreatic polypeptide family in the nervous system of the adult human blood fluke, *Schistosoma mansoni*. *Cell and Tissue Research* **261**, 573–581.

TURNER, A. J. (1987). *Neuropeptides and their Peptidases*. Ellis Horwood Ltd, Chichester, UK.

VANDE WAA, E. A., MILLS, G., CHEN, G. Z. & BENNETT, J. L. (1989). Physiological role of HMG-CoA reductase in regulating egg production by *S. mansoni*. *American Journal of Physiology* **253**, R618–R625.

WASILEWSKI, M. M., LIM, K. C., PHILLIPS, J. & McKERROW, J. H. (1996). Cysteine protease inhibitors block schistosome hemoglobin degradation *in vitro* and decrease worm burden and egg production *in vivo*. *Molecular and Biochemical Parasitology* **81**, 179–189.

XU, Y. Z. & DRESDEN, M. H. (1986). Leucine amino-peptidase and hatching of *Schistosoma mansoni* eggs. *Journal of Parasitology* **72**, 507–511.

XU, Y. Z., SHAWAR, S. M. & DRESDEN, M. H. (1990). *Schistosoma mansoni*: purification and characterization of a membrane-associated leucine aminopeptidase. *Experimental Parasitology* **70**, 124–133.