AD-A040 614 BAYLOR COLL OF MEDICINE HOUSTON TEX DEPT OF BIOCHEMISTRY F/G 6/5
MECHANISMS OF SKIN PENETRATION BY 'SCHISTOSOMA MANSONI' CERCARI--ETC(U)
APR 77 M H DRESDEN DADA17-72-C-2184

UNCLASSIFIED NL

1 OF 1
ADA
040614

END

DATE
FILMED
7-77

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
Mechanisms of Skin Penetration by Schistosoma mansoni Cercariae

5. TYPE OF REPORT & PERIOD COVERED
Final Report - July 1, 1972 to March 30, 1977

6. PERFORMING ORG. REPORT NUMBER
1 Jul 72-30 Mar 77.

7. AUTHOR(S)
Marc H. Dresden, Ph.D.

8. CONTRACT OR GRANT NUMBER(s)
DADA 17-72-C-2184

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Dr. Marc H. Dresden, Associate Professor
Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
25 Apr 77

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development Command
Washington, D.C. 20314

12. REPORT DATE
April 25, 1977

13. NUMBER OF PAGES
12 17p.

14. MONITORING AGENCY NAME & ADDRESS (If different from Controlling Office)

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Distribution of this document is unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)
Distribution of this document is unlimited.

DDC
JUN 14 1977
B

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Schistosoma mansoni	Zinc
Schistosomiasis	Antisera
Cercariae	Alpha-1-antitrypsin
Proteolytic enzymes	Antiproteases
Calcium	Secretory granules

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report discusses the final status of studies of the mechanisms by which cercariae of the human parasite, Schistosoma mansoni penetrate the skin of vertebrate hosts. These studies were undertaken to investigate means by which the processes can be controlled for possible therapeutic and prophylactic intervention.

The penetration processes are mediated by secretion of proteolytic enzymes from the preacetabular glands of the cercariae. Isolation and partial characterization of these enzymes has been

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

410228

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. Abstract

achieved, and it is concluded that these enzymes resemble the vertebrate chymotrypsins. The enzymes are inhibited by several of the human serum antiproteases as well as by specific chymotrypsin and serine protease inhibitors. Studies on the possible inhibitory activity of γ-globulins from sera of immunized animals are being continued.

The role of these proteolytic enzymes in the penetration of skin has been studied using model connective tissue substrates in vitro. Major proteolytic effects are observed on the protein backbone of proteoglycans and on non-collagenous basement membrane proteins. Minor proteolytic effects are observed on elastic fibers and on the keratinaceous layer of the outer skin. No effect of these enzymes on polysaccharides or on collagen fibers of the dermis has been demonstrated. These studies suggest the major role of these cercarial enzymes in penetration is a proteolytic action on the epidermal basement membrane and on the proteoglycans of the dermis.

The preacetabular glands also contain calcium and the localization and quantitation of the calcium content of these glands has been reported. The calcium has been shown to be present in the preacetabular glands as calcium carbonate. We have suggested that these calcium deposits function as in situ inhibitors of the cercarial proteases during storage in the preacetabular glands. Further studies are currently being carried out to isolate the protease- and calcium-containing granules of these preacetabular glands by sucrose gradient centrifugation techniques.

We have reported that zinc salts were very effective in inhibition of the protease activity from these preacetabular glands. Furthermore, we have demonstrated that zinc is effective in killing both cercariae and schistosomules. These results suggest that the use of zinc salts in a prophylactic manner in infected waters and streams may open new avenues for control of schistosomiasis.

(12)

A D_____

Mechanisms of Skin Penetration by *Schistosoma mansoni* Cercariae

Final Report

Marc H. Dresden, Ph.D.
Department of Biochemistry

April 1977

Supported by
U.S. Army Medical Research and Development Command
Washington, D.C. 20314

Contract No. DADA-17-72-C-2184

Baylor College of Medicine

DDC Availability Statement
"Approved for public release: distribution unlimited"

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

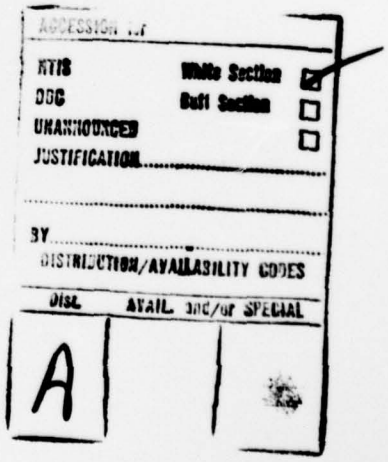
Final Report

Mechanisms of Skin Penetration by *Schistosoma mansoni* Cercariae

Contract No. DADA 17-72-C-2184

Marc H. Dresden, Ph.D.
Department of Biochemistry
Baylor College of Medicine
Houston, Texas 77030

