THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ENZYME STUDIES WITH HATCHING FLUID AND DEVELOPMENTAL STAGES OF *ASCARIS SUUM*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1971

ENZYME STUDIES WITH HATCHING FLUID AND

DEVELOPMENTAL STAGES OF *ASCARIS SUUM*

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Michael H. Ivey for his invaluable guidance and advice throughout the course of this investigation and preparation of the manuscript. Credit also goes to Dr. Gilbert Castro and other faculty members of the Department of Parasitology and Laboratory Practice, School of Health for their helpful assistance. Special thanks go to my wife, Carol, for her support and patient indulgence and for her typing of the manuscript.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

The process of development of parasitic organisms and the manner in which they infect their host has received the attention of workers for many years. While much has been learned about processes of development and infection, numerous questions remain unanswered. Greater knowledge is necessary in order to understand clearly the biology of parasites and to develop better means of diagnosis, treatment, and control.

With certain helminth parasites, the active role of the organism in the infectious process begins when the larva emerges from its egg or sheath following ingestion by the host. Previous studies with several intestinal nematode species suggested that the hatching process was triggered by local stimuli from the host, inducing the larva to secrete substances that act on the egg shell or sheath to break it down, thus enabling escape. *Ascaris lumbricoides* which parasitizes the small intestine of man and pigs is an example of such a nematode.

Infective eggs of *Ascaris* can be induced to hatch by placing them in an *in vitro* environment physiologically similar to that in the

intestine. Rupture of the egg during the hatching process liberates a biologically complex substance called hatching fluid (Rogers, 1958). Evidence indicated that this substance aids the larva in emerging from the egg, since hatching fluid alone causes infective eggs to hatch.

In the egg, the second stage infective larva of Ascaris is surrounded by a more or less impermeable lipid membrane, which in turn is surrounded by a middle layer composed primarily of chitin, and, finally, an outer protein coat. If hatching fluid aids the hatching process, quite likely it contains enzymes capable of attacking the components of the surrounding membrane as well as the chitin and protein layers. Rogers (1958) demonstrated the presence of a chitinase and an esterase in the hatching fluid. The esterase could conceivably alter the permeability of the inner membrane allowing the chitinase to pass through the membrane and hydrolyze the chitin layer. Rogers, however, failed to demonstrate proteinase activity in the fluid but suggested that proteinase might be present.

Upon escaping from the egg, the infective Ascaris larva must penetrate the wall of the intestine and begin a migration that will carry it through the lungs and eventually back to the small intestine where maturation is completed. To facilitate its migration the larva probably also secretes enzymes that attack structural materials in the host tissues. This has not yet been shown with Ascaris; however, penetration factors have been demonstrated for a variety of other helminth parasites (Lewert and Lee, 1954, 1955, 1956).

Immunologic studies of metabolites from various parasites suggest that enzymes and other physiologically active substances might

serve as antigens for inducing immunity and may also have value for diagnostic serological tests (Campbell, 1955; Thorson, 1956). Rabbits experimentally infected with Ascaris were found to produce precipitating antibodies to Ascaris hatching fluid (Soulsby, 1963), but the protective value of such antibodies was not determined. Immunoelectrophoretic analysis indicated that hatching fluid contained a minimum of five antigenic components (Justus, 1968). Also chitinase activity in hatching fluid can be completely inhibited by rabbit antisera prepared against the unaltered fluid (Justus, 1968).

Although earlier studies have revealed some interesting information on the antigenic and enzymatic nature of hatching fluid, there have been no critical analyses of its individual components. Also, it is not really clear to what extent the various components are involved in the mechanism of hatching and the over-all infectious process.

It was the purpose of the present investigation to:

1. develop better assays for chitinase and esterase and to devise an assay system capable of demonstrating proteinase in hatching fluid.
2. analyze the relationship these enzymes may have with various stages of egg development, the hatching process, and post-hatching existence.
3. determine the possible role(s) that hatching fluid enzymes may play in invasion of the host and whether the host produces antibodies against enzymatically active components of the hatching fluid.

Literature Review

Numerous investigations have been made into the chemical and physical composition of the egg envelope of ascarids. As early as 1852, Nelson suggested the presence of three surrounding layers in the eggs of Ascaris mystax. Ackert (1931), Wottge (1937), and Chitwood (1938) described a three layered envelope for the eggs of Ascaridia lineata, Ascaris megalocephala and Ascaris lumbricoides var. suum, respectively. Reports by various workers on the chemical nature of the envelope also indicated that there were three layers: a lipoidal inner membrane, a chitinous middle layer, and an outer coat of protein (Faure-Fremiet, 1913, 1913a; Chitwood, 1938; Christenson, et al., 1942; Timm, 1950; Monne and Honig, 1954). Kreuzer (1953) and Frenzen (1954), however, included an additional protein layer (termed the upper membrane) between the chitin shell and outer coat. Rogers (1956) published the results of electron microscope studies of the eggs of Ascaris lumbricoides var. suum in which he found a thin, very electron dense zone between the thick chitin shell and the outer coat. Based on higher magnifications, he regarded it as having a "typical chitin structure" and considered it to be a part of the chitin shell.

Fairbairn and Passey (1955) showed ascaryl alcohol to be the major lipid component of the inner membrane but suggested that the membrane was chemically more complex. By the use of paper chromatography, Jaskoski (1962) identified nine amino acids from the inner membrane and judged it to be a lipo-protein. Kreuzer (1954) and Monne and Honig (1954) indicated this layer to be a permeability barrier to protect the egg from chemical damage.

Kreuzer (1954) suggested that the rigid chitin layer provided structural stability and resistance to mechanical harm. Jaskoski (1962) employed paper chromatography to demonstrate the presence of at least seven different amino acids in this layer. He regarded this amino acid fraction as probably corresponding to the upper membrane described by Kreuzer rather than an integral part of the chitin zone itself.

Kreuzer (1953) found the outer coat to be resistant to pepsin and trypsin. Upon identifying ten amino acids from this layer by paper chromatography, she concluded that it is a scleroprotein. Ammon and Debusmann-Morgenroth (1953) showed it to be resistant to papain as well. Again by the use of paper chromatography, Jaskoski (1962) found sixteen amino acids occurring in the coat. Monne and Honig (1954) considered the protein in the outer coat to be quinone-tanned and to have acquired its inert, resistant nature during passage through the intestines of the host. It appears then that the egg envelope of an organism such as Ascaris consists of a relatively impermeable inner membrane, a very tough middle shell, and a rather chemically inert outer coat.

Experiments aimed at inducing infective eggs to hatch in vitro have been conducted with ascarids and many other parasitic helminths. Early reports of in vitro hatching of ascarid eggs were described by McRae (1935) as having been the result of mechanical injury to the shell from laboratory manipulation rather than real biological hatching. Yoshida and Toyoda (1938) reported the hatching of eggs of Ascaris lumbricoides var. suum when maintained at 40 C for five or six days in a Ringer-Tyrode's solution containing tissue extracts and other complex materials. Fenwick (1939) was able to hatch Ascaris eggs by treating

them first with sodium hypochlorite and then pressing them between a slide and a coverslip. Pitts (1948) demonstrated that when Ascaris eggs were pretreated in sodium hypochlorite they could be hatched by incubation in physiological saline for four weeks at 37.5 C or by physical treatments such as centrifugation. He failed to hatch eggs by the methods of Yoshida and Toyoda (1938) and suggested that they had probably used sodium hypochlorite as a disinfectant which could have accounted for their positive results. O'Connor (1950), however, did achieve success with the techniques of Yoshida and Toyoda and did so without employing sodium hypochlorite. He further reported that the eggs hatched equally well in Tyrode's solution with or without the addition of trypsin, sodium glycocholate, gastric mucin, and pancreatic extract. He concluded from the microscopic observation of hatching larvae that disintegration of the egg shell occurred from the inside of the egg by the action of embryo secretions.

Hatching of helminth larvae such as ascarids prior to ingestion by the host renders them less capable of establishing an infection than when an intact egg is swallowed (Hansen, et al., 1955). Infective Ascaris eggs typically remain dormant until ingested by the host (Passey and Fairbairn, 1955). Rogers (1958) considered that conditions within the gut of the host stimulate the unhatched larvae to produce secretions which would digest the egg envelope. He induced in vitro hatching within only a few hours by incubating infective eggs in a buffer solution containing reducing agents, undissociated carbonic acid, and dissolved gaseous CO_2. He showed chitinase and esterase to be present in the hatching fluid which was released from the egg but was unable to show a

proteinase. He suggested that the role of the esterase might be to alter the permeability of the inner membrane so that the chitinase and proteinase (if present) could attack the middle and outer zones of the envelope. Fairbairn (1961) further refined the techniques for in vitro hatching of Ascaris lumbricoides and reported that hatching of 80-95 per cent of infective eggs could be obtained in 3 hours under conditions similar to those used by Rogers (1958). He also showed the stimulus to be ineffective when applied to preinfective stages. Hass and Todd (1962) extended the techniques of Fairbairn to accomplish hatching of several other ascarids as well as two species of Trichuris. Jaskoski and Colucci (1964) obtained nearly 100 per cent hatching of Ascaris suum within a 3 hour period in a medium devoid of reducing agents but saturated with CO_2. However, eggs which had not been previously "deshelled" in sodium hypochlorite to remove all but the inner membrane would not hatch, in contrast to the experiments of Rogers (1958, 1960) and Fairbairn (1961). Fairbairn considered the true physiological conditions for the stimulus to be: (1) a pH of near 7.0, (2) a temperature near that of warm-blooded animals, (3) reducing conditions, (4) and a partial pressure of CO_2 approximating 5 volume per cent.

Secretions which might aid in the penetration or migration through host tissue have been demonstrated with several helminths. From histochemical studies, Lewert and Lee (1954) reported that skin penetration by cercariae of Schistosoma mansoni and Schistosomatium douthitti and by filariform larvae of Ancylostoma caninum, Strongyloides ratti, and Strongyloides simiae caused changes in the tissue indicative of glycoprotein depolymerization. These workers described a similar phenomenon in

the vicinity of developing Taenia taeniaeformis larvae in the livers of rats (1955). Beckett (1961) reported histochemical changes in mouse muscle fibers injected with Trichinella spiralis, suggesting the secretion of lytic enzymes by this parasite.

Lewert and Lee (1954) showed the presence of collagenase-like enzymes in extracts of the filariform larvae of several nematodes as well as in extracts of schistosome cercariae. They also showed that certain of these extracts possessed gelatinolytic activity. Lewert (1958) and Stirewalt (1963) also reported proteinase activity in schistosome cercarial preparations when tested against hemoglobin and casein.

Several workers (Stirewalt and Evans, 1952; Lincicome, 1953; Lee and Lewert, 1957) have presented indirect evidence for hyaluronidase activity in extracts of nematode larvae and schisotosome cercariae by employing the streptococcal decapsulation test. Hyaluronidase activity in these extracts was not shown, however, when purified hyaluronic acid was used as a substrate (Lee and Lewert, 1957). On the other hand, Levine, et al., (1948) reported that preparations of schistosome cercariae hydrolyzed an unpurified umbilical cord hyaluronic acid preparation.

The possibility that helminth enzyme secretions may have an important role in bringing about a protective immune response was suggested by Chandler in 1932. Taliaferro and Sarles (1937) reported the occurence of precipitates at the ends of Nippostrongylus muris larvae located in the skin and lungs of rats. They also demonstrated the formation of oral and anal precipitates on Nippostrongylus larvae that were incubated in homologous antiserum (1939). These results strongly

suggested an antigenic role for larval excretions and secretions produced at these orifices. Campbell (1955) found that animals immunized against excretions and secretions of Trichinella spiralis acquired a partial immunity to the worm. Thorson (1956) showed that sera from immune dogs inhibited the activity of proteinase in esophageal extracts of adult Ancylostoma caninum. He also showed that immunization of non-infected dogs with these extracts produced an immunity which resulted in milder infections than those of non-immunized controls (1956a). Rhodes, et al., (1965) demonstrated the production of a partial immunity to Ascaris in guinea pigs that had been immunized with purified Ascaris malic dehydrogenase. However, the proteinase (Thorson, 1956) and malic dehydrogenase (Rhodes, et al., 1965) were both prepared from extracts and were not shown to have been secreted by the worms. Soulsby (1963) reported precipitin bands by agar-gel diffusion when Ascaris hatching fluid was reacted with antisera from rabbits infected with Ascaris. He also pointed to the possible diagnostic value of such antigenic materials.

