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Metalloproteases of Infective *Ancylostoma* Hookworm Larvae and Their Possible Functions in Tissue Invasion and Ecdysis

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To infect their hosts, hookworm larvae must exsheath and migrate through connective tissue. A modified in vitro skin chamber was used to show that the human hookworm *Ancylostoma duodenale* and the zoonotic canine hookworm *Ancylostoma caninum* penetrate epidermis, basement membrane, and dermis in similar ways. These similarities in tissue invasion properties reflect the observed biochemical similarities in parasite protease composition. The larvae of both species contain protease activity that is inhibited by *o*-phenanthroline; this identifies the proteases as metalloproteases. The enzyme activities exhibit an alkaline pH optimum between pH 9 and 10. During modified sodium dodecyl sulfate-polyacrylamide gel electrophoresis in which a protein substrate (either casein or gelatin) was used, the protease activities resolved into a major band at an M_r of 68,000 and a minor band at an M_r of 38,000. Proteases were released by living *A. caninum* larvae in vitro and degraded purified and radiolabeled casein to smaller peptides. Motile hookworm larvae were also incubated with purified and radiolabeled connective tissue macromolecules in vitro. Both *Ancylostoma* species degraded human fibronectin to a 60,000-M_r polypeptide intermediate, but could not degrade solubilized bovine elastin or human laminin. In contrast, the obligate skin-penetrating nematode *Strongyloides stercoralis* degraded all three substrates. This biochemical difference may explain some observed differences in invasiveness.

Nematode larvae of the genus *Ancylostoma* produce a range of cutaneous and systemic manifestations in humans that occur consequent to their migratory behavior in connective tissue. Anthropophilic (human) and zoonotic *Ancylostoma* third-stage larvae can penetrate human epidermis as a prelude to infection, but ultimately they suffer different fates depending on when and where they meet resistance and succumb to the host inflammatory response. Infective larvae of this genus exhibit at least three general patterns of migratory behavior. They enter the epidermis and migrate laterally to produce cutaneous larva migrans (16), they migrate from the skin to reach the viscera and elicit visceral larva migrans (2, 15, 22, 31, 32), or they develop into adults in the intestine to produce a classical human hookworm infection (12, 29). There is overlap between the patterns of tissue migration exhibited by anthropophilic and zoonotic hookworms, as the human species *Ancylostoma duodenale* can elicit larva migrans (1, 2), while the canine species *Ancylostoma caninum* can develop to adulthood in humans (3). Hookworms of the genus *Ancylostoma* also infect humans orally and are therefore facultative skin penetrators (18, 29).

The biochemical mechanisms of tissue invasion by infective larval stages of the genus *Ancylostoma*, as well as other soil-transmitted nematodes, are not well understood, although they appear to depend on parasite-derived proteases. As helminth larvae migrate through connective tissue, they release proteases that degrade extracellular matrix macromolecules in vitro and presumably function in histolysis (26). Proteases also mediate ecdysis in nematode larvae that cast off their loosely retained second-stage cuticles upon host entry (8). Infective *Ancylostoma* larvae undergo both connective tissue invasion and ecdysis at approximately the same time.

In this study, we found that a human hookworm species and a zoonotic hookworm species exhibit similarities in their patterns of migration through human skin and connective tissue in vitro. We also observed that these two species produce metalloproteases which have similar molecular weights and properties that might mediate histolysis and ecdysis.

MATERIALS AND METHODS

Collection of nematode larvae. Feces containing embryonated eggs of *A. caninum* or *A. duodenale* were obtained from experimentally infected dogs (33). Third-stage larvae were reared in coprocultures prepared with bone charcoal or by using a modification of the Harada-Mori filter paper culture method, as described elsewhere (P. J. Hotez, J. Hawdon, N. Cox, G. A. Schad, and F. F. Richards, J. Helminthol. Soc. Washington, in press). For comparison, third-stage *Strongyloides stercoralis* larvae were obtained from charcoal cultures made from the feces of immunosuppressed dogs (34). Larvae were recovered from the coprocultures with a Baermann apparatus. Water containing the larvae was passed through a 60-mesh sieve and then through cheesecloth to remove minor particulates. The sedimented larvae were washed four or five times with RPMI 1640 containing antibiotics (1,000 U of penicillin per ml and 1 mg

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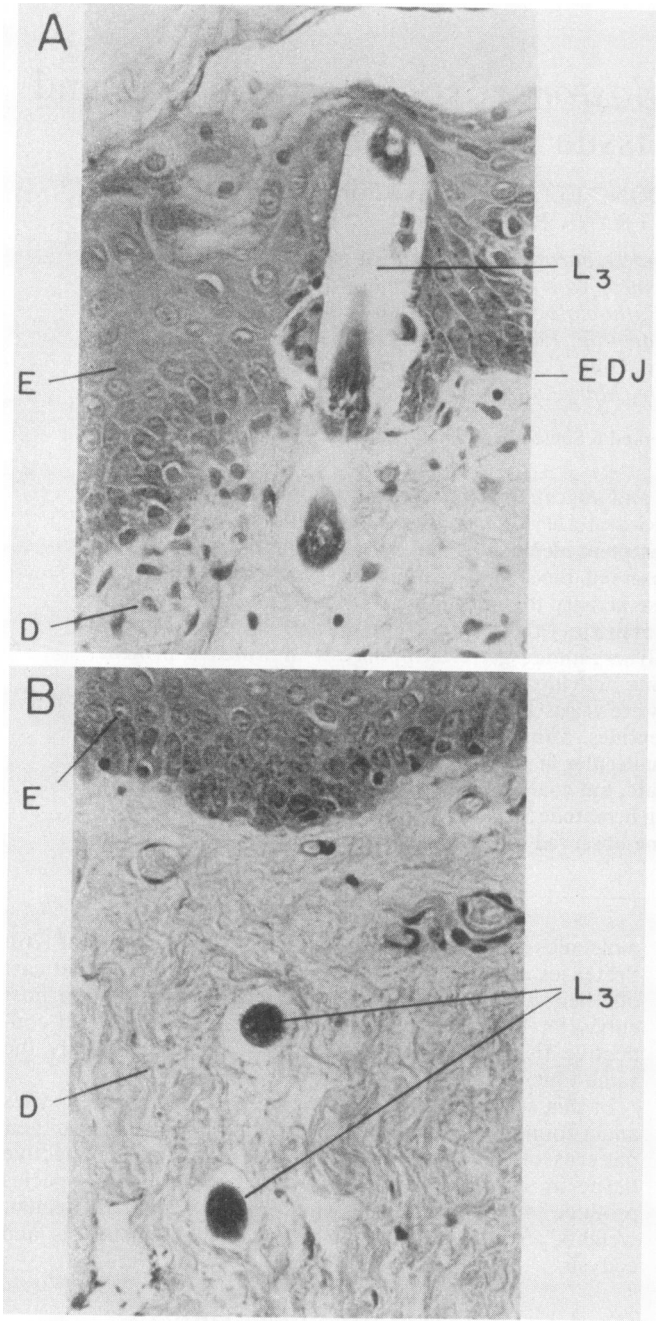