April 1977



Summary

This report discusses the final status of studies of the mechanisms by which cercariae of the human parasite, Schistosoma mansoni penetrate the skin of vertebrate hosts. These studies were undertaken to investigate means by which the processes can be controlled for possible therapeutic and prophylactic intervention.

The penetration processes are mediated by secretion of proteolytic enzymes from the preacetabular glands of the cercariae. Isolation and partial characterization of these enzymes has been achieved, and it is concluded that these enzymes resemble the vertebrate chymotrypsins. The enzymes are inhibited by several of the human serum antiproteases as well as by specific chymotrypsin and serine protease inhibitors. Studies on the possible inhibitory activity of γ-globulins from sera of immunized animals are being continued.

The role of these proteolytic enzymes in the penetration of skin has been studied using model connective tissue substrates in vitro. Major proteolytic effects are observed on the protein backbone of proteoglycans and on non-collagenous basement membrane proteins. Minor proteolytic effects are observed on elastic fibers and on the keratinaceous layer of the outer skin. No effect of these enzymes on polysaccharides or on collagen fibers of the dermis has been demonstrated. These studies suggest the major role of these cercarial enzymes in penetration is a proteolytic action on the epidermal basement membrane and on the proteoglycans of the dermis.

The preacetabular glands also contain calcium and the localization and quantitation of the calcium content of these glands has been reported. The calcium has been shown to be present in the preacetabular glands as calcium carbonate. We have suggested that these calcium deposits function as in situ inhibitors of the cercarial proteases during storage in the preacetabular glands. Further studies are currently being carried out to isolate the protease- and calcium-containing granules of these preacetabular glands by sucrose gradient centrifugation techniques.

We have reported that zinc salts were very effective in inhibition of the protease activity from these preacetabular glands. Furthermore, we have demonstrated that zinc is effective in killing both cercariae and schistosomules. These results suggest that the use of zinc salts in a proplylactic manner in infected waters and streams may open new avenues for control of schistosomiasis.

FINAL PROGRESS REPORT 1972-1977

During the term of this project we have studied several aspects of the biochemistry of schistosome penetration through host skin.

A. Enzymology

The enzymology and biochemistry of the proteolytic enzymes found in cercarial secretions and homogenates has been investigated. We have reported partial characterization of these proteases in two separate publications (1,2). These studies are being continued using a variety of chromatographic techniques in order to isolate these enzymes in pure form. We have utilized Sephadex G-150, molecular sieve chromatography, preparative isoelectric focusing, ion-exchange chromatography and affinity chromatography in order to purify these enzymes. The present status of this aspect of the project is that the schistosomes appear to contain one major protease species and possibly two minor species. All species appear to consist of serine proteases of molecular weight 25,000 to 28,000 daltons: they differ in isoelectric point, but respond similarly to certain inhibitors and divalent cations. The amino acid composition of these enzymes is not known at the present time.

We have utilized specific protease inhibitors in order to characterize the preacetabular proteases. The results of these studies have been presented at several meetings (3,4) and are summarized in Tables 1 and 2. They indicate that: 1) Cercarial proteases are not trypsin-like nor are they elastase-like, although these enzymes have been previously shown to digest elastin substrates (5,1). 2) Their inhibition spectra is similar but not identical to those of the vertebrate chymotrypsins. The peptide derivative acetyl-phenylalanyl-glycyl-alanyl-leucyl-chloromethylketone is the most effective inhibitor of the cercarial proteases we have found to date. Furthermore, studies using the oxidized B-chain of insulin as a model substrate has furthermore strengthened our contention that we are dealing with a chymotryptic-like protease because the cleavage pattern of this substrate resembles that found for pancreatic chymotrypsin.

B. Serum inhibitors

Determination of whether infected hosts develop antibodies to these proteases may provide information not only on the role of these enzymes in penetration but also their relationships to pathogenesis and to immune-related phenomena. Their purification, for example, might lead to a sensitive serological or skin test (see for example: 6). Also, some authors have considered cercarial and schistosomule stages to be significant in

immunity to schistosomiasis and these enzymes may be important antigens in this respect. Such information may also be relevant to the dermatitis observed in the infection of humans with non-human schistosomes (swimmer's itch).