CHAPTER II

MATERIALS AND METHODS

Cleaning of Glassware

Glassware, with the exception of pipettes, was washed in a Heineke laboratory washer with rinses in both tap water and distilled water. This was followed by three rinses with deionized water. Pipets were cleaned by soaking for 3 to 4 hours in a dichromate-sulfuric acid cleaning solution. They were then rinsed for at least 8 hours in running tap water followed by two rinses in distilled water and three final rinses in deionized water.

Embryonation and Hatching of Eggs

Mature female Ascaris worms were collected from a local slaughterhouse, and the terminal one-third of the uterus of each was resected. The uterine sections were placed in 50 ml conical centrifuge tubes to which approximately three volumes of 2 per cent sodium hydroxide was then added. These were maintained at 4 C for a period of 20-24 hours with four or five centrifugations and changes of sodium hydroxide. Centrifugation was done, as in all other instances, at the lowest speed (less than 2,000 X G) that would sediment the eggs in 5 minutes. This treatment was sufficient to dissolve the uterus and the outer protein coat of the eggs. The decoated eggs were repeatedly washed with

distilled water by centrifugation until the pH of the supernatant fluid was that of distilled water.

Following the water washings, an equal volume of 0.1 Normal sulfuric acid (containing 1:10,000 aqueous merthiolate to inhibit the growth of microbial contaminants) was added to each tube of packed eggs. One ml aliquots of the egg suspension was pipetted into sterile disposable plastic petri dishes to which approximately 8.0 ml of 0.1 N sulfuric acid with merthiolate was subsequently added. The dishes with lids in place were put in sterile petri dish cans, and blotter discs soaked in 1:10,000 aqueous merthiolate were positioned between every two or three dishes to provide a humid environment. The cans, with ventilation holes open, were incubated at 30 C for 28 days in an incubator, the inside of which had been previously exposed to short-wave ultraviolet light for 5 minutes or longer. The blotter discs were replaced by freshly wetted ones at the end of 14 days at which time the incubator was once again treated with ultraviolet light. At the end of 28 days the eggs were examined microscopically. If at least 90 per cent of the eggs were fully embryonated and viable, the eggs were collected by pouring the petri dish contents through a large glass funnel into 50 ml centrifuge tubes. A rubber spatula was employed to loosen those eggs which adhered to the dish bottoms. These eggs were centrifuged and washed once with distilled water followed again by centrifugation leaving the eggs at a slightly acid pH. The subsequent treatment of the eggs with sodium hypochlorite was more effective at an acid pH.

With certain modifications, techniques described by Fairbairn (1961) were then used for the <u>in vitro</u> hatching of the eggs. The chitin

shells were removed from the eggs by placing approximately 10 ml of eggs along with three to five volumes of fresh 6 per cent sodium hypochlorite (Purex) in 500 or 1000 ml erlenmeyer flasks followed by gentle shaking in a 35 C water bath. Deshelling normally was complete within 2 hours but occasionally required 3 or 4 hours. When microscopic observation revealed the complete removal of shells, the sodium hypochlorite was diluted at least 1:1 with water and the eggs were centrifuged. The eggs were washed twice with water (carefully suspending them by means of a stream of water from a squeeze bottle or by gentle stirring with a glass rod) by centrifugation and aspiration of the supernatant fluid. They were then dialyzed overnight against cold running tap water or careful washings and centrifugations were continued (six or eight times) to remove most of the hypochlorite. Next the eggs were washed with distilled water until no odor of hypochlorite could be detected and then washed twice more with deionized water.

Hatching was accomplished in 500 or 1000 ml erlenmeyer or side-arm flasks equipped with holed-rubber stoppers and glass and rubber tubing so that gas could be bubbled through the hatching medium inside. Approximately 10 ml of the washed, deshelled eggs were placed in each flask along with four volumes of a 0.25 Molar sodium chloride - 0.1 M sodium bisulfite solution and incubated at 38-39 C for about 5 minutes in a water bath shaker. A 0.1 M sodium bicarbonate solution was gassed in a separate flask at 39 C with a 95 per cent nitrogen - 5 per cent carbon dioxide mixture until the pH fell to 8.5 or slightly lower. An equal volume of the gassed $NaHCO_3$ solution was added to the hatching flasks containing the $NaCl$-$NaHSO_3$ egg suspension. The complete hatching medium

was then flushed with the same gas mixture for 5 minutes at which time all tubes were pinched closed, trapping the gas inside the flasks. Shaking was then begun and continued until hatching was completed. At the end of 2 hours hatching was arbitrarily terminated to limit the contamination of hatching fluid with excretions and secretions produced by larvae after hatching. The hatch rate was often 90 per cent or more and seldom less than 80 per cent as determined by microscopic examination.

Following the hatch, the larvae, ruptured egg membranes, and unhatched eggs were removed by centrifugation in conical 50 ml centrifuge tubes at 2,000 X G (1,500 X G if the larvae were to be saved for experiments) for 10 minutes. The supernatant which contained the hatching fluid was centrifuged at 15,000 X G for at least 30 minutes at 4 C. The supernatant was carefully drawn off and dialyzed against four changes of deionized water at 4 C for 24 hours. The dialysate was then lyophilized and kept in a vial in a dessicator jar at 4 C until protein determinations and enzyme assays were conducted.

Protein Determination

With slight modification, the method of Lang (1958) was employed for the microdetermination of Kjeldahl nitrogen using a sample of 1.0 mg dry weight or 1.0 ml volume. The nitrogen value (mg) was multiplied by the factor 6.25 to give milligrams protein. In the current studies, 0.2 ml of 30 percent H_2O_2 was included in the digestion mixture to eliminate turbidity by oxidizing nonprotein materials.

Preparation of Antisera

Antisera against hatching fluid was prepared in albino rabbits weighing 2.5-3.2 kg. A single lot of hatching fluid known to have high activity with respect to chitinase, esterase, and proteinase was used for the immunizations. Lyophilized hatching fluid dissolved in 0.5 ml of 0.85 per cent sodium chloride was mixed with an equal volume of incomplete Freund's adjuvant. Rabbits were injected subcutaneously on the back with an initial dose of 3.0 mg (dry weight) followed by subsequent injections of 1.0 mg at two week intervals. Once the animals showed strong Arthus reactions, immunizations were discontinued and intracardial bleedings were commenced.

Infection antisera prepared in rabbits against *Ascaris suum* and *Toxocara canis* was kindly supplied by Dr. M. H. Ivey and Dr. T. I. Aljeboori.

Chitinase Assay

Three mg of chitin were placed in a conical 15 ml centrifuge tube. The substrate employed was a lyophilized chitin colloid prepared from *Ascaris* egg shells as described in a later section. One mg of a lyophilized enzyme preparation suspended in 1.0 ml of 0.1 M phosphate buffer, pH 6.0, was added to the tube. Appropriate substrate controls were always included. The tubes were stoppered and slanted in a water bath shaker and agitated for 15 minutes at 38 C. At the end of the incubation period all tubes were placed immediately in crushed ice and then centrifuged at 2,000 to 2,300 X G for 3 minutes at 4 C. Finally 0.5 ml of the supernatant was drawn off and assayed for free N-acetylglucosamine by the method of Reissig, *et al.*, (1955).

Preparation of Substrates for Chitinase Assay

Several lobster chitin and *Ascaris* egg shell chitin preparations were compared as to their value as a substrate for chitinase. Crude, technical lobster chitin and lobster chitin purified according to the method of Reynolds (1954) to remove calcium salts and other nonchitinous materials were employed as finely ground and coarse, gramular forms. Also, colloids were prepared from crude and purified lobster chitin as described by Skujens, *et al.*, (1965) and then lyophilized to yield a dry, fluffy preparation.

Chitin from *Ascaris* eggs was prepared by the following procedure. Eggs were collected from female worms by dissolving the entire length of resected uteri in 2 per cent sodium hydroxide. The treatment in sodium hydroxide with several changes over a period of 24 hours dissolved the uterus as well as the protein coat of the eggs. The eggs were then caused to burst with a French press cell at 20,000 psi and extracted repeatedly with sodium hydroxide using centrifugation during a period of about 6 hours to remove protein and other materials. The extraction was terminated when the absorbance of the supernatant read at 280 mμ was essentially zero. Optical density measurements were made in a Beckman Model DB-G Spectrophotometer. To remove lipid materials, the egg shells were next extracted twice with acetone in a separatory funnel with an acetone to egg shell ratio of about 3:1 for a period of 3 to 5 minutes with frequent shaking. They were allowed to settle and were drained through the stopcock. Three additional lipid extractions were performed using diethyl ether and water. The chitin settled in the water layer and was drained through the stopcock as before. The

remaining water and ether were discarded. Following the ether-water extractions the egg shells were washed two or three times with distilled water in a separatory funnel and then dialyzed for 24 hours against three changes of distilled water and one change of deionized water. Finally, the purified shells were lyophilized. Some of the material was further purified by the method of Reynolds (1954). The purified and repurified Ascaris egg shell chitin preparations were used in the dry form and also prepared into colloids and relyophilized.

All substrates tested were in a dry form and 3 mg samples of each were used along with 1.0 mg hatching fluid per tube. The assays were conducted as previously described.

Influence of pH and Type of Buffer on Chitinase Activity

Four different types of buffers and a wide range of pH values were used to determine which buffer and pH provided optimal conditions for the assay of hatching fluid chitinase. The buffers and pH values utilized were: 0.1 M acetate, pH 4.0-5.0; 0.1 M phthalate, pH 5.0-6.0; 0.1 M phosphate, pH 5.5-8.0; 0.1 M borate, pH 8.0-9.0. Hatching fluid was used as the source of chitinase and was made up in a concentration of 1.0 mg per ml of deionized water. A 3 mg sample of lyophilized chitin substrate was placed in each tube followed by the addition of 1.0 ml of a particular buffer. The enzyme preparation was then added to each tube, and the tubes were incubated at 38 C for 15 minutes in a water bath shaker. The remainder of the assay was carried out as described earlier.

Chitinase Temperature Curve

Assays were conducted at temperatures ranging from 25 C to 65 C to explore the effect that incubation temperature might have on chitinase activity. A 1.2 mg sample of hatching fluid was dissolved in 1.2 ml of 0.1 M phosphate buffer, pH 6.0, in each of several tubes and held at the appropriate temperature for 1 minute. Then 1.0 ml aliquots were transferred to tubes containing 3 mg chitin substrate. These were incubated at the chosen temperature for 15 minutes and assayed according to the standard procedure.

Chitinase Stability

Fresh hatching fluid was placed in dialysis tubing and concentrated to a small volume over a 5 hour period at 4 C by the use of dry polyvinylpyrrolidone. The concentrated hatching fluid was then dialyzed for 24 hours at 4 C against four changes of deionized water.

The following treatments were done in duplicate. Three tubes containing 0.5 ml of hatching fluid each were frozen at -20 C in a deep freeze. One was frozen only once, while a second was thawed at 25 C and refrozen, and a third was thawed and refrozen twice. During the period when the freezing experiments were conducted a tube with 0.5 ml of hatching fluid was heated at 65 C for 30 minutes and another was heated for 5 minutes at 95 C. At the end of the heating periods, the tubes were cooled immediately and held at 4 C until assayed for chitinase. A tube containing 0.5 ml of hatching fluid was held at 4 C to serve as an untreated control sample. The three frozen samples were thawed together at 25 C to be assayed for chitinase along with the heated and untreated samples. The total time elapsed during the above operations was less

than 1 hour. To each of the tubes was added 3.0 mg of chitin substrate followed by 0.5 ml of 0.1 M phosphate buffer, pH 6.0. These were reacted at 38 C according to the chitinase assay procedure for 15 minutes after which 0.5 ml aliquots of supernatant were removed to separate tubes that were then tightly stoppered and frozen to be held for N-acetylglucosamine determinations.