FIG. 1. Light microscopy of *A. caninum* larvae. Approximately 140 larvae were applied to a human neonatal foreskin for 30 min prior to histologic sectioning and staining with periodic acid-Schiff stain. (A) Third-stage larva (L_3) traversing the epidermis (E), epidermis-dermis junction (EDJ), and dermis (D). Magnification, ×340. (B) Larva in the dermis. Magnification, ×340.

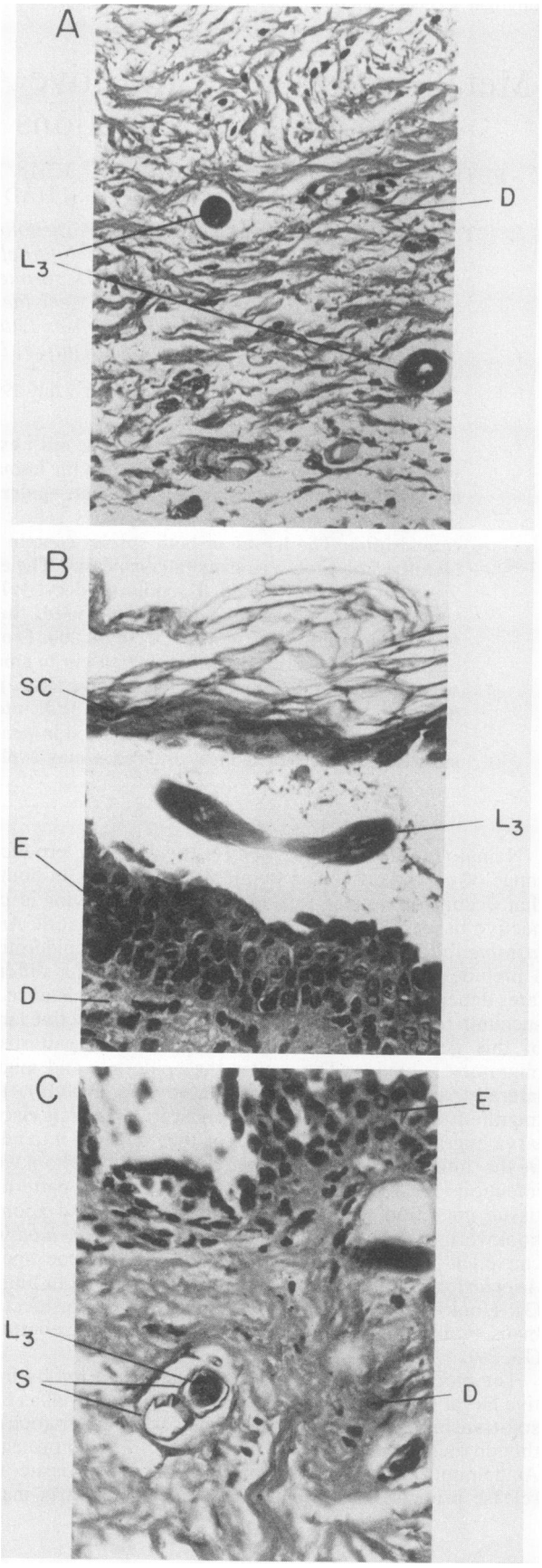

FIG. 2. Light microscopy of *A. duodenale* larvae. Approximately 640 larvae were applied to a human neonatal foreskin for 10 and 15 min prior to histologic sectioning and staining with periodic acid-Schiff stain. (A) Third-stage Larvae (L_3) in the dermis (D) after 15 min. Magnification, ×400. (B) Larva underneath the stratum corneum (SC) after 15 min. E, Epidermis; D, dermis. Magnification, ×400. (C) Larva with retained sheath (S) at the site of broken skin after 10 min. Magnification, ×400.

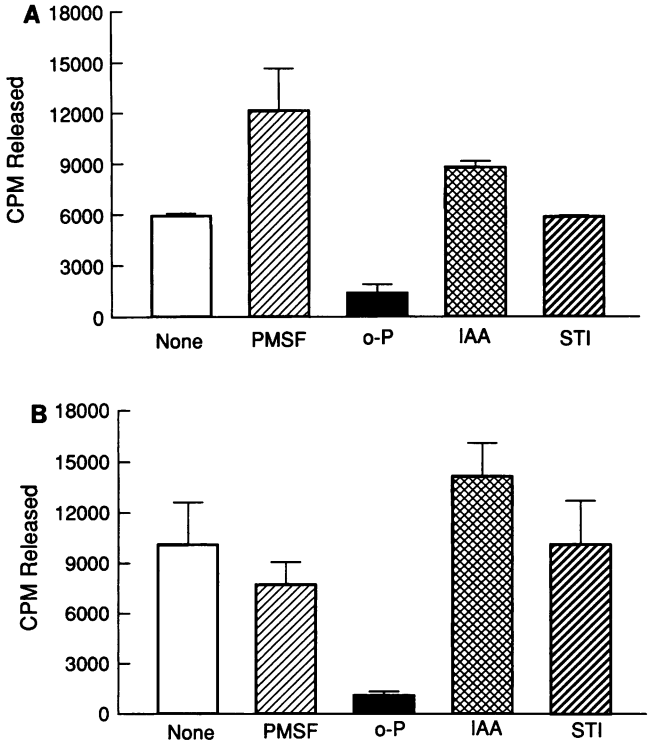

FIG. 3. Inhibitory profiles of *Ancylostoma* protease activities. (A) *A. caninum*. Approximately 1.4 μg of homogenate protein was placed in duplicate wells of ^{125}I-radiolabeled fibrinogen plates containing 300 μl of 0.1 M Tris hydrochloride buffer (pH 8.5) supplemented with either no inhibitor (None), 1 mM *o*-phenanthroline hydrochloride (o-P), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM iodoacetic acid (IAA), or 6 μg of soybean trypsin inhibitor per ml (STI). Aliquots (100 μl) were removed after 20 h of incubation at 37°C and counted with a gamma counter for 2 min. Background counts were subtracted. (B) *A. duodenale*. This experiment was similar to the experiment described above; 0.4 μg of *A. duodenale* homogenate protein was used.