We have previously reported that normal human serum inhibits protease activity. This is to be expected in view of the multiple protease inhibitors reported to be present in the serum (7). This work was continued by examination of the various Cohn fractions of human serum and it was demonstrated that only Cohn fraction 4 inhibited cercarial protease activity. This fraction contains the α_1 and α_2 globulins. We have now shown that purified α_2-macroglobulin and α_1-antitrypsin fractions inhibit protease activity. Furthermore, two other purified human serum antiproteases, α_1-antichymotrypsin and C'-1 inactivator, also inhibit cercarial protease activity. On the other hand, the two remaining serum protease inhibitors, inter-α-inhibitor and antithrombin-III have no effect on the cercarial proteases. Some 92% of the inhibitory activity in normal human serum can be accounted for by the effects of α_1-antitrypsin and another 6% by α_2-macroglobulin. This work is in press (8).

We have recently examined a number of sera from infected animals provided to us by Drs. Patricia Minard and Darwin Murrell from the Naval Medical Research Institute, in order to determine whether the γ-globulin fractions of infected and noninfected animals might contain antiprotease activity. We have determined that the γ-globulins from noninfected sera do not contain antiprotease activity. On the other hand, a single serum prepared against schistosomes has been shown to contain antiprotease activity in the γ-globulin fraction. We are currently continuing these studies in order to determine whether the IgG fractions from immune sera might be effective in inhibiting cercarial protease activity and therefore cercarial penetration.

C. Effects of Divalent Cations

We previously demonstrated that divalent cations affect dramatically the protease activity of cercarial homogenates and secretions (9). Dual effects of Ca^{++} and Mg^{++} on cercarial proteases (stimulation at low concentrations, 0-10mm. and inhibition at high concentrations, above 10mm) were intriguing in view of the observations of Stirewalt (10) and Lewert (11) and their collaborators that the preacetabular glands of cercariae contain large amounts of calcium. The cercarial proteases

are also found in these glands. Using the technique of electron probe spectroscopy we demonstrated that indeed, most of the calcium of the cercariae is localized in the preacetabular glands (12). Using atomic absorption spectroscopy it was possible to quantitate the levels of calcium; approximately 0.01 to 0.15µg of calcium are found per cercariae. More recent studies using chemical assays have been used to demonstrate that the majority of this calcium is present in the form of calcium carbonate (13). Because of our previous results showing that insoluble calcium carbonate can bind the cercarial proteases reversibly we have suggested that the preacetabular calcium deposits serve to maintain the proteases in an inactive form prior to secretion of the preacetabular gland contents. This hypothesis is being further investigated by attempts to isolate the secretion granules from these preacetabular glands. The techniques used for these studies include sonication of cercariae, followed by low speed centrifugation on sucrose gradients. Fractions which exhibited protease activity when treated by the neutral detergent Triton X-100 are then removed from the gradient, and layered on a second set of sucrose gradients which are spun at high speeds (100,000 xg). Active fractions from these gradients were removed and subjected to fixation by glutaraldehyde and prepared for electron microscopy. Our preliminary results suggest that we have been successful in isolating the preacetabular secretory granules from the schistosome cercariae as shown in Figure 1. These studies are continuing.

In the course of our studies on the effects of divalent cations, inhibition by low concentrations of zinc salts was particularly striking. Lewert and his coworkers previously reported that zinc salts inhibited cercarial infectivity (11). We have reinvestigated the question of which of the steps in infectivity might be inhibited by zinc. The results of these studies have been recently published (14). In brief, zinc salts at low levels (0.05-5mm) dramatically decrease cercarial longevity and produce crenation of cercarial bodies and decaudation. Zinc salts also appear to decrease survival of schistosomules. In efforts to determine whether zinc salts may prove effective in combating schistosomiasis, we are currently carrying out in vivo studies on the effects of zinc on schistosomule activity and survival.