A tube containing 0.5 ml of hatching fluid was tightly stoppered and maintained at 4 C for 24 hours while a second was kept at that temperature for 72 hours. Tubes were also maintained for 24 and 72 hours at 25 C. At the end of each time period, the hatching fluid was incubated with chitin. Following centrifugation to remove the unhydrolyzed substrate, the supernatants were frozen.

To investigate the effect of lyophilization, 3.0 ml of hatching fluid was placed in a serum bottle and lyophilized. It was reconstituted with exactly 3.0 ml of deionized water and a 0.5 ml sample was removed for assaying. The 2.5 ml of hatching fluid remaining in the bottle was relyophilized, then reconstituted with 2.5 ml of deionized water. A 0.5 ml aliquot was subsequently withdrawn for assaying.

N-acetylglucosamine determinations were performed at one time for the samples from all the various treatments.

Antibody Inhibition of Hatching Fluid Chitinase

Inhibition of chitinase by antibody was studied by the methods of Justus (1968) except for slight modifications. In the present study 3.0 mg of lyophilized Ascaris egg shell chitin colloid were used per tube (Justus had used 1.0 mg of lobster chitin preparation per tube). Also, the period of incubation of chitinase with the substrate was

reduced from 3 hours to 90 minutes. Finally, in the actual determination of free N-acetylglucosamine, the potassium tetraborate heating step was carried out at 65 C for 1 hour rather than 70 C for 45 minutes. This change eliminated all serum coagulation.

The serum sample tested included Toxocara infection antisera, Ascaris infection antisera, antisera prepared against Ascaris hatching fluid, and normal serum. Hatching fluid in 125 and 250 µg amounts was used with each serum. Controls in 0.1 M phosphate buffer, pH 6.0, were included for both concentrations of enzyme. Also, controls for each of the sera were set up without hatching fluid since the sera themselves gave rather high optical density readings.

Esterase Assay

The esterase assay was based on the methods of Huggins and Lapides (1947). The substrate was prepared by dissolving 44 mg of p-nitrophenyl acetate in 4.0 ml of methanol. Two ml of this solution were then added very slowly to 98.0 ml of deionized water in a 250 ml erlenmeyer flask on a magnetic stirrer. The preparation was stable for one week or longer at 4 C.

Either 1 mg of lyophilized hatching fluid or 0.5 ml of hatching fluid in solution was placed in a test tube along with 1.0 ml of 0.067 M phosphate buffer, pH 7.0, and 3.0 ml of deionized water (2.5 ml if the hatching fluid was added as a solution). This was followed by 1.0 ml of substrate. The tubes were incubated in a water bath at 38 C for 15 minutes after which they were cooled in crushed ice for about 1 minute. Optical density readings were made in a Beckman Model DB-G Spectrophotometer. Readings were taken against a substrate control that had also

been incubated at 38 C for 15 minutes. A standard curve of p-nitrophenol was constructed with a range of 0.1 to 1.0 micromoles.

Esterase Temperature Curve

Assays were performed at temperatures ranging from 25 C to 40 C to determine if the incubation temperature has an effect on hatching fluid esterase activity. For each temperature studied, a test tube containing 1.0 ml of p-nitrophenyl acetate substrate, 1.0 ml of 0.067 M phosphate buffer, pH 7.0, and 2.5 ml of deionized water was incubated in a water bath at the chosen temperature for 5 minutes along with a second tube containing 1.2 mg of hatching fluid in 0.6 ml of deionized water. This permitted the substrate solution and hatching fluid mixture to equilibrate with the water bath temperature. Next 0.5 ml of the hatching fluid suspension was withdrawn from the second tube and was added to the substrate tube which was then incubated at the same temperature for 15 minutes. Substrate controls were included for each temperature. The remainder of the assay was performed as previously described (Esterase Assay).

Anson Proteinase Assay

With modifications, the techniques described by Anson (1938) were employed as one method for the demonstration and assay of proteinase. Casein was the substrate routinely used. A 1.0 per cent casein solution was prepared by heating 1.0 g of powdered casein with 100 ml of 0.1 M phosphate buffer, pH 8.0, at 100 C for 15 to 30 minutes until dissolved. One or 2.0 mg of enzyme preparation (eg. hatching fluid in 2.0 ml of 0.1 M phosphate buffer, pH 8.0) were placed in a test tube followed by

1.0 ml of the casein solution. Controls consisting of casein only in buffer and enzyme preparation only in buffer were also included. The tubes were incubated in a water bath at 38 C for 15 to 30 minutes at which time the reaction was stopped by adding 5.0 ml of 0.3 N trichloroacetic acid. The mixture was then filtered through Whatman No. 3 filter paper, and 2.5 ml of the filtrate was transferred to another tube. Five ml of 0.5 N NaOH were added to the filtrate. With the tube held in place on a Vortex mixer, 1.5 ml of Folin phenol reagent (diluted with twice its volume of distilled water) was added slowly. The phenol reagent was added at the fastest rate that still permitted it to fall as discrete drops. The tubes were allowed to set approximately 10 minutes for color development after which optical density readings were taken at 750 mμ on a Beckman DB-G Spectrophotometer.

Azocoll Proteinase Assay

Proteinase was also assayed by the use of dye-containing collagen substrate Azocoll (Calbiochem) roughly according to the procedure described by the manufacturer. Twenty-five mg of Azocoll were placed in a 25 ml or 50 ml erlenmeyer flask in a water bath set at 38 C. Then 4.0 ml of 0.05 M borate buffer, pH 8.8, was carefully added so that the insoluble Azocoll floated on the surface. The enzyme preparation (normally 1.0 mg) was added in 1.0 ml of borate buffer, and the flask was incubated at 38 C with very slow, gentle shaking for 15 minutes. A substrate control was always included. The reaction was stopped when the substrate was removed by filtration with Whatman No. 1 filter paper. The optical density of the filtrate was read at 520 mμ on a Beckman DB-G Spectrophotometer.

Influence of pH and Type of Buffer on Proteinase Activity

The possible effect that different pH values and different buffers might have on proteinase activity as determined by the Anson method was investigated. Assays were conducted essentially as described (Anson Proteinase Assay). The substrate, however, consisted of 10 mg of powdered casein per assay tube rather than 1.0 ml of a 1 per cent casein solution. The buffers studied were 0.10 M phosphate (pH 6.0-8.0) and 0.05 M borate buffer (pH 8.0-9.0).

Into each test tube a 10.0 mg sample of powdered casein was placed followed by 2.0 ml of a particular buffer at a given pH. Next 1.0 mg of hatching fluid in 1.0 ml of deionized water was added to each tube. The tubes were stoppered and slanted in a water bath shaker. They were incubated at 36 C with shaking for a period of 30 minutes at which time the assay was completed as described earlier. For each pH and buffer, both substrate and hatching fluid controls were included.

Assays were also carried out to see what effect varying pH values and different buffers might have on activity when the Azocoll method was used. A 25 mg amount of Azocoll was placed in each 50 ml erlenmeyer flask followed by 4.0 ml of the appropriate buffer (0.05 M borate, pH 8.0-10.0; 0.05 M phosphate, pH 6.0-8.0). A 1.0 ml aliquot of deionized water containing 1.0 mg of hatching fluid was then added, and the assay was completed as described (Azocoll Proteinase Assay). Substrate controls were included for each pH and buffer tested.

Proteinase Temperature Curve

Assays were conducted by the Azocoll procedure at temperatures ranging from 25 C to 65 C. A 1.5 ml sample of buffer containing 0.75 mg

of hatching fluid was pipetted into each of ten test tubes. Into each of ten 50 ml erlenmeyer flasks were placed 25 mg of Azocoll followed by 4.0 ml of borate buffer. The tubes and flasks were incubated in water baths at the various temperatures for 1 minute. Then 1.0 ml aliquots of the hatching fluid were transferred from the test tubes to the corresponding flasks which were incubated for 15 minutes at the chosen temperatures with intermittent gentle shaking. Substrate controls were set up for each temperature. The remainder of the assay was carried out as described (Azocoll Proteinase Assay).

Comparison of Substrates for Anson Proteinase Assay

Samples of both denatured hemoglobin and casein weighing 5.0 mg and 10.0 mg were placed in test tubes followed by 2.0 ml of 0.067 M phosphate buffer, pH 7.5. The tubes were mixed for 10 seconds each at the slowest speed on a Vortex Genie mixer. Next 1.0 ml aliquots of phosphate buffer containing 2.0 mg of hatching fluid was added. The tubes were slanted in a water bath shaker and incubated for 30 minutes at 38 C with shaking. The remainder of the assay was conducted according to the Anson method described earlier.

Antibody Inhibition of Proteinase

Prior to use, all sera were cleared by centrifugation at 25,000 X G for 45 minutes at 4 C (Campbell, et al., 1964). Each serum sample (diluted 1:2 in 0.85 per cent sodium chloride) was placed in a separate test tube along with a 1.0 mg amount of hatching fluid known to possess proteinase. These were incubated in a water bath at 37 C for 1 hour and then held for 2 hours at 4 C. One ml of 0.1 M phosphate buffer, pH 8.0

was added to each tube. The tubes were adjusted to pH 8.0, when necessary, by adding a small amount of 0.5 N sodium hydroxide. The volumes were equalized in all tubes by the addition of the required amounts of phosphate buffer. Next 1.0 ml of a 1 per cent casein solution in phosphate buffer was pipetted into each tube. All tubes were incubated in a water bath at 38 C for 30 minutes. For each of the serum samples a control consisting of hatching fluid (denatured by heating at 100 C for 5 minutes), serum, and casein was included.

The assay for proteinase was conducted according to the Anson method previously described with one exception. Two and one-half ml of 1.0 N sodium hydroxide was added to the filtrate following TCA protein precipitation rather than the 5.0 ml of 0.5 N sodium hydroxide. This modification was somewhat more sensitive since there was less dilution with the smaller volume of sodium hydroxide.

Collection of Second-stage Larval Excretions and Secretions (E.S.)

Infective eggs were hatched, and the freed second-stage larvae were washed several times with a lactated Ringers solution (Eli Lilly) by low speed centrifugation. In some experiments, larvae were separated from the small percentage of unhatched eggs that were present by a modified Baermann apparatus employing a cotton pad on a sheet of lens paper (Scientific Products) as the filter for removing the eggs. The washed larvae were placed in a 250 ml erlenmeyer flask along with a lactated Ringers solution containing 200 µg of penicillin and streptomycin each per ml. They were incubated with gentle shaking at 38 C for 12 hours in a water bath shaker after which time the larvae were removed by centrifugation at 2,000 X G for 5 minutes at 4 C. The supernatant was withdrawn

and centriguged at 15,000 X G for 30 minutes or longer at 4 C. Following the high speed centrifugation, the supernatant was recovered and dialyzed at 4 C for 24 hours against four changes of deionized water after which time it was lyophilized.

Preparation of Sonicates

Deshelled eggs or hatched second-stage larvae were placed in physiological saline and subjected to sonic oscillation for 3 to 5 minutes in a Raytheon Sonic Oscillator set at 0.75 amperes. This treatment was sufficient to break up about 90 per cent of the eggs or larvae. Ice water was circulated through the cooling jacket of the oscillation chamber during operation to prevent the denaturing of proteins by excessive heat.

The sonicated material was transferred to a 50 ml conical centrifuge tube and centrifuged at 2,000 X G for approximately 5 minutes at 4 C to remove most of the particulate material. The supernatant, which was extremely turbid, was withdrawn and centrifuged at 20,000 X G for 1 hour at 4 C. Following centrifugation, a white pellicle that formed on the surface, was removed by careful suction using a Pasteur pipet connected to a vacuum line. The supernatant was further cleared by centrifugation at 25,000 X G for 2 hours at 4 C. Any surface pellicle was removed as described and discarded. If the supernatant did not appear quite clear, centrifugations at 25,000 X G were continued. After all centrifugations were completed, the supernatant was dialyzed against four changes of deionized water at 4 C during a period of 24 hours. The sonicate was then lyophilized and held at 4 C as described for hatching fluid (Embryonation and Hatching of Eggs).