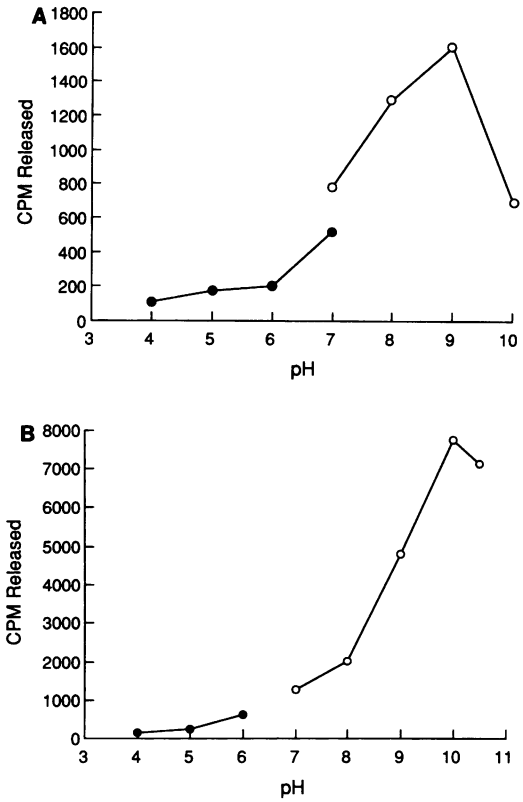

FIG. 4. pH optima of two *Ancylostoma* protease activities. (A) *A. caninum*. Approximately 5.0 μg of homogenate protein was placed in wells of radiolabeled fibrinogen plates containing 250 μl of either 0.05 M sodium acetate buffer (pH 4 to 7) (●) or 0.1 M Tris hydrochloride buffer (pH 7 to 10) (○). Aliquots (40 μl) were removed after 20 h of incubation at 37°C and counted with a gamma counter for 1 min. (B) *A. duodenale*. Approximately 0.2 μg of homogenate protein was placed in wells containing 300 μl of either 0.05 M sodium acetate buffer (pH 4 to 6) (●) or 0.1 M Tris hydrochloride buffer (pH 7 to 10.5) (○). Aliquots (100 μl) were removed after 16 h of incubation at 37°C and counted with a gamma counter for 2 min.

of streptomycin per ml) by using low-speed centrifugation prior to biochemical analysis. The washings from the final centrifugation were saved as a control. Living larvae were either used directly or stored frozen at −70°C. Homogenates from freeze-thawed larvae were prepared by grinding the larvae in 0.1 M Tris hydrochloride buffer (pH 8.0) at 4°C in a glass homogenizer. For comparative studies exsheathing fluid (EF) was also obtained from the ruminant trichostrongyle *Haemonchus contortus* (7, 8).

Skin penetration studies. Individual pieces of human neonatal foreskin were mounted in a modified glass diffusion (Franz) chamber (6). The skin was held between two ball joints on a Nytex filter by using a pinch type, ground joint clamp. The epidermal side of the skin was exposed to ambient air, while the dermal side was bathed in Dulbecco modified Eagle medium without serum. At time zero a small volume of RPMI 1640 containing nematode larvae was gently pipetted onto the epidermal surface. After 5, 10, 15, or 30 min of incubation the pieces of skin were removed, fixed in 10% Formalin, sectioned, and stained by using the periodic acid-Schiff technique.

Protease assays. (i) Solid-phase fibrinogen degradation. Bovine fibrinogen (Sigma Chemical Co., St. Louis, Mo.) was further purified by using ammonium sulfate and ethanol precipitation. This material was resuspended in phosphate-buffered saline (0.01 M sodium phosphate [pH 7.2] containing 0.15 M NaCl) and radiolabeled in iodogen-coated tubes (Pierce Chemical Co., Rockford, Ill.) with Na^{125}I (5). The radiolabeled protein was separated from free NaI by gel filtration chromatography with a type PD-10 column containing Sephadex G-25M (Pharmacia, Uppsala, Sweden) and dried onto Linbro plates containing 24 flat-bottom wells (1.7 by 1.6 cm; Flow Laboratories, McLean, Va.) at 55°C (13, 35). Baking the denatured protein onto plastic resulted in irreversible binding. Prior to use the wells were washed three times with either RPMI 1640 or 0.1 M Tris hydrochloride buffer to remove unbound radiolabeled protein; larval homogenates were added to 250 μl of RPMI 1640 supplemented with antibiotics or to 300 μl of 0.1 M Tris hydrochloride buffer, respectively. Protease activity was measured as the release of radiolabeled fibrinopeptides in 40 to 100 μl of solution after 16 to 20 h at 37°C by counting with a gamma scintillation counter for 2 min. The assays were also carried out in the presence of enzyme inhibitors, including phenylmethanesulfonyl fluoride, iodoacetic acid, *o*-phenanthroline hydrochloride, and soybean trypsin inhibitor, and at pHs ranging from 4 to 10 with either 0.05 M sodium acetate buffer or 0.1 M Tris hydrochloride buffer.