D. Role of proteases in penetration

Our studies to determine the role of the schistosomal proteases in penetration have been directed by utilizing connective tissue substrates. These studies demonstrated that cercarial proteases were able to hydrolyze basement membrane proteins isolated from glomerular basement membrane (15). This substrate is hydrolyzed only in its

non-collagenous portions, but not in its collagenous fractions. In addition, major hydrolytic activity was observed against the protein backbone of the proteoglycan from cartilage, chondromucoprotein (1). Further, a significant hydrolysis of elastin (1,5) and keratin (15) could be demonstrated by these in vitro tests. On the other hand, the carbohydrate components of chondromucoprotein, i.e. haluronic acid, chondroitin sulfates a, c, and d and dermatan sulfate were not hydrolyzed by the preparations from cercarial secretions or homogenates. In addition, skin collagen, either in soluble or fibrous form, was not hydrolyzed by the cercarial secretions. These studies have strongly suggested that the major activity of the proteases in penetration of cercariae through host skin is by proteolytic action on the non-collagenous protein of the basement membrane between epidermis and dermis and by hydrolysis of the protein backbone of the proteoglycans which make up the dermal matrix. These studies are supportive of earlier histological observations of Lewert and his collaborators. Currently attempts are underway to utilize specific inhibitors of these proteases in skin preparations in order to determine whether this approach might be fruitful for preventing penetration of schistosome cercariae through host skin.

Literature Cited

1. M.H. Dresden and H.L. Asch, Proteolytic enzymes in extracts of Schistosoma mansoni cercariae, Biochim. Biophys. Acta 289, 378 (1972).

2. D.L. Campbell, P.J.F. Frappaolo, M.A. Stirewalt and M.H. Dresden, Schistosoma mansoni: Partial characterization of enzyme(s) secreted from the preacetabular glands of cercariae, Exptl. Parasitol. 40, 33 (1976).

3. M.H. Dresden and H.L. Asch, Schistosome cercarial proteases: Substrates and inhibitors, Am. Soc. Parasitol. (1975).

4. M.H. Dresden and H.L. Asch, The proteases of Schistosoma mansoni cercariae, Fed. Proc. 35, 1475 (1976).

5. G. Gazzinelli and J. Pellegrino, Elastolytic activity of Schistosoma mansoni cercarial extracts, J. Parasitol. 591 (1964).

6. A.W. Senft and S.E. Maddison, Hypersensitivity to parasite proteolytic enzyme in schistosomiasis, Am. J. Trop. Med. Hyg. 24, 83 (1975).

7. H.L. Asch and M.H. Dresden, Schistosome cercarial proteases: Inhibition by serum components, Am. Soc. Parasitol. (1975).

8. H.L. Asch and M.H. Dresden, Schistosoma mansoni: Inhibition of cercarial "penetration" proteases by components of mammalian blood, Comp. Biochem. Physiol. (in press).

9. M.H. Dresden and E.M. Edlin, Schistosoma mansoni: Effects of some cations on the proteolytic enzymes of cercariae, Exptl. Parasitol. 35, 299 (1974).

10. M.A. Stirewalt, Isolation and Characterization of deposits of secretion from the acetabular gland complex of cercariae of Schistosoma mansoni, Exptl. Parasitol. 8, 199 (1959).

11. R.M. Lewert, D.R. Hopkins, and S. Mandlowitz, The role of calcium and magnesium in invasiveness of schistosome cercariae, Am. J. Trop. Med. Hyg. 15, 314 (1966).

12. M.H. Dresden and E.M. Edlin, Schistosoma mansoni: Calcium content of cercariae and its effects on protease activity in vitro, J. Parasitol. 61, 398 (1975).

13. M.H. Dresden and H.L. Asch, Calcium carbonate content of the preacetabular glands of Schistosoma mansoni cercariae, J. Parasitol. 63, 163 (1977).

14. H.L. Asch and M.H. Dresden, Schistosoma mansoni: Effects of zinc on cercarial and schistosomule viability, J. Parasitol. 63, 80 (1977).

15. M.H. Dresden, J.C. Lewis and I. Krisko, Proteolytic action of Schistosoma mansoni cercarial proteases in keratin and basement membrane proteins, J. Parasitol. (in press).