Hyaluronidase Assay of Ascaris Materials

A turbidimetric assay procedure described in the Worthington Enzymes Manual (1968) and based on the methods of Kass and Seastone (1944) and Tolksdorf, et al., (1949) was employed with slight modification for the assay of hyaluronidase. A hyaluronic acid (Nutritional Biochemical Corp.) solution of 0.4 mg/ml was prepared in a buffer of 0.1 M monosodium phosphate and 0.15 M sodium chloride at pH 5.3. A 3.0 mg sample of enzyme preparation was placed in a test tube along with 0.5 ml of buffer. Following the addition of 0.5 ml of substrate, the tube was incubated for 2 hours at 38 C in a water bath. A substrate control tube was also incubated. The assay was completed according to the procedure mentioned above. The turbidity change occurring in the control was subtracted from the change in the test samples.

The materials tested for presence of hyaluronidase included hatching fluid, second-stage larval excretions and secretions, and extracts from sonicates of hatched second-stage larvae and deshelled infective eggs.

Comparison of Different Hatching Techniques in Terms of the Enzyme Composition of Hatching Fluid and Excretions and Secretions (E.S.) of Hatched Larvae

Infective eggs from the same lot were stimulated to hatch by the method previously described (Embryonation and Hatching of Eggs) and by the technique of Jaskoski (1964) which employed 100 per cent carbon dioxide but no reducing agent. The hatching stimulus was applied for 2 hours after which the percentage of hatching was determined microscopically. Hatching fluid and E.S. were collected and assays for chitinase, esterase, and proteinase were conducted as previously described.

Collection and Enzyme Analysis of Egg Fluid

The fluid bathing the unhatched second-stage larvae was secured by chemical and mechanical methods. Infective eggs were deshelled as described (Embryonation and Hatching of Eggs). In the chemical method adapted from the procedure of Haskins and Weinstein (1957), eggs were suspended in 0.85 per cent sodium chloride in a 50 ml erlenmeyer flask. A few drops of xylene were added, and the flask was incubated in a water bath shaker at 35 C for 1 hour with gentle shaking. This was sufficient to dissolve nearly 100 per cent of the membranes. The membranes were also ruptured mechanically by forcing a suspension of the deshelled eggs through a 30 gauge hypodermic needle. This resulted in the liberation of approximately 25 per cent of the larvae. In both procedures, the larvae and remaining deshelled eggs were removed by centrifugation at 2,000 X G. The supernatant was recentrifuged at 15,000 X G for 30 minutes at 4 C. The supernatant was then dialyzed and lyophilized. Assays were conducted for chitinase, esterase, and proteinase.

Enzyme Analysis of Developmental Stages

Eggs were set up for embryonating at intervals of approximately 3 days so that after 28 days there were eggs in various stages of embryonation. A sample was held at 4 C for the full 28 days to serve as the unembryonated control sample. At the end of the 28 days, the eggs were deshelled by the sodium hypochlorite method (Embryonation and Hatching of Eggs). Sonicates of the various stages were prepared and processed according to the procedure previously described (Preparation of Sonicates). Also an attempt was made to hatch eggs from the 14-, 18-, 21-,

and 28-day samples. The sonicates and hatching fluid samples were assayed for chitinase, esterase, and proteinase.

Preliminary Attempts to Isolate the Enzymes in Hatching Fluid

Isolation of Chitinase by Adsorption on Lyophilized Chitin

Twenty mg of lyophilized hatching fluid, previously shown to have strong chitinase activity, was divided into two 10.0 mg samples which were treated identically. A 10.0 mg sample was placed in a 15 ml conical centrifuge tube along with 3.0 ml of 0.1 M phosphate buffer, pH 6.0, and 10 mg of lyophilized Ascaris egg shell chitin substrate. The hatching fluid and chitin were allowed to react at 38 C for 10 minutes with the tube slanted and agitated in a water bath shaker. The tube was subsequently centrifuged at 4 C for 3 minutes at 2,000 X G. The supernatant was transferred to a second centrifuge tube and incubated with 5.0 mg of chitin. Following centrifugation, the supernatant was incubated a third time with chitin (5.0 mg). The chitinase-chitin complex precipitates from the three centrifugations were pooled and washed several times with phosphate buffer until the optical density reading of the washing was nearly zero at 280 mμ. The washings were pooled. Chitinase remaining in the enzyme-substrate complex was recovered by two consecutive elutions with 12 per cent ammonium sulfate. The eluent was separated from the chitin by centrifugation at 2,000 X G for 3 minutes. The precipitate was washed twice with phosphate buffer to remove any chitinase that might have precipitated because of high salt concentration. These washings, along with those above, were pooled with the eluent and then dialyzed and lyophilized. The lyophilized preparation was tested for

chitinase, proteinase, and esterase activity and was also examined by polyacrylamide gel electrophoresis (Davis, 1964).

Fractionation on Sephadex G-100 and DEAE Sephadex A-50

Hatching fluid known to exhibit good chitinase, esterase, and proteinase activity was fractionated on a Sephadex G-100 column. A 60 mg sample in 2.0 ml phosphate buffer was applied to a 1.5 x 30.0 inch gel bed in a water-jacketed lucite (acrylic) plastic column. Coolant, circulated through the column jacket from a Buechler refrigerated fraction collector, kept the column at 3-4 C at all times. The gel was eluted at a rate of approximately 30 ml per hour, employing 1500 ml of 0.1 M phosphate buffer, pH 7.5, with 5 ml volumes being collected per tube. Optical density readings at 280 mμ were used to detect protein-containing fractions, which were then dialyzed and lyophilized. The lyophilized fractions were assayed for chitinase, esterase, and proteinase and were further studied by polyacrylamide gel electrophoresis.

An attempt was made to further purify the chitinase found in one of the Sephadex fractions by fractionation on DEAE Sephadex A-50 at 4 C in the column described above. The column was eluted with 1500 ml of a sodium chloride gradient (0.05-0.20 M) in 0.1 M phosphate buffer, pH 7.5. The elution rate and volumes collected were the same as with Sephadex G-100. Fractions were treated as above and were assayed for chitinase.

Analysis of Enzyme Preparations by Polyacrylamide Gel Disc Electrophoresis

Several enzyme preparations were examined by polyacrylamide gel disc electrophoresis. Equipment for electrophoresis was constructed and

the electrophoresis performed according to specifications and procedures outlined by Davis (1964). Samples of 150 or 200 µg protein were electrophoresed in each tube with a current of 2 ma per tube. Electrophoresis in a tube was halted when the free Bromphenol blue dye reached a point of 2 mm from the bottom of the separation gel. Staining and further treatment of the gels were done according to the methods of Davis (1964).

CHAPTER III

RESULTS

Comparison of Substrates for Chitinase Assay

Several chitin preparations were tested as substrates for *Ascaris* hatching fluid chitinase. One mg of hatching fluid was reacted against each substrate listed in Table 1 at pH 6.0 and 38 C for 15 minutes. The results indicate that *Ascaris* egg shell chitin was a better substrate than lobster chitin. The colloid preparations, especially in the case of lobster chitin, were better substrates than non-colloid preparations. Also, the lobster chitin preparations purified by the method of Reynolds (1964) yielded better results than preparations of crude lobster chitin.

Influence of pH and Type of Buffer on Chitinase Activity

The activity of hatching fluid chitinase against *Ascaris* egg shell chitin was determined at different pH values using acetate, phthalate, phosphate, and borate buffers to vary the pH. Tests were done using 1 mg amounts of hatching fluid at 38 C for 15 minutes. The results (Table 2) showed the greatest enzyme activity occurred between pH 4.5 and 6.5. Optimal activity was achieved in a phosphate buffer at pH 5.8.

TABLE 1

COMPARISON OF SUBSTRATES FOR CHITINASE ASSAY

Substrate Preparation	mμ moles N-Acetylglucosamine/mg protein/minute[a]
Ascaris Egg Shell Chitin Colloid[b]	6.60
Ascaris Egg Shell Chitin[b]	6.54
Ascaris Egg Shell Chitin Colloid[b,c]	5.53
Ascaris Egg Shell Chitin[b,c]	5.47
Lobster Chitin Colloid[c]	5.33
Crude Lobster Chitin Colloid	4.50
Finely ground Lobster Chitin[c]	1.11
Unground Lobster Chitin[c]	0.93
Finely ground Crude Lobster Chitin	0.87
Unground Crude Lobster Chitin	0.81

[a]Mean of three individual determinations.
[b]Purified by method described on page 15.
[c]Purified by method of Reynolds (1964).

TABLE 2

INFLUENCE OF pH ON *ASCARIS* HATCHING FLUID CHITINASE ACTIVITY AGAINST *ASCARIS* EGG SHELL CHITIN

Buffer	pH	mμ moles N-Acetylglucosamine/mg protein/minute[a]
Acetate	4.0	2.88
"	4.5	3.33
"	5.0	3.28
Phthalate	5.0	3.12
"	5.5	3.33
"	5.8	3.61
"	6.0	3.47
Phosphate	5.5	3.34
"	5.8	3.80
"	6.0	3.75
"	6.2	3.49
"	6.5	3.12
"	7.0	2.39
"	7.5	1.84
"	8.0	1.46
Borate	8.0	1.59
"	8.5	1.23
"	9.0	1.04

[a]Mean of three individual determinations.

Chitinase Temperature Curve

Chitinase assays carried out using 1 mg amounts of enzyme at pH 6.0 through a temperature range of 25 C to 65 C revealed an increase in enzyme activity against Ascaris egg shell chitin as the temperature increased from 25 C to 50 C, with activity diminishing with further rise in temperature. The results are shown in Figure 1.

Chitinase Stability

Hatching fluid chitinase received a variety of treatments listed in Table 3. Subsequently 0.5 ml amounts of hatching fluid were reacted against Ascaris egg shell chitin at pH 6.0 and a temperature of 38 C. The data in Table 3 show the chitinase activity to have been affected little, if any, by repeated freezing and thawing, by storage at 4 C for 24 hours, or by hyophilization of the hatching fluid. Temperatures of 25 C for 24 or 72 hours, 65 C for 30 minutes, and 95 C for 5 minutes resulted in a loss of activity ranging from 11.1 per cent to 95.6 per cent.

Antibody Inhibition of Hatching Fluid Chitinase

Experiments were performed to ascertain if antisera from Ascaris- or Toxocara-infected animals would inhibit the activity of Ascaris hatching fluid chitinase. Results given in Table 4 indicate the chitinase is inhibited somewhat by both anti-Ascaris and anti-Toxocara infection antisera with the greater degree of inhibition by the homologous antiserum. Antisera prepared by injecting rabbits with hatching fluid gave virtually complete inhibition.

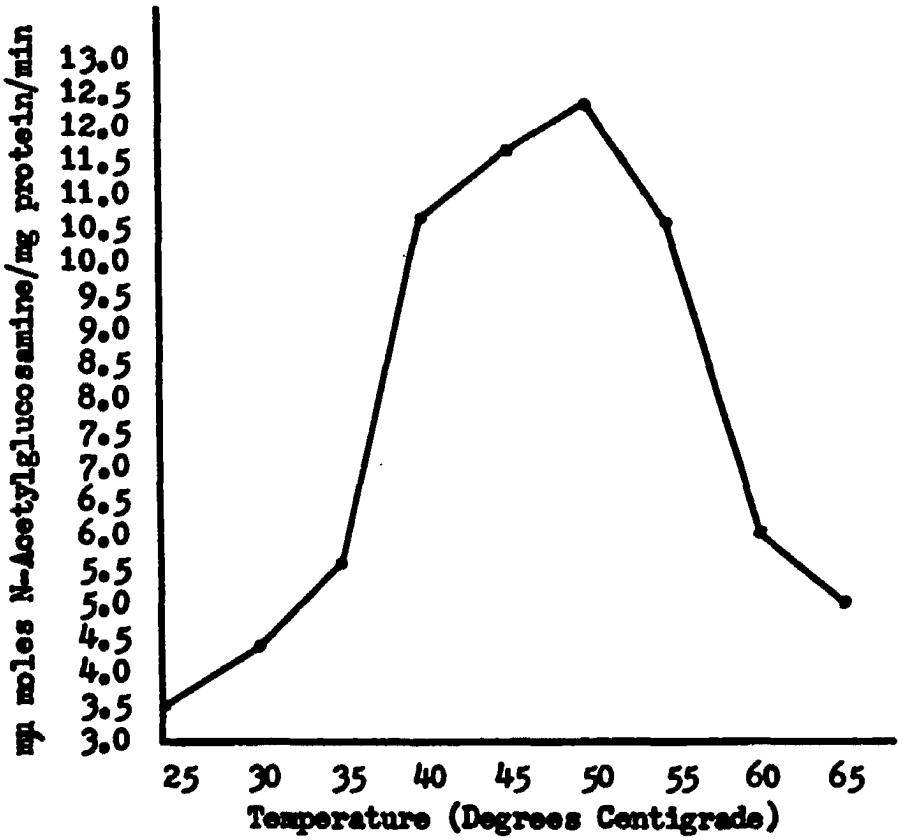

Figure 1. Influence of temperature on Ascaris hatching fluid chitinase activity against Ascaris egg shell chitin. Each point represents the mean of two individual determinations.