(ii) SDS-PAGE casein underlay. For the sodium dodecyl

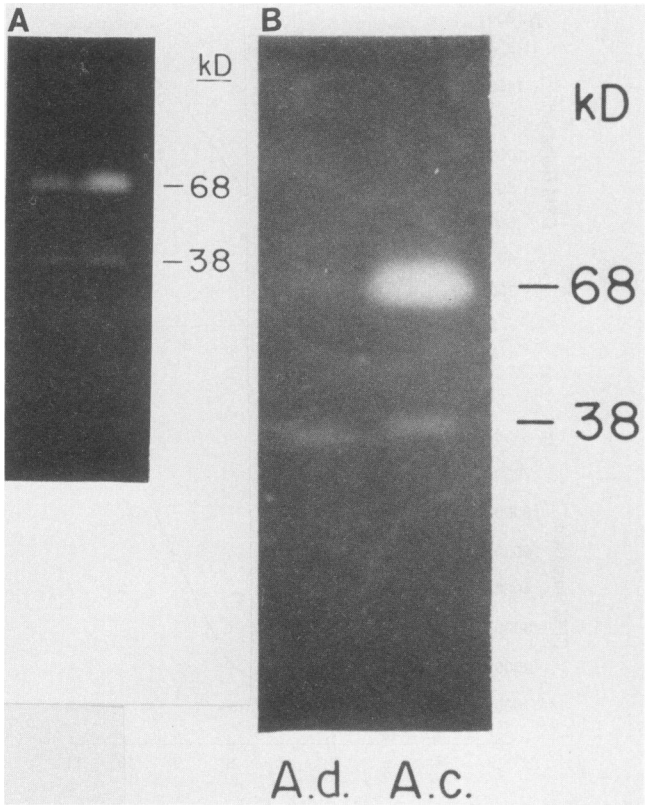

FIG. 5. SDS-PAGE of *Ancylostoma* homogenate with casein underlay. (A) *A. caninum*. Approximately 12 μg of homogenate protein was applied in the presence of SDS to a 0.75-mm-thick nonreducing gel without heating and at low voltage. Upon completion the gel was sliced into lanes containing duplicate samples. After the gel slices were washed in 2.5% Triton X-100, they were washed three times (10 min each) in water. The gels were blotted onto substrate overnight at 37°C. Protease activity is shown as a clear zone. (B) *A. duodenale*. Homogenates of either 0.5 μg of *A. duodenale* protein (A.d.) or 6.5 μg of *A. caninum* protein (A.c.) were applied to SDS-PAGE gels as described above. kD, Kilodalton.

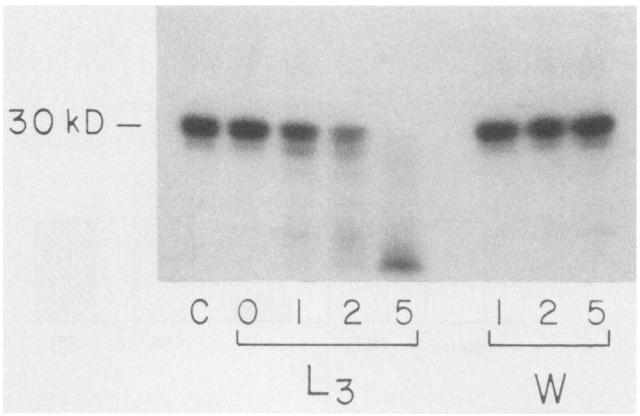

FIG. 6. Degradation of ^{125}I-radiolabeled casein by *A. caninum* larvae. Approximately 100 third-stage larvae (L_3) were placed in 90 μl of RPMI 1640 supplemented with penicillin and streptomycin, and then we added 10 μl of phosphate-buffered saline containing 0.2 μg of radiolabeled casein (C) (approximate specific radioactivity, 2.5 μCi/μg of protein). The reaction mixture was incubated at 37°C, and 10-μl aliquots were removed at 0, 0.5, 1, 2, and 5 h, placed in sample buffer, and stored at −20°C. An equal volume of larval washings (W) was incubated with radiolabeled casein as a control. The samples were thawed, heated to 100°C for 1 min, and cooled prior to application onto a 15% SDS–PAGE gel. The gel was autoradiographed. kD, Kilodaltons.

sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) casein underlay assay, larval homogenates in 1% SDS were applied without heating to 10% SDS–polyacrylamide gels (17) and run in one dimension under nonreducing conditions. Low voltage (100 to 150 V) was used to prevent significant heating. Gels that were not more than 0.75 mm thick were used. The gels were washed once in 2.5% Triton X-100 for 40 min and then three times in water (10 min each). Each gel was then placed onto casein agar (5.2 ml of 0.1 M Tris hydrochloride buffer [pH 8.0], 3.6 ml of 2.5% agar, and 2.0 ml of 8% Carnation Instant Milk boiled in Tris buffer) and incubated overnight at 37°C (10, 13). Bands of proteolysis that appeared within 4 to 12 h were visualized better after background staining with amido black, followed by destaining with methanol-acetic acid-water (70:10:20). Activity was inhibited by washing the gel in water containing 10 mM *o*-phenanthroline before it was placed onto casein agar.

(iii) SDS-PAGE gelatin gels. Larval homogenates or EF preparations in sample buffer containing 65 mM Tris hydrochloride (pH 6.8), 10% glycerol, and 3% SDS were applied without heating to a 12.5% polyacrylamide gel containing 0.1% gelatin. Electrophoresis was carried out at 50 V. The gel was washed three times (20 min each) in 2.5% Triton X-100 and then three times (20 min each) in 0.1 M glycine buffer (pH 8.0) containing 1 mM calcium chloride. The gel was incubated overnight in the same buffer at 37°C. The gel was stained in 0.2% Coomassie blue in 50% methanol and then destained in 50% methanol, followed by 10% methanol and 10% glycerol.

(iv) SDS-PAGE autoradiography of skin macromolecule degradation products. Soluble human fibronectin, human laminin, bovine casein, bovine albumin (Sigma), and bovine elastin (Elastin Products, Owensville, Mo.) were radiolabeled with NaI by the iodogen method and separated from free radioactivity by gel filtration chromatography in the presence of phosphate-buffered saline. Living third-stage larvae in RPMI 1640 supplemented with antibiotics, larval homogenates, or EF preparations were incubated at 37°C in the presence of the radiolabeled macromolecules in an Eppendorf tube. Aliquots of each reaction mixture were removed at 0, 30, 60, 90, and 120 min, added to sample buffer, and stored at −20°C. As a negative control, the macromolecules were incubated with an equal volume of larval washings. The samples were heated at 100°C for 45 s before being applied to SDS-PAGE gels under reducing conditions. The gels were dried and autoradiographed by exposing them to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Skin penetration studies. A modified Franz chamber, which was constructed to examine the effects of chemicals applied to the epidermal side of skin in vitro, also permitted study of skin penetration by nematode larvae. Light microscopy showed that both *A. caninum* and *A. duodenale* larvae penetrated epidermis and basement membrane. By 30 min *A. caninum* larvae were identified traversing the epidermis-