Table 1. Effect of Low-Molecular Weight Inhibitors on Cercarial Protease Secretions

No inhibition (at 1mM or greater)

EDTA - Metalloproteins

Tosyllysine chloromethyl ketone - Trypsin

Ac-Ala-Pro-Ala-Ala-CH_2Cl-Leucocyte Elastase

Ac-Ala-Ala-Pro-Ala-CH_2Cl-Leucocyte and Pancreatic Elastase

Pepstatin-Acidic Proteases

Inhibition (concentration to achieve 50% inhibition)

DIFP, PMSF - Serine Proteases - 1 mM

TPCK - Chymotrypsin - 0.8 mM

Z-Gly-Leu-Phe-CH_2Cl-Chymotrypsin - 0.11 mM

Z-Phe-CH_2CL-Chymotrypsin - 0.08 mM

Ac-Phe-Gly-Ala-Leu-CH_2Cl-Chymotrypsin - 0.005-0.1 mM

Assays of proteolytic activity were carried out at 35°C for 16-20 hours in a volume of 1.0 ml glycine-NaOH buffer, pH 8.8, containing 2 mM $CaCl_2$. Inhibitors were suspended in this buffer and their activity assessed at 3-5 different concentrations. The concentration of inhibitor required to achieve 50% inhibition of protease activity was determined.

Table 2. Macromolecular Inhibitors of Cercarial Proteases

Significant Inhibition (at < 1μM)

Human Alpha-1-Antitrypsin

Human Alpha-2-Macroglobulin

Human C'-1-Inactivator

Human Alpha-1-antichymotrypsin

Potato Chymotrypsin Inhibitors I and II

No significant Inhibition

Human Inter-Alpha-Trypsin Inhibitor

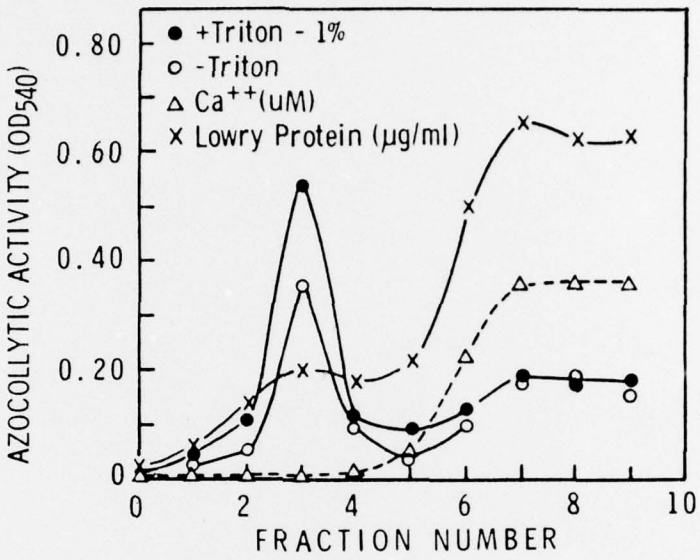
Human Antithrombin III

Soybean Trypsin Inhibitor

Penguin-Ovomucoid-Subtilisin Inhibitor

Assays of proteolytic activity were carried out at 35°C for 16-20 hours in a volume of 1.0 ml glycine-NaOH buffer, pH 8.8, containing 2 mM $CaCl_2$. Inhibitors were suspended in this buffer and their activity assessed at 3-5 different concentrations. The concentration of inhibitor required to achieve 50% inhibition of protease activity was determined.

Sucrose Gradient Fractionation of Proteases and Calcium from Cercarial Homogenates



Approximate 10^5 cercariae (in 10% sucrose, 0.05M glycine-NaOH, pH 8.8) were sonicated and the debris was removed by centrifugation at 600 xg for 6 minutes. The supernatant was layered on a discontinuous sucrose gradient (final volume 5 ml, 10% to 80% sucrose). Following centrifugation at 100,000 xg for 55 min., 0.5 ml fractions were collected and assayed for protein content (x-x) , calcium content (Δ-Δ) and protease activity. For the latter assay each tube contained 2 mg Azocoll, 7.5 µl sample and the sucrose-glycine buffer, with (●-●) or without (o-o) Triton X-100. Incubations were carried out for 18 hrs. at 36°C.

Bibliography of Research Supported by this Contract

Published and In-Press Manuscripts

1. Marc H. Dresden and Harold L. Asch, "Proteolytic Enzymes in Extracts of Schistosoma mansoni Cercariae," Biochimica et Biophysica Acta 289:378-384 (1972).

2. Marc H. Dresden and Edith M. Edlin, "Schistosoma mansoni: Effect of Some Cations on the Proteolytic Enzymes of Cercariae," Experimental Parasitology 35:299-303 (1974).

3. Harold L. Asch and Clark P. Read, "Transtegumental Absorption of Amino Acids by Male Schistosoma mansoni," Journal of Parasitology 61:378-379 (1975).