TABLE 3

STABILITY OF ASCARIS HATCHING FLUID CHITINASE SUBJECTED TO VARIOUS TREATMENTS

Treatment	mμ moles N-Acetylglucosamine/mg protein/min[a]	% Loss of Activity
Untreated Control	9.02	-
65 C/30 min	1.17	77.0
95 C/5 min	0.40	95.6
4 C/24 hr	8.77	2.8
4 C/72 hr	8.10	11.1
25 C/24 hr	7.63	15.4
25 C/72 hr	6.60	26.8
Frozen Once	8.98	-
Frozen Twice	9.04	-
Frozen Thrice	8.98	-
Lyophilized	8.70	3.6
Relyophilized	8.50	5.8

[a]Mean of two determinations run simultaneously.

TABLE 4

INHIBITION OF ASCARIS HATCHING FLUID CHITINASE
WITH HOMOLOGOUS OR HETEROLOGOUS ANTIBODY

Sample	µg Hatching Fluid	O.D.[a,b]	O.D. difference from Control	% Inhibition
Buffer	125	0.047	-	-
"	250	0.105	-	-
Normal Serum Control	-	0.196	-	-
" " Test	125	0.288	0.092	-
" " Test	250	0.340	0.144	-
Anti-Hatching Fluid Antisera Control	-	0.205	-	-
" " " " Test	125	0.205	0.000	100
" " " " Test	250	0.209	0.004	97.2
Ascaris Infection Antisera Control	-	0.214	-	-
" " " Test	125	0.240	0.026	71.7
" " " Test	250	0.298	0.084	41.7
Toxocara Infection Antisera Control	-	0.126[c]	-	-
" " " Test	125	0.198[c]	0.072	21.7
" " " Test	250	0.248[c]	0.122	15.2

[a] O.D. represents measurement of N-Acetylglucosamine.
[b] Mean of three individual determinations.
[c] Mean of two individual determinations.

Esterase Temperature Curve

Temperatures from 25 C to 40 C were employed to investigate the effect assay temperature might have on the activity of hatching fluid esterase at pH 7.0. The data in Figure 2 indicate that enzyme activity increases up to 40 C. The activity at 30 C or less is very low when compared to the activity at 38 C or 40 C.

Influence of pH and Type of Buffer on Proteinase Activity

Assays were conducted to determine the effect of varying pH values and different buffers on proteinase activity as measured by the Anson method. The results in Table 5 show a maximal activity at pH 8.0 in a phosphate buffer. The activity at the same pH in borate buffer was only about 13.5 per cent of the activity demonstrated in phosphate buffer.

Similar assays carried out by the Azocoll method gave decidedly different results. Data in Table 6 revealed that a borate buffer at pH 8.8 provided maximal proteinase activity.

Proteinase Temperature Curve

The effect of assay temperature on proteinase activity was determined for a temperature range of 25 C to 65 C. The Azocoll method was employed for the assays. There was virtually no reaction at 25 C and activity increased with the temperature to 55 C. Higher temperatures caused a rapid drop in enzyme activity.

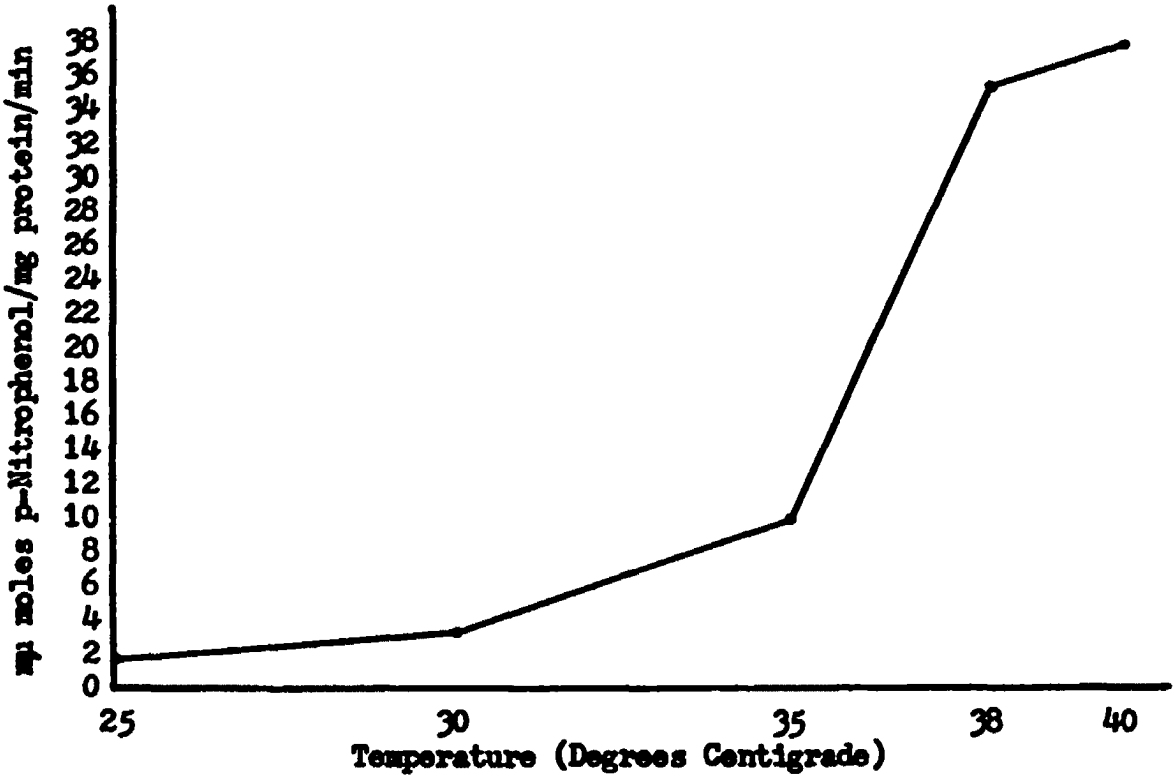

Figure 2. Influence of temperature on Ascaris hatching fluid esterase activity. Each point represents the mean of two individual determinations.

TABLE 5

INFLUENCE OF pH AND BUFFER ON *ASCARIS* HATCHING FLUID PROTEINASE ACTIVITY[a]

Buffer	pH	mμ moles Tyrosine/mg protein/minute[b]
Phosphate	6.0	0.048
"	6.5	1.440
"	7.0	2.800
"	7.5	3.320
"	7.8	4.040
"	8.0	4.250
Borate	8.0	0.570
"	8.5	0.450
"	9.0	0.495

[a]Proteinase activity determined by Anson method.
[b]Mean of three individual determinations.

TABLE 6

INFLUENCE OF pH AND BUFFER ON *ASCARIS* HATCHING FLUID PROTEINASE ACTIVITY[a]

Buffer	pH	O.D. units/mg protein/minute[b]
Phosphate	6.0	0.006
"	6.5	0.011
"	7.0	0.016
"	7.2	0.018
"	7.5	0.027
"	7.8	0.036
"	8.0	0.053
Borate	8.0	0.086
"	8.5	0.107
"	8.8	0.118
"	9.0	0.114
"	9.3	0.059
"	9.5	0.031
"	10.0	0.017

[a]Proteinase activity determined by Azocoll method.
[b]Mean of three individual determinations.

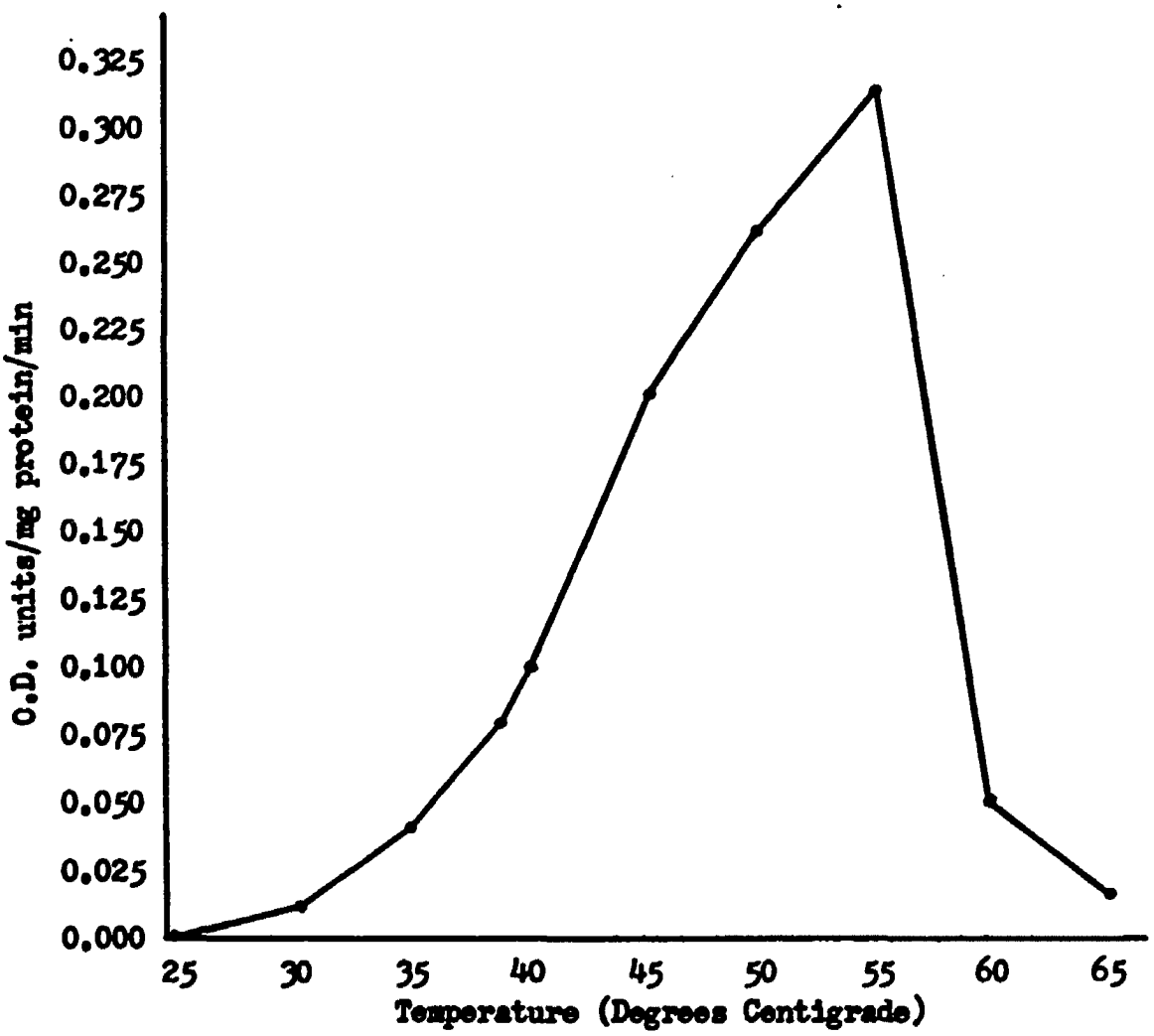

Figure 3. Influence of temperature on Ascaris hatching fluid proteinase activity. Each point represents the mean of two individual determinations.

Comparison of Substrates for Anson Proteinase Assay

Denatured hemoglobin and casein were compared to find which might serve as the better substrate for the assay of hatching fluid proteinase by the Anson method, at pH 7.5 and 38 C. Casein, as seen by the results in Table 7, proved to be the better substrate, although proteinase activity was readily demonstrable by the use of denatured hemoglobin.