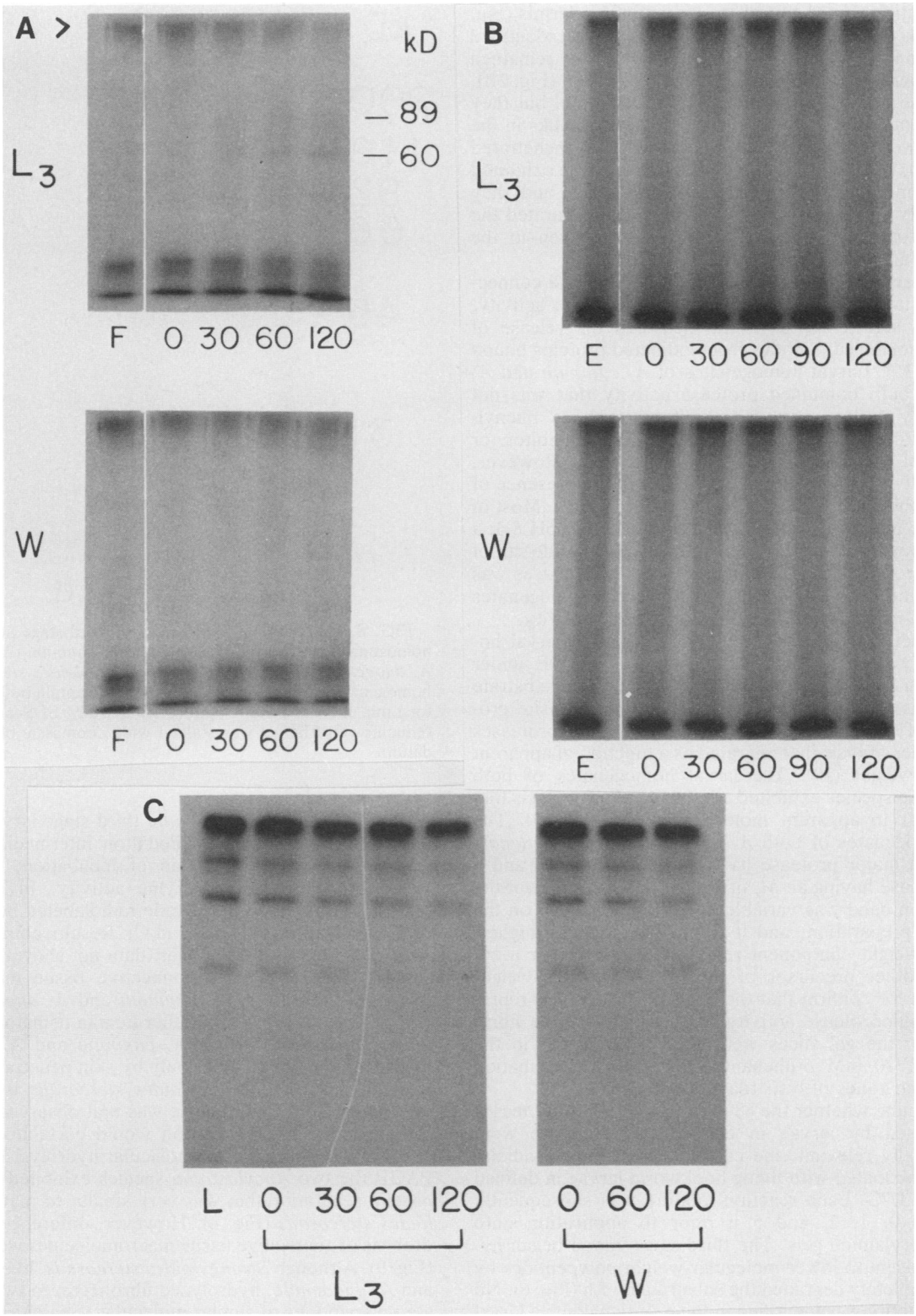

FIG. 7. Degradation of connective tissue macromolecules by *A. caninum*. Third-stage larvae (L_3) were added to buffer containing ^{125}I-radiolabeled substrate and incubated at 37°C. Aliquots were removed at 0, 30, 60, 90, and 120 min and added to sample buffer. Larval washings (W) in identical volumes were used as controls. Samples were subjected to autoradiographic analysis after application to 10% SDS–polyacrylamide gels. (A) Fibronectin. Approximately 200 larvae in 100 μl of RPMI 1640 were added to 200 μl of radiolabeled fibronectin (F), and 20-μl aliquots were removed. The arrowhead indicates the position of fibronectin undergoing degradation. The 90-min aliquot is not shown. (B) Elastin. Approximately 585 larvae in 90 μl of RPMI 1640 were added to 10 μl of radiolabeled elastin (E), and 10-μl aliquots were removed. (C) Laminin. Approximately 500 larvae in 60 μl of RPMI 1640 were added to 10 μl of radiolabeled laminin (L), and 7-μl aliquots were removed. kD, Kilodalton.

dermis junction (Fig. 1A) and were seen in the dermis (Fig. 1B). Similarly, by 15 min *A. duodenale* larvae were identified in the dermis (Fig. 2A), although some larvae remained above the dermis underneath the stratum corneum (Fig. 2B). Some larvae retained their sheath on penetration, but they were predominantly in regions where obvious breaks in the epidermis were identified (i.e., sheathed larvae penetrated broken skin) (Fig. 2C). All of the hookworm larvae exhibited intense staining with periodic acid-Schiff stain, and they were usually surrounded by a clear halo that separated the helminth from dermal tissue. This was true even in the absence of a sheath.

Protease activity. To determine whether skin and connective tissue invasion was associated with protease activity, we used a sensitive assay that measures the release of soluble radiolabeled peptides from iodinated proteins bound to plastic (35). Larval homogenates of *A. caninum* and *A. duodenale* both contained protease activity that was not inhibited by serine protease inhibitors, such as phenylmethanesulfonyl fluoride and soybean trypsin inhibitor, or by the thiol protease inhibitor iodoacetic acid. However, enzyme activity was inhibited up to 90% in the presence of 1.0 mM *o*-phenanthroline, a metal chelator (Fig. 3). Most of the enzyme inhibition studies were carried out at pH 8.3; a pH of 7.9 was used in the presence the hydrochloride salt of *o*-phenanthroline. The pH optimum for both proteases was between 9 and 10 (Fig. 4). Therefore, the larval homogenates contained a neutral (alkaline) metalloprotease activity.

To characterize the protease activity further, larval homogenate proteins were separated by SDS-PAGE under nonreducing conditions and blotted onto a protein substrate matrix. This technique facilitated diffusion of parasite proteins out of the gel and into the matrix, where proteases created zones of lysis that migrated as a function of apparent molecular weight (10). The larval homogenates of both *Ancylostoma* species exhibited two bands of proteolysis that were similar in apparent molecular weight (Fig. 5). The larval homogenates of both *A. caninum* and *A. duodenale* contained a major protease having an M_r of 68,000 and a minor protease having an M_r of 38,000. The presence of the 38-kilodalton band was variable and often depended on the age of the preparation, and it is possible that the higher-molecular-weight component represented a dimer or a zymogen or other precursor of the lower-molecular-weight component. To confirm that the bands of proteolysis represented metalloprotease activity as measured by the fibrin plate assay, the gel slices were also preincubated in the presence of 10 mM *o*-phenanthroline; this preincubation abolished the zones of lysis (data not shown).