4. Marc H. Dresden and Edith M. Edlin, "Schistosoma mansoni: Calcium Content of Cercariae and Its Effects on Protease Activity in vitro," Journal of Parasitology 61:398-402 (1975).

5. Dale L. Campbell and P.J.F. Frappaolo, M.A. Stirewalt and Marc H. Dresden, "Schistosoma mansoni: Partial Characterization of Enzyme(s) Secreted from the Preacetabular Glands of Cercariae," Experimental Parasitology 40:33-40 (1976).

6. Harold L. Asch and Marc H. Dresden, "Schistosoma mansoni: Effects of Zinc on Cercarial and Schistosomule Viability," Journal of Parasitology 63:80-86 (1977).

7. M.H. Dresden and H.L. Asch, "Calcium Content of the Preacetabular Glands of Schistosoma mansoni Cercariae," Journal of Parasitology 63:163-165 (1977).

8. Harold L. Asch and Marc H. Dresden, "Schistosoma mansoni: Inhibition of Cercarial "Penetration" Proteases by Components of Mammalian Blood," Comparative Biochemistry and Physiology (in press).

9. Marc H. Dresden, J.C. Lewis, and I. Krisko, "Proteolytic Action of Schistosoma mansoni Cercarial Proteases on Keratin and Basement Membrane Proteins," Journal of Parasitology (in press).

Two additional manuscripts supported by this grant are currently in preparation.

Abstracts - National and International Meetings

1. H.L. Asch and M.H. Dresden, "Schistosoma mansoni: Biochemical Aspects of Morphogenesis from Cercariae to Adult," American Society of Parasitologists (1974).

2. M.H. Dresden, E.M. Edlin and H.L. Asch, "The Penetration Protease of Schistosoma mansoni Cercariae," Fed. Proc. 34:533 (1975).

3. H.L. Asch and M.H. Dresden, "Schistosome Cercarial Proteases: Inhibition by Serum Components," American Society of Parasitologists (1975).

4. M.H. Dresden and H.L. Asch, "Schistosome Cercarial Proteases: Substrates and Inhibitors," American Society of Parasitology (1975).

5. M.H. Dresden and H.L. Asch, "Proteases of Schistosoma mansoni Cercariae," International Congress on Schistosomiasis, Cairo, (1975).

6. M.H. Dresden and H.L. Asch, "The Proteases of Schistosoma mansoni Cercariae," Fed. Proc. 35:1475 (1976).

7. H.L. Asch and M.H. Dresden, "Schistosoma mansoni Cercarial Proteases: Inhibition by Specific Serum Inhibitors and by an IgG Fraction from Blood of Immunized Mice," American Society of Parasitologists (1976).

8. H.L. Asch and M.H. Dresden, "Effects of Specific Components from Immune and Nonimmune Serum on the Proteolytic Enzymes of Schistosoma mansoni Cercariae," Presented at the IVth Latin American Congress of Parasitology, San Jose, Costa Rica (1976).

9. H.L. Asch and M.H. Dresden, "Isolation of Membrane-Bound Secretory Proteases from Schistosoma mansoni Cercariae," American Society of Parasitologists (1977).

Personnel Employed on Grant

Professional Personnel

Harold L. Asch, Ph.D. - Research Associate

Technical Personnel

Edith M. Edlin - Research Assistant
Dora M. Payne - Research Technician
Mary L. Mailman - Technician
Valory R. Munroe - Technician
Sydney A. Westphal - Laboratory Assistant

Undergraduate Trainees and Laboratory Helpers

Steven Lopez - Undergraduate Trainee
Jay B. Murry - Laboratory Helper
Lamar Pittmann - Laboratory Helper
Maria A. Rodriguez - Laboratory Helper

Clerical and Service Personnel

Allie F. Holman - Technician
Victoria A. Lynch - Technician
Linda K. Reader - Research Assistant
Georgia West - Secretary

DISTRIBUTION LIST

4 copies	HQDA (SGRD-RP) WASH DC 20314
12 copies	Defense Documentation Center (DDC) ATTN: DDC-TCA Cameron Station Alexandria, Virginia 22314
1 copy	Superintendent Academy of Health Sciences, US Army ATTN: AHS-COM Fort Sam Houston, Texas 78234
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences Office of the Secretary of Defense 6917 Arlington Road Bethesda, MD 20014