Antibody Inhibition of Hatching Fluid Proteinase

The results of experiments conducted to determine if *Ascaris* hatching fluid proteinase was inhibited by anti-hatching fluid antisera or antisera from *Ascaris*- or *Toxocara*-infected animals are shown in Table 8. Enzyme activity was determined at 38 C and pH 8.0 using the Anson method. These results indicate that the proteinase activity was almost completely inhibited by the anti-hatching fluid antisera while the *Ascaris* infection antisera and the *Toxocara* infection antisera both gave partial inhibition. Of the two infection antisera, the stronger inhibition was observed with the homologous antisera.

Hyaluronidase Assay of Ascaris Materials

Experiments performed to test for the presence of hyaluronidase in *Ascaris* preparations revealed that hyaluronidase activity is present in extracts of sonicated hatched larvae and infective eggs. Activity was present to a much less extent in Excretions and Secretions (E.S.) recovered from hatched larvae but was not demonstrated in hatching fluid. The results are given in Table 9.

TABLE 7

COMPARISON OF SUBSTRATES FOR ANSON ASSAY
OF ASCARIS HATCHING FLUID PROTEINASE

Substrate	mg Substrate	mμ mole Tyrosine/mg protein/min[a]
Denatured Hemoglobin	5.0	0.62
Denatured Hemoglobin	10.0	1.10
Casein	5.0	1.32
Casein	10.0	1.93

[a]Mean of three individual determinations.

TABLE 8

ANTIBODY INHIBITION OF *ASCARIS* HATCHING FLUID PROTEINASE

Sample	O.D.[a,b]	O.D. Difference from Control	% Inhibition
Normal Serum Control	0.132	-	-
Normal Serum Test	0.222	0.090	-
Anti-Hatching Fluid Antisera Control	0.126	-	-
Anti-Hatching Fluid Antisera Test	0.127	0.001	98.9
Ascaris Infection Antisera Control	0.142	-	-
Ascaris Infection Antisera Test	0.181	0.039	56.7
Toxocara Infection Antisera Control	0.168	-	-
Toxocara Infection Antisera Test	0.227	0.059	34.5

[a] O.D. represents measurement of Tyrosine.
[b] Mean of three individual determinations.

TABLE 9

HYALURONIDASE ASSAY OF *ASCARIS* MATERIALS

Sample	µg Hyaluronic acid digested in 2 hr[a]
Second stage larvae (hatched)	128
Infective eggs	100
Hatching fluid	none
Second larval E.S.	20
Second larval E.S. (Baermann)	14

[a]Values represent the mean of three separate experiments (two individual determinations each).

Comparison of Different Hatching Techniques in Terms of the Enzyme Composition of Hatching Fluid and Excretions and Secretions (E.S.) of Hatched Larvae

The enzyme activity of hatching fluid and E.S. collected after hatching by the modified Fairbairn method described earlier (Embryonation and Hatching of Eggs) and of hatching fluid and E.S. collected after hatching by the method of Jaskoski (1964) were compared with respect to chitinase, esterase, and proteinase. The results of the proteinase assays (Table 10) show a little difference between the hatching fluid collected by the Fairbairn method and by the Jaskoski method and between the E.S. samples. Esterase assays also revealed essentially no difference between the hatching fluid samples or the E.S. samples collected by the two methods (Table 11). However, the results of the chitinase assays (Table 12) revealed a consistently higher degree of enzyme activity in the hatching fluid recovered by the Fairbairn method than by the Jaskoski method. No difference was found between the two E.S. preparations.

Enzyme Analysis of Egg Fluid

The egg fluid that bathes the unhatched second-stage infective larvae was collected and tested for chitinase, esterase, and proteinase activity. The results (Table 13) showed a somewhat lower degree of chitinase activity in the hatching fluid than in either egg fluid preparation. Table 14 reveals no esterase activity in the egg fluid preparations but a considerable degree of activity in the hatching fluid. The results of the proteinase assays (Table 15) also show activity in the hatching fluid but none in the egg fluid.

TABLE 10

COMPARISON OF THE PROTEINASE ACTIVITY IN ASCARIS HATCHING FLUID (H.F.) AND EXCRETIONS AND SECRETIONS (E.S.) OF LARVAE HATCHED BY DIFFERENT METHODS

Sample	Hatching Technique	O.D./mg protein/min[a]
H.F.	Fairbairn Method	0.092
H.F.	Jaskoski Method	0.084
E.S.	Fairbairn Method	0.088
E.S.	Jaskoski Method	0.095

[a]Values represent the mean of three separate experiments (two individual determinations each).

TABLE 11

COMPARISON OF THE ESTERASE ACTIVITY IN ASCARIS HATCHING FLUID (H.F.) AND EXCRETIONS AND SECRETIONS (E.S.) OF LARVAE HATCHED BY DIFFERENT METHODS

Sample	Hatching Technique	mμ moles p-Nitrophenol/mg protein/min[a]
H.F.	Fairbairn Method	31.2
H.F.	Jaskoski Method	28.9
E.S.	Fairbairn Method	8.4
E.S.	Jaskoski Method	8.0

[a]Values represent the mean of three separate experiments (two individual determinations each).

TABLE 12

COMPARISON OF THE CHITINASE ACTIVITY IN ASCARIS HATCHING FLUID (H.F.) AND EXCRETIONS AND SECRETIONS (E.S.) OF LARVAE HATCHED BY DIFFERENT METHODS

Sample	Hatching Technique	mμ moles N-Acetylglucosamine/mg protein/min[a]
H.F.	Fairbairn Method	8.2
H.F.	Jaskoski Method	5.1
E.S.	Fairbairn Method	2.2
E.S.	Jaskoski Method	2.3

[a]Values represent the mean of three separate experiments (two individual determinations each).

TABLE 13

COMPARISON OF CHITINASE ACTIVITY IN ASCARIS EGG FLUID AND HATCHING FLUID

Sample	mμ moles N-Acetylglucosamine/mg protein/min[a]
Egg Fluid (collected by Xylene Method)	9.85
Egg Fluid (collected by Needle Method)	9.02
Hatching Fluid	8.12

[a]Values represent the mean of three separate experiments.

TABLE 14

COMPARISON OF ESTERASE ACTIVITY IN ASCARIS EGG FLUID AND HATCHING FLUID

Sample	mμ moles p-Nitrophenol/mg protein/min[a]
Egg Fluid (collected by Xylene Method)	-
Egg Fluid (collected by Needle Method)	-
Hatching Fluid	32.1

[a]Values represent the mean of three separate experiments.

TABLE 15

COMPARISON OF PROTEINASE[a] ACTIVITY IN *ASCARIS* EGG FLUID AND HATCHING FLUID

Sample	O.D. units/mg protein/min[b]
Egg Fluid (collected by Xylene Method)	-
Egg Fluid (collected by Needle Method)	-
Hatching Fluid	0.098

[a]Proteinase activity determined by Anson11 assay.
[b]Values represent the mean of three separate experiments.

Enzyme Analysis of Developmental Stages

Chitinase, esterase, and proteinase assays were performed on sonicates of deshelled eggs in various stages of embryonation. Assays were also carried out on any hatching fluid collected from the 14-, 18-, 21-, or 28-day samples that had received the hatching stimulus. Results on Table 16 show that chitinase was detectable on the tenth day of embryonation with only very slight activity shown prior to that. The activity continued to increase until the twenty-first day. Hatching was achieved with 18-day or older samples, and the hatching fluid in each case possessed strong chitinase activity.

Esterase activity (Table 17) of high levels was present in all embryonic stages as well as in all of the hatching fluid preparations. The 14- and 18-day samples produced the highest levels of esterase activity.

Table 18 shows low levels of proteinase activity in all preparations prior to the 21-day sample. There was a slight increase in activity at 18 days. The activity at 21 and 28 days far exceeded that of any of the other sonicate preparations but was still considerably less than the values for any of the hatching fluid samples. In the hatching fluid, proteinase activity increased sharply with an increase in the age of the eggs employed.

TABLE 16

CHITINASE ACTIVITY IN SONICATES AND HATCHING FLUID FROM ASCARIS DEVELOPMENTAL STAGES

Sample	Days of Embryonation	mμ moles N-Acetylglucosamine/mg protein/min[a]
Sonicate	0	0.00
"	3	0.00
"	7	0.08
"	10	0.74
"	14	1.87
"	18	2.50
"	21	3.58
"	28	3.46
Hatching Fluid	14	-[b]
" "	18	6.33
" "	21	6.21
" "	28	6.64

[a]Values represent a mean of two separate experiments (three individual determinations each) with the exception of those for the 18-day hatching fluid for which there was a single determination for each experiment due to insufficient material.

[b]No hatching achieved.

TABLE 17

ESTERASE ACTIVITY IN SONICATES AND HATCHING FLUID FROM *ASCARIS* DEVELOPMENTAL STAGES

Sample	Days of Embryonation	mμ moles p-Nitrophenol/mg protein/min[a]
Sonicate	0	40.2
"	3	42.4
"	7	38.1
"	10	32.0
"	14	63.6
"	18	74.4
"	21	39.4
"	28	35.7
Hatching Fluid	14	-[b]
" "	18	26.6
" "	21	28.6
" "	28	32.5

[a]Values represent a mean of two separate experiments (three individual determinations each) with the exception of those for the 18-day hatching fluid for which there was a single determination for each experiment due to insufficient material.

[b]No hatching achieved.

TABLE 18

PROTEINASE ACTIVITY IN SONICATES AND HATCHING FLUID FROM *ASCARIS* DEVELOPMENTAL STAGES

Sample	Days of Embryonation	O.D. units/mg protein/min[a]
Sonicate	0	0.003
"	3	0.004
"	7	0.003
"	10	0.004
"	14	0.004
"	18	0.007
"	21	0.019
"	28	0.021
Hatching Fluid	14	-[b]
" "	18	0.035
" "	21	0.065
" "	28	0.102

[a]Values represent a mean of two separate experiments (three individual determinations each) with the exception of those for the 18-day hatching fluid for which there was a single determination for each experiment due to insufficient material.

[b]No hatching achieved.

Preliminary Attempts to Isolate the Enzymes in Hatching Fluid

Isolation of Chitinase by Adsorption on Lyophilized Chitin

An attempt was made to separate the chitinase from hatching fluid by adsorption onto colloidal chitin. The process yielded 5.20 mg of lyophilized material. The preparation possessed strong chitinase activity with no trace of proteinase and esterase. There was an increase in chitinase activity of slightly more than three-fold over that present in the hatching fluid prior to adsorption.

Similar results were obtained when this technique was employed to isolate the chitinase from a sonicate preparation of infective larvae.

Fractionation on Sephadex G-100 and DEAE Sephadex A-50

Fractionation of hatching fluid on Sephadex G-100 produced two major protein fractions and three minor ones (Figure 4). Analyses revealed no esterase activity in any of the fractions. Chitinase activity was detected only in fraction II. The values in Table 19 show that fraction II also contained proteinase with lesser amounts in fractions I and III.

An attempt to separate the chitinase and proteinase in fraction II by ion-exchange chromatography on DEAE Sephadex gave two protein fractions. Chitinase and proteinase could not be detected in either fraction.