To determine whether the 68- and 38-kilodalton proteases were released by larvae in culture and therefore were physiologically relevant, the casein substrate was radiolabeled and incubated with living hookworm larvae in defined medium at 37°C. Each reaction mixture was subsequently sampled at 0, 1, 2, and 5 h prior to application onto SDS-polyacrylamide gels. The third-stage larvae began hydrolyzing casein to lower-molecular-weight polypeptides by 1 h and completely degraded the substrate by 5 h (Fig. 6). No protease activity was identified in the negative control larval washings over the same incubation period.

Additional experiments were undertaken to determine whether the caseinolytic proteases released by larvae in vitro also degraded the connective tissue macromolecules essential for tissue invasion. In an experiment similar to the casein hydrolysis experiment, larvae were also incubated at 37°C with radiolabeled connective tissue macromolecules.

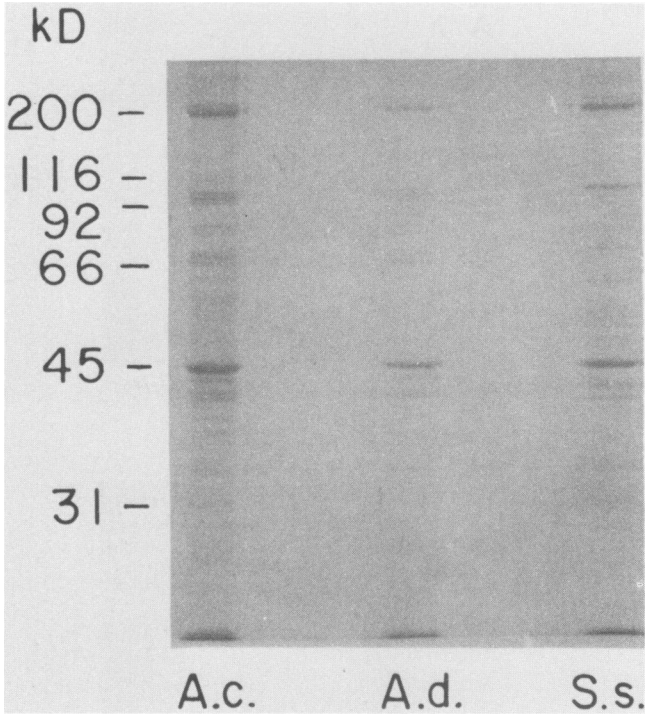

FIG. 8. Comparative SDS-PAGE of third-stage nematode larva homogenate proteins. Either 1.8 μg of *A. caninum* (A.c.), 0.5 μg of *A. duodenale* (A.d.), or 0.6 μg of *Strongyloides stercoralis* (S.s.) homogenate protein was placed in 5 μl of sample buffer and heated for 1 min at 100°C prior to application to a 10% SDS–polyacrylamide reducing gel. The gel was stained with Coomassie blue. kD, Kilodalton.

As Fig. 7A shows, *A. caninum* third-stage larvae degraded fibronectin to 60- and 89-kilodalton intermediate polypeptides after less than 30 min of incubation, while larval washings exhibited no enzyme activity. In contrast, *A. caninum* larvae did not degrade radiolabeled bovine elastin or human laminin (Fig. 7B and C). Results obtained with *A. duodenale* larvae were similar (data not shown).

Next, the patterns of connective tissue migration and hydrolysis caused by *A. caninum* and *A. duodenale* were compared with those of another human nematode, *Strongyloides stercoralis*. Unlike *A. caninum* and *A. duodenale*, which can enter a host not only by skin penetration but also by oral ingestion, *Strongyloides stercoralis* is an obligate skin penetrator. Our premise was that observed differences in connective tissue invasion would correlate with differences in patterns of macromolecular hydrolysis. After SDS-PAGE the two *Ancylostoma* species exhibited a pattern of protein separation that was very similar to that of *Strongyloides stercoralis* (Fig. 8). However, differences in the hydrolysis of connective tissue macromolecules were observed (Fig. 9). Although *Strongyloides stercoralis*, like *A. caninum* and *A. duodenale*, hydrolyzed fibronectin to two intermediate polypeptides of similar molecular weights (Fig. 9A), the obligate skin-penetrating species also degraded human laminin to a 90-kilodalton polypeptide (Fig. 9C) and was more effective at degrading soluble bovine elastin (Fig. 9B). No protease activity was identified in the negative control larval washings.

Because the protease activity from *A. caninum* or *A. duodenale* is less effective against connective tissue sub-

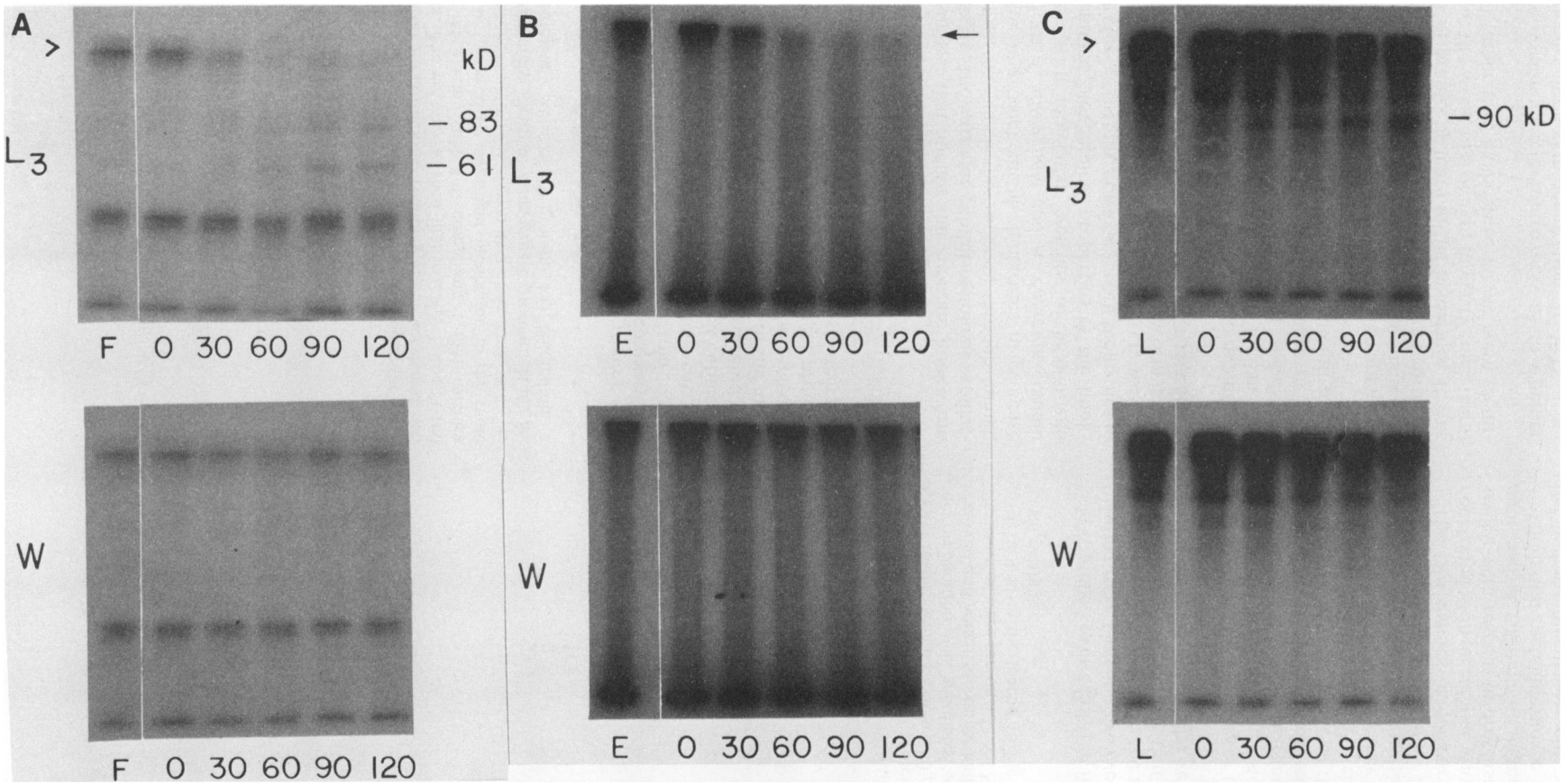

FIG. 9. Degradation of connective tissue macromolecules by *Strongyloides stercoralis*. Third-stage larvae (L_3) were added to buffer containing ^{125}I-radiolabeled substrate and incubated at 37°C. Aliquots were removed at 0, 30, 60, 90, and 120 min and added to sample buffer. Larval washings (W) in identical volumes were used as controls. Samples were subjected to autoradiographic analysis after application to 10% SDS–PAGE gels. The arrow and the arrowheads indicate the positions of macromolecules undergoing degradation. (A) Fibronectin. Approximately 520 larvae in 40 μl of RPMI 1640 were added to 80 μl of radiolabeled fibronectin (F), and 12-μl aliquots were removed. (B) Elastin. Approximately 520 larvae in 40 μl of RPMI 1640 were added to 10 μl of radiolabeled elastin (E), and 5-μl aliquots were removed. (C) Laminin. Approximately 520 third-stage larvae in 40 μl of RPMI 1640 were added to 80 μl of radiolabeled laminin (L), and 12-μl aliquots were removed. kD, Kilodalton.

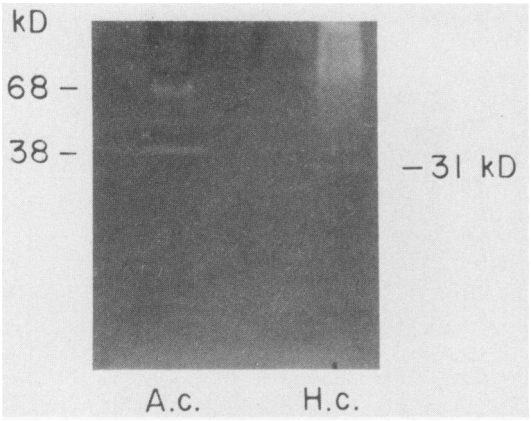

FIG 10. SDS-PAGE gelatin gel. Protein was applied in the presence of SDS to a 0.75-mm-thick nonreducing 12.5% acrylamide gel containing gelatin. The gel was washed first in 2.5% Triton X-100 and then in 0.1 M glycine (pH 8.0) containing 1 mM calcium chloride; this was followed by overnight incubation in the same buffer at 37°C. Protease activity is shown as a clear zone. A.c., *A. caninum* (Approximately 12 μg of homogenate protein); H.c., *H. contortus* (Approximately 20 μg of EF protein); kD, Kilodalton.

strates than the protease activity from *Strongyloides stercoralis* is, experiments were undertaken to compare the *Ancylostoma*-derived proteases with proteases from the EF of *H. contortus*. The infective larvae of the latter nematode species undergo ecdysis by casting their retained cuticles or sheaths upon oral ingestion, a process that is associated with a metalloprotease (7, 8). The molecular weight of the major protease of *H. contortus* EF was similar to the molecular weight of the *A. caninum* lower-molecular-weight protease (Fig. 10). The protease contained in EF also degraded soluble elastin (Fig. 11).

DISCUSSION

Our results suggest that human and zoonotic hookworm larvae of the genus *Ancylostoma* exhibit histologic similarities in the manner by which they migrate through human connective tissue and biochemical similarities in protease composition. Both *A. duodenale* and *A. caninum* contain metalloproteases with molecular weights of 68,000 and 38,000. We identified biochemical properties of the hookworm metalloproteases that are consistent with their roles in mediating histolysis or ecdysis or both. However, we also identified biochemical differences between hookworm larvae and other infective nematode larvae that may account for differences in invasiveness and migratory behavior.

Observations via light microscopy revealed no qualitative differences in skin penetration between the human and zoonotic canine species. Within 30 min both *A. duodenale* and *A. caninum* were engaged in various stages of skin penetration. By this time larvae could be identified as underneath the stratum corneum, entering the epidermis-dermis junction, or in the dermis. The larvae often appeared to follow the path of least resistance, so that increased numbers were found beneath breaks in the stratum corneum and at the sites of breaks or tears in the epidermis. It was predominantly at the violations of skin integrity (i.e., tears) that ensheathed rather than exsheathed larvae were identified.