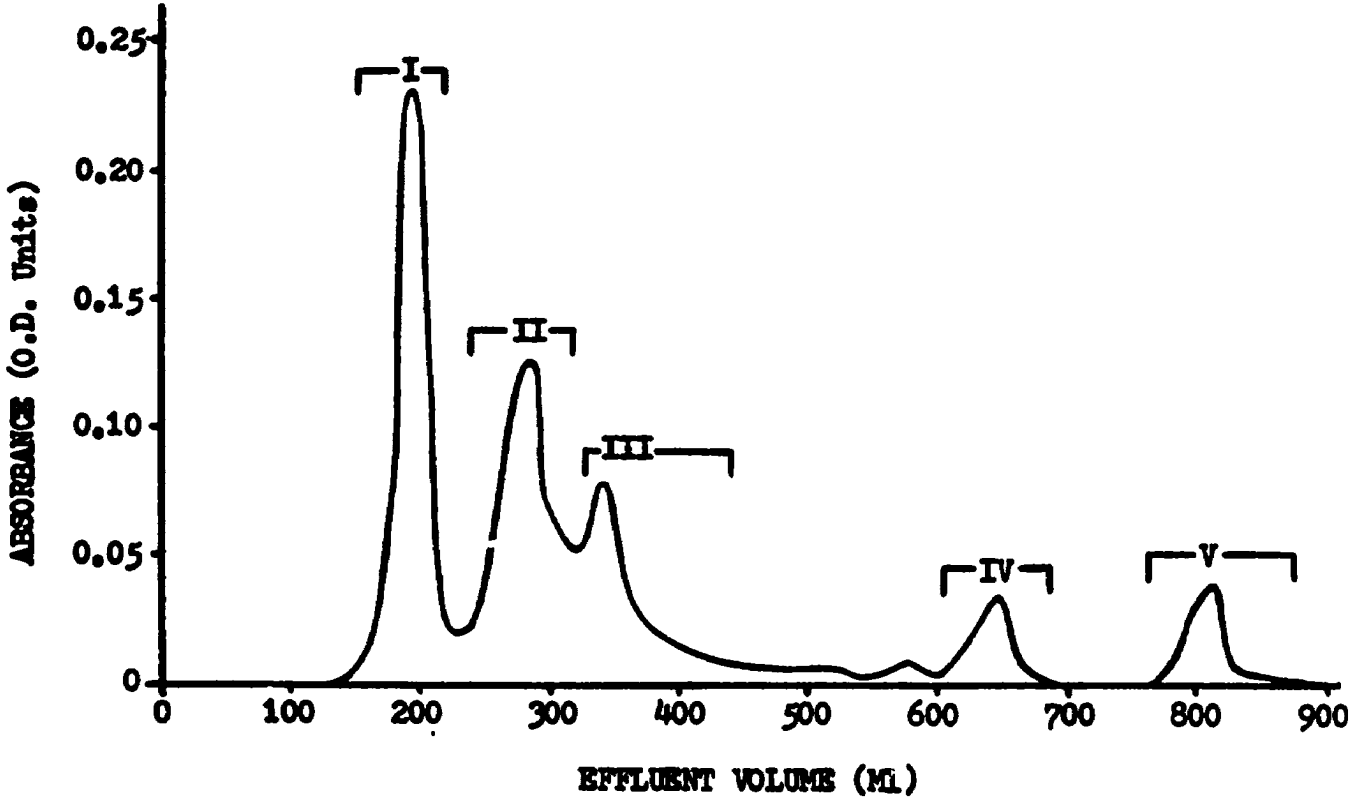

Figure 4. Fractionation of hatching fluid proteins on Sephadex G-100.

TABLE 19

PROTEINASE ACTIVITY IN SEPHADEX G-100 PROTEIN FRACTIONS OF *ASCARIS* HATCHING FLUID

Sample	mμ moles Tyrosine/mg protein/min[a]
Fraction I	2.5
Fraction II	4.8
Fraction III	1.4
Fraction IV	none
Fraction V	none

[a]Values represent a mean of two individual determinations.

Analysis of Enzyme Preparations by Polyacrylamide Gel Disc Electrophoresis

Polyacrylamide gel disc electrophoretic analyses were performed on several enzyme preparations. Hatching fluid (H.F.) collected after *in vitro* hatching by the Fairbairn method revealed seven distinct protein bands (Figure 5 A). Two of these bands (3 and 6) were fairly wide and stained quite dark. Hatching fluid collected after *in vitro* hatching by the Jaskoski method showed six protein bands (Figure 5 B). Four of these bands, including bands 3 and 6, appeared at the same locations as bands seen in the H.F. from the Fairbairn procedure and received the same numerical designation. The other two bands were assigned numbers 8 and 9. Larval bathing fluid collected from infective deshelled eggs by the xylene technique produced only three protein bands when subjected to electrophoresis (Figure 5 C). They corresponded in appearance and location to bands 3, 5, and 6 seen in the H.F. gel (Figure 5 A) and were so numbered. Electrophoresis of excretions and secretions (E.S.) collected after hatching by the Fairbairn procedure showed a total of eight bands (Figure 5 D). Six were given designations in common with bands found in hatching fluid. Once again, these included bands 3 and 6. Two additional bands (10 and 11) were seen. The E.S. collected after hatching by the Jaskoski method (Figure 5 E) appeared nearly identical to the other E.S. The only difference observed was the addition of a ninth band designated number 12. Hatching fluid chitinase purified by adsorption on colloidal chitin revealed two protein bands (Figure 5 F). These appeared at the some locations and, in general, looked the same as bands 3 and 6 found in complete hatching fluid.

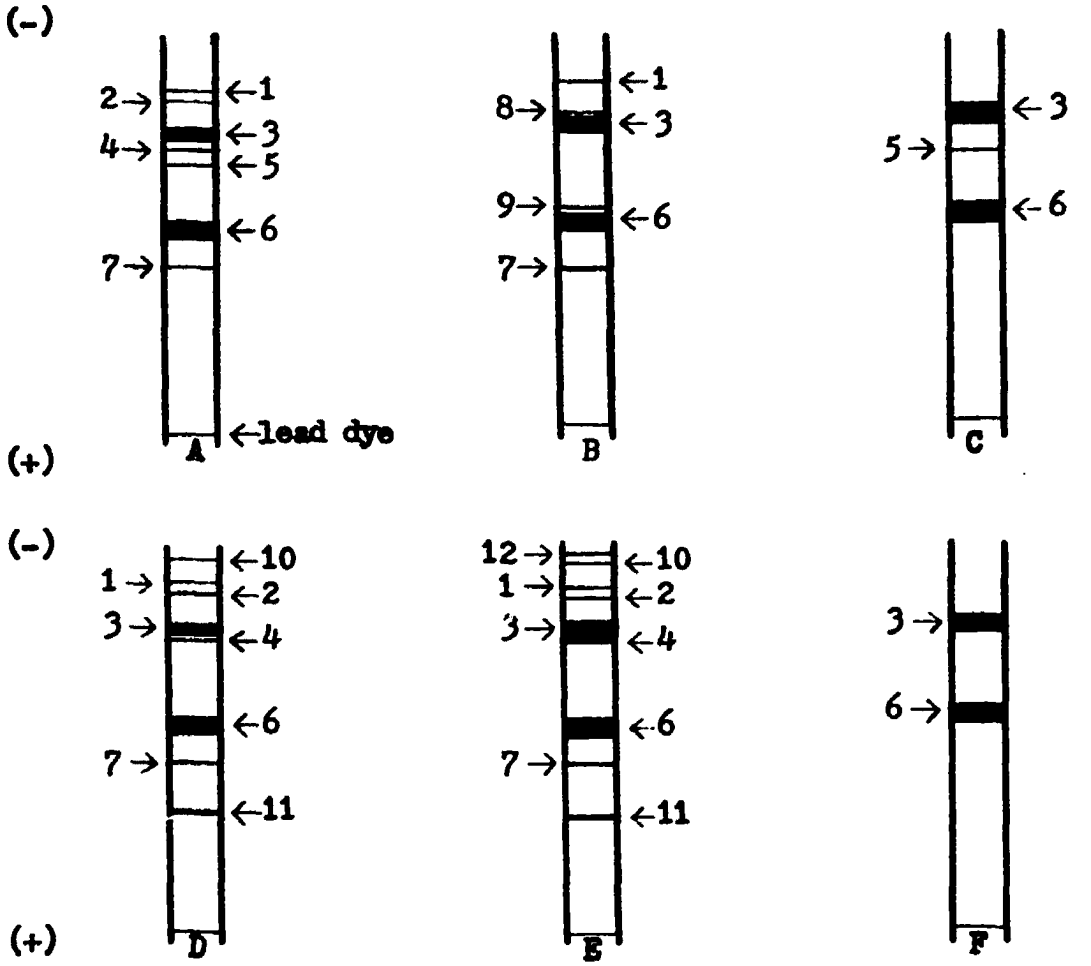

Figure 5. Polyacrylamide gel electrophoresis of hatching fluid, Fairbairn method (A); hatching fluid, Jaskoski CO_2 method (B); larval bathing fluid, infective egg (C); excretions and secretions of second stage larvae hatched by Fairbairn method (D); excretions and secretions of second stage larvae hatched by Jaskoski CO_2 method (E); chitinase purified by adsorption on chitin (F). Only the separation gels are shown.

CHAPTER IV

DISCUSSION

Comparative tests showed that *Ascaris* egg shell chitin was superior to lobster chitin for the determination of hatching fluid chitinase activity (Table 1). Additional "purification" of *Ascaris* egg shell chitin by a method designed to remove mineral salts (Reynolds, 1964) produced a less suitable substrate. However, this treatment made lobster chitin comparable in value to *Ascaris* egg shell chitin treated in the same manner. Although not proven, these results suggest that *Ascaris* egg shell chitin possesses structural or chemical differences which render it a better substrate for the homologous chitinase.

Lyophilization of colloidal chitin provided a substrate of very fine particles which could be accurately measured and easily stored. Colloid preparations of lobster chitin proved much superior to non-colloid preparations while little difference was seen between colloid and non-colloid *Ascaris* egg shell preparations. The lack of difference in the case of *Ascaris* chitin probably was due to the fact that the chitin was already in the form of very fine particles prior to colloid preparation.

Hatching fluid chitinase showed its greatest activity under acid conditions. The highest activity occurred at pH 5.8 (Table 2). This agrees fairly closely with the results of Rogers (1963) who found

a pH of 6.0 to be optimal. The tests also revealed that the type of buffer was important, as well, since greater activity was demonstrated in phosphate buffers than in phthalate buffers at the same pH values.

As expected, hatching fluid chitinase activity was greatly influenced by the reaction temperature (Figure 1). Little reaction occurred at 25 C, the temperature often employed for assaying Streptomyces chitinase. Surprisingly, the greatest activity occurred at 50 C, a temperature considerably above that at which hatching is possible. Nevertheless, the physiological temperature of 38 C was retained as the standard assay temperature for Ascaris chitinase, because the data from other tests would be more meaningful if collected at the physiological temperature.

The chitinase was shown to be fairly stable (Table 3). Freezing and thawing the enzyme three times failed to diminish activity. The activity was only slightly reduced by lyophilizing and relyophilizing. It should also be noted that marked chitinase activity was still present in one lot of hatching fluid stored for 18 months in the lyophilized state at 4 C and in another lot frozen at -20 C for 11 months. Obviously either method would be suitable for preserving this enzyme. The loss of activity after storage for 72 hours at 4 C was 11.1 per cent while storage at 25 C for 72 hours resulted in loss of 27 per cent of activity. Like most enzymes, the chitinase was susceptible to elevated temperatures since heating at 65 C for 30 minutes resulted in a 77 per cent activity loss, while heating at 95 C caused a loss of 95.6 per cent of the activity.

Studies were attempted to determine the effect of pH on the assay of esterase. Since the end product of the reaction, p-nitrophenol, is itself a colorimetric indicator of pH and thus gives different intensities of color with different degrees of acidity, the results were very difficult to analyze. It was possible, however, to study the effect of temperature on the assay of the enzyme. At temperatures above 40 C the substrate, p-nitrophenol acetate, decomposes spontaneously at a rapid rate resulting in extremely high blank readings. Therefore, esterase activity was measured only at temperatures ranging from 25 C to 40 C. Figure 2 shows that the reaction is very slow at lower temperatures. The measured activity at 25 C was only about 5.3 per cent as high as the activity at the hatching temperature, 38 C.

A preliminary investigation (data not reported) conducted to determine the stability of esterase indicates that it is less stable than chitinase but can be dialyzed for 24 hours at 4 C and then lyophilized with only slight loss of activity.

Proteinase activity in hatching fluid was demonstrated by the Anson method using denatured hemoglobin and casein as substrates (Table 7). Better activity was achieved with casein than with hemoglobin. Perhaps the proteinase is more specific for a protein like casein, or it might simply be a reflection of the higher percentage of tyrosine in casein than in hemoglobin.