The literature contains some controversy as to whether exsheathment occurs upon skin penetration. In 1925, using

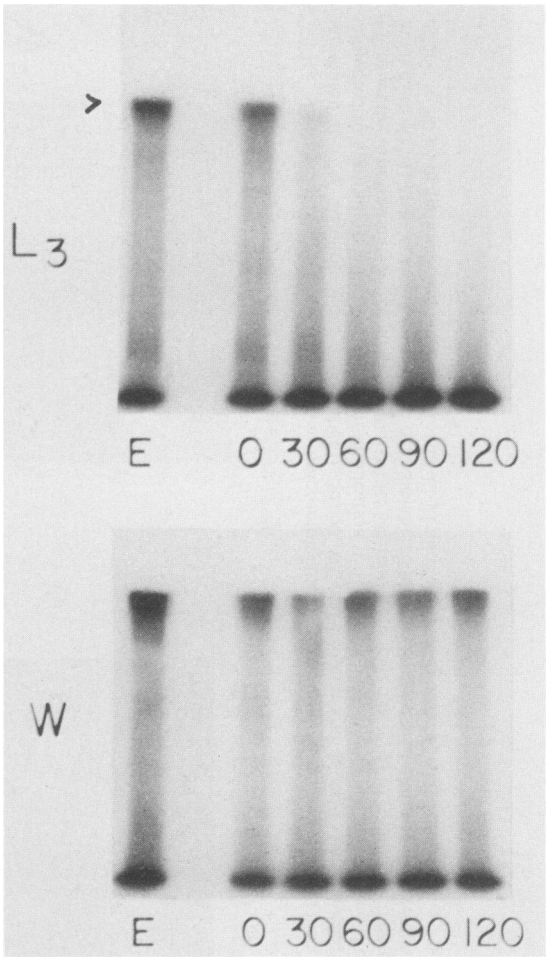

FIG. 11. Degradation of ^{125}I-radiolabeled elastin by *H. contortus* EF. EF containing 34 μg of protein in 20 μl was added to 20 μl of RPMI 1640 to which we added 80 μl of radiolabeled elastin (E), and the preparation was incubated at 37°C. Aliquots (12 μl) were removed and added to sample buffer at 0, 30, 60, 90, and 120 min. The samples were run on a 12.5% SDS–PAGE gel containing gelatin as described in the legend to Fig. 10. L_3, Third-stage larvae; W, larval washings.

skin stretched over a hole cut into cork that floated on saline (floating raft), Goodey observed large numbers of discarded sheaths of *A. caninum* on the skin surface, leading him and other workers to propose that exsheathment must occur at the point of entry (9). The results of more recent studies suggest that ensheathed *Ancylostoma tubaeforme* larvae may also penetrate, however (23, 24). Our results failed to resolve this point but suggested that exsheathment occurs when larvae encounter resistance (i.e., unbroken skin) and that with large tears ensheathed larvae achieve some degree of penetration. As rupture of the sheath may be required to release nematode larval proteases, the timing of exsheathment may determine whether proteases are released upon entry into the skin (penetration) or at some later point in connective tissue migration.

The two species of hookworm larvae which we studied penetrated skin in similar ways, and they were also very similar with respect to protease composition. Both *A. caninum* and *A. duodenale* homogenates contained 68- and 38-kilodalton metalloprotease activities. It is possible that the higher-molecular-weight component was a dimer, zy-

mogen, or some other precursor form. However, metalloproteases frequently autolyze to lower-molecular-weight species (19), particularly during purification.

The hookworm proteases may represent the equivalent of other alkaline metalloproteases that have been identified from infective soil-transmitted nematode larvae (4, 8, 20, 27). However, the exact function of these metalloproteases remains unclear. McKerrow et al. offered compelling evidence that for *Strongyloides stercoralis* they effect skin and connective tissue invasion, analogous to the serine protease from the preacetabular glands of *Schistosoma mansoni* cercariae (26–28). Skin invasion by *Strongyloides stercoralis* is inhibited by coincubation of larvae with 2 mM *o*-phenanthroline (27). Similarly, cellular destruction through the epidermis has been observed to be correlated with an alkaline protease activity from *Necator americanus* (25).

Ancylostoma larval metalloproteases may also function to achieve connective tissue invasion. In our studies both *A. caninum* and *A. duodenale* larvae degraded radiolabeled fibronectin as well as a comparable number of *Strongyloides stercoralis* larvae did. The degradative pattern was similar to the pattern of interdomain cleavage of fibronectin produced by a zinc metalloprotease from *Serratia marcescens* (30). Although neither *Ancylostoma* species degraded laminin in vitro, this does not appear to be a prerequisite for basement membrane penetration, as metastatic tumor cells also release metalloproteases that facilitate passage yet cannot degrade laminin (21). However, *Strongyloides stercoralis* larvae were more efficient at degrading the elastin substrate and even the laminin substrate. This finding may reflect the observation that *Strongyloides stercoralis* is an obligate skin penetrator, whereas both *Ancylostoma* species are facultative skin penetrators, since they are also orally infective. Indeed, both *Ancylostoma* species have been passaged many times via gastric tubes or gelatin capsules in laboratory animals (18), so that a less efficient skin-penetrating population of larvae may have been selected. Matthews has suggested that skin penetration by the cat hookworm *A. tubaeforme* may be mechanical and not require proteases because he found that this species cannot degrade radiolabeled bovine serum albumin (24). Our findings are similar, but we also determined that bovine serum albumin is particularly resistant to degradation by either *Strongyloides stercoralis* or *A. caninum* and is therefore not an acceptable substrate for detecting nematode larval protease activity (data not shown).

The *Ancylostoma* metalloproteases may also mediate exsheathment and ecdysis. The infective larval stage of the ruminant trichostrongyle *H. contortus* releases a zinc alkaline metalloprotease into the EF that mediates breakdown of the cuticle (sheath) in a region that is 20 μm from the anterior end (7, 8). This breakdown permits the release of an anterior cuticular cap that permits the subsequent escape of the larvae through the resultant opening (7, 8). The exsheathment process is a prerequisite for further development within the vertebrate host. The major protease from *H. contortus* EF migrated with an apparent molecular weight on gelatin gels that was similar to the molecular weight of the lower-molecular-weight protease from *Ancylostoma* larvae. Like *Ancylostoma* species, *H. contortus* EF contained higher-molecular-weight proteases. The functions of histolysis and ecdysis may not be mutually exclusive, as the EF protease degraded connective tissue macromolecules in vitro.

The *Ancylostoma* proteases may also mediate digestion to coincide with feeding when feeding resumes after larval penetration (11). Feeding continues through two parasitic molts and into adulthood in the small intestine. The association of protease activity with feeding is consistent with the finding that polyclonal antibody to a protease in adult worms immunologically cross-reacts with larval homogenates (14). Studies are under way to purify the larval *Ancylostoma* metalloproteases and to examine their function in parasite invasion and development.

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