When proteinase was studied to determine the effects of pH and type of buffer on the measurement of enzyme activity, strikingly different results were observed depending on the assay method (Tables 5 and 6). Using the Anson method, the highest activity was recorded at

pH 8.0 in a phosphate buffer while the activity at pH 8.0 in a borate buffer was only 13.5 per cent as high. On the other hand, when the Azocoll method was employed, the greatest activity was found at pH 8.8 in a borate buffer. At pH 8.0 with this system, the activity in the borate buffer was approximately 62 per cent higher than in the phosphate buffer. This poses the question of why would a certain buffer work well in one system but not in another. Possibly there is more than one proteinase. In this case one or more proteinases may be most active in the Anson system while a different enzyme(s) that might be termed a collagenase-like enzyme(s) may be responsible for most of the activity measured in the Azocoll system. Possibly the former proteinase(s) operates best in a phosphate buffer with the collagenase-like enzyme(s) being more active in a borate buffer. Another explanation could be that the buffer(s) might actually enter into the reaction in some way and either inhibit or enhance the reaction in a particular system while not doing so in another.

As with chitinase and esterase, temperature was found to be an important factor in measuring the activity of proteinase. At 25 C almost no activity was detected (Figure 3). However, a sharp increase in activity accompanied rising temperatures. Temperatures above 55 C produced a rapid loss of activity. A preliminary stability study (data not reported) indicated the enzyme is generally less stable than the chitinase and loses a considerable amount of activity after repeated freezing and thawing. Dialysis at 4 C followed by lyophilization caused only a small loss of activity.

The definition of conditions for handling and storing hatching fluid enzymes along with the establishment or refinement of procedures for their assay, especially chitinase and proteinase, should permit future studies of these enzymes to be conducted with greater ease.

As previously shown, Ascaris infection antisera from rabbits contained precipitin antibodies against hatching fluid (Soulsby, 1963). Justus (1968) observed that rabbit anti-hatching fluid antisera inhibited the activity of hatching fluid chitinase. Results of the present study (Table 4) agreed with the work of Justus in that anti-hatching fluid antisera gave nearly complete inhibition of the hatching fluid chitinase. When hatching fluid chitinase was tested against Ascaris infection antisera, a partial inhibition was observed. With the lower concentration of hatching fluid, the inhibition was approximately 71.7 per cent. However, when the chitinase was tested against infection antisera prepared against Toxocara canis, an organism closely related to Ascaris, the degree of inhibition was much less. The degree of inhibition at the lower concentration of hatching fluid was only 21.7 per cent in this case. Although these results indicate that the infected animals developed antibody to hatching fluid chitinase, one cannot necessarily infer that this antibody renders any degree of protective immunity. Since hatching takes place in the lumen of the gut, it is unlikely that there would be a sufficient level of antibody present to interfere with the hatching process. The presence of chitinase in the excretions and secretions of hatched second-stage larvae suggests that the enzyme might be released for a period of time as the larvae migrate through the tissues. If this is true, possibly the reaction between the enzyme and

antibody results in the formation on the worm of an antigen-antibody precipitate which could act as a physical barrier to interfere with feeding or enzyme secretion processes. Protective immunity studies with laboratory animals using purified chitinase would have to be conducted to test its role in stimulating a protective immune response.

Similar results were observed when hatching fluid proteinase was tested against the above antisera (Table 8). Anti-hatching fluid antisera completely inhibited proteinase activity while the two infection antisera gave a partial inhibition. With Ascaris infection antisera a considerably higher level of inhibition was achieved than with Toxocara infection antisera. Whether the inhibition produced in vitro reflects any degree of protective immunity is not known. Proteinase might aid the larvae in penetrating and migrating through tissues. If this enzyme does function as a penetration-migration factor, then its inhibition by specific antibody would likely reduce the invasiveness of the larvae. In vivo protection studies using purified Ascaris proteinase would be required to establish if the enzyme is involved in the development of a protective immunity.

Antibody inhibition of esterase was not studied because of technical difficulties. The method employed for esterase assay in our experiments could not be adapted to an enzyme inhibition study of this type since the antisera or normal serum caused the breakdown of the substrate p-nitrophenyl acetate. Apparently there was a sufficient level of esterase present in the sera to produce this effect.

The production of chitinase by hatched infective larvae (Table 12) suggested the possibility that the enzyme might also act as a

penetration-migration factor by attacking hyaluronic acid which is structurally similar to chitin. Hyaluronidase activity was not detected when hatching fluid was tested against hyaluronic acid. However, both the extracts of sonicated hatched larvae and the excretions and secretions (E.S.) collected from hatched larvae gave positive tests for hyaluronidase. Since hatching fluid gave negative hyaluronidase tests in spite of stronger chitinase activity than the extracts of sonicated hatched larvae or E.S. products of hatched larvae, it seems unlikely that chitinase was responsible for the hyaluronidase activity demonstrated. It is perhaps noteworthy that in some experiments the hatching fluid test sample gave higher turbidity readings than the substrate control which contained no enzyme. Since this assay method depends upon the reaction of hyaluronic acid with albumin to form a turbid suspension, the degree of turbidity is proportional to the amount of hyaluronic acid present. If some component in the hatching fluid also produced this turbidity by reacting with the albumin, then the disappearance of hyaluronic acid might well have been masked. It would be necessary to test the separate protein fractions of hatching fluid to determine for certain if any of its constituents possessed hyaluronidase activity.

Surprisingly, the hyaluronidase activity of the E.S. was considerably lower than that of the sonicate. It appears that, although hyaluronidase activity is readily measurable in the larval extract, little of the enzyme is released <u>in vitro</u> by the hatched larvae. Perhaps it is necessary for the larvae to encounter the substrate while migrating through the tissues before a really significant amount of the enzyme is released. Larvae need to be collected from the tissues in order to

test this possibility. It might also prove worthwhile to test for activity against some other mucopolysaccharide constituents of tissue such as chondroitin sulfate.

Jaskoski and Colucci (1964) reported that *in vitro* hatching of *Ascaris* eggs could be achieved in a medium saturated with CO_2 and containing no reducing agents if the eggs were first deshelled in sodium hypochlorite. The removal of the chitinous shells was not required for hatching with the techniques (Rogers, 1958 and Fairbairn, 1961) which included both reducing agents and CO_2 in the hatching medium. This presented the possibility that the technique of Jaskoski and Colucci provided an incomplete stimulus which did not elicit the production or release of sufficient chitinase to weaken the chitin shell. Hatching experiments were, therefore, conducted using the method of Jaskoski and Colucci and the method of Fairbairn. Hatching fluid and excretions and secretions (E.S.) collected after hatching by each method were analyzed for chitinase, esterase, and proteinase activity. The results show almost no difference in proteinase activity (Table 10) or esterase activity (Table 11) when the two methods were compared. However, the chitinase activity in the hatching fluid recovered after hatching by the Fairbairn method was approximately 61 per cent greater than activity in the hatching fluid collected after hatching by the Jaskoski method. The lower chitinase activity in the latter hatching fluid might be interpreted as indicating that a lowered redox potential is a necessary part of the stimulus. Perhaps in the absence of reducing agents, the organism does not produce or release enough chitinase for hatching from eggs which have intact shells. These results lend support to the idea that

the infective larva responds to the proper hatching stimulus by secreting chitinase as suggested by Rogers (1958) and Fairbairn (1961). The results might also explain why the 100 per cent CO_2 hatching experiments of Jaskoski and Colucci (1964) were successful only when deshelled eggs were used. Thus, it appears that a lowered redox potential stimulates the larva to increase its chitinase production. However, the stimuli necessary for inducing proteinase and esterase release apparently does not depend on a lowered redox potential. The stimuli responsible for eliciting proteinase and esterase release are probably CO_2 and the proper temperature.

In the present studies, measurable chitinase activity was found in extracts of sonicated eggs which had been developing for only 10 days (Table 16). This confirms the work of Justus (1968) who reported chitinase activity in extracts of 14 day old eggs and suggested that the production of chitinase did not necessarily depend on the hatching stimulus. Since hatching cannot be achieved with eggs incubated less than 16 days (Fairbairn, 1961), the presence of chitinase in 10 day old eggs makes it apparent that the enzyme is not produced solely at the time of hatching. The current studies showed essentially no chitinase activity in eggs less than 10 days old. An increase in activity occurred as development progressed to the twenty-first day. Hatching was successful with the 18-, 21-, and 28-day eggs, and the hatching fluid collected from the three samples showed little difference in chitinase activity. This does not conflict with the results from the extracts, however, as there was an increase in percentage of hatching as development time advanced, especially from the 18-day to the 21-day sample with the

difference between the 21- and the 28-day samples being less dramatic. This indicates that there were simply fewer fully developed infective larvae in the 18-day and 21-day samples and that those larvae which did hatch after these development times were capable of the full level of chitinase production necessary for hatching. In other words, the ability to produce chitinase is essentially the same for all infective larvae regardless of their age.

Esterase activity in the sonicates of the various egg samples mentioned above cannot be related to the hatching potential of the samples (Table 17). In fact, the activity in the undeveloped sample (0 days embryonation) was greater than that of the 28-day sample which gives the highest percentage of hatching. It seems unlikely that the same esterase which is involved in hatching was responsible for the activity detected in the undeveloped eggs, especially in view of the fact that there was less activity in the 28-day-old eggs. As might be expected, considering the chitinase results, the esterase activity did not vary a great deal between the different hatching fluid samples although there was some increase in activity with increased embryonation time. The esterase activity in the various sonicates was probably a measurement of metabolic esterases rather than a hatching enzyme.

The enzyme whose activity correlates the closest with age of development and hatchability is proteinase (Table 18). Only low levels of proteinase were recorded in the extracts of eggs incubated less than 21 days. Some increase is seen at 18 days, the earliest sample in which any hatching occurred in the present studies. The activity was much higher in the 21- and 28-day samples which gave good percentages of

hatching when stimulated *in vitro*. Correspondingly, the activity in the hatching fluid also increased with the length of embryonation time. These results differ markedly from those observed with chitinase and esterase in hatching fluid. An enzyme such as proteinase could serve the hatched larvae in their penetration and migration through the tissues. Thus, the proteinase(s) might aid the larva in escaping from the egg as well as in migrating through the tissues. If the organism has a well-developed capacity for proteinase production then its chances for a successful migration would seemingly be better. Possibly the increased proteinase activity in the hatching fluid is related to the older larva's better preparedness for migration through the tissues rather than an increased hatchability.

An attempt was made to examine further the enzymes present in unhatched, unstimulated infective eggs. When the internal egg fluid that bathes the unhatched second-stage larvae was collected and analyzed, high levels of chitinase were detected in both xylene method and needle method samples (Table 13). In fact, the chitinase activity was even higher in the egg fluid than in the hatching fluid. The presence of chitinase in the egg fluid suggests that the infective larvae had secreted (or simply leaked) chitinase into the bathing fluid surrounding them in advance of hatching. It should be noted, however, that the amount of lyophilized material recovered from the egg fluid was considerably less than that which could be recovered from the hatching fluid from an equal number of eggs. Thus, the total quantity of chitinase in the hatching fluid from a given number of stimulated eggs is much greater than that which occurred in the internal egg fluid. Since infective eggs stimulated by the

100 per cent CO_2 method would not hatch unless their chitin shells were artificially removed (Jaskoski and Colucci, 1964), it is doubtful that the amount of chitinase present in the bathing fluid prior to stimulation is adequate to permit hatching.

Analyses of the egg fluid revealed no esterase (Table 14) or proteinase (Table 15) activity in either the sample collected by the needle method or the xylene method. Controls run with hatching fluid showed a sizable loss of esterase activity following the xylene treatment but relatively little loss of activity from the needle treatment. Neither treatment caused very much loss of proteinase activity. These results indicated that neither proteinase nor esterase was present in the egg fluid prior to hatching stimulation. It is conceivable that either enzyme could attack the lipoprotein inner membrane surrounding the unhatched larva and consequently alter the permeability of the membrane to allow other enzymes to pass through it and attack the outer layers. It appears that secretion of these two enzymes into the fluid contained within the egg is induced by the hatching stimulus and, therefore, is released only during the hatching process. However, this stimulus does not seem to be a reduced redox potential since high levels of proteinase and esterase were detected in hatching fluid from eggs hatched by the Jaskoski and Colucci method which lacks a reducing agent.

Thus, the study revealed that chitinase, proteinase and esterase were present in unstimulated infective eggs, but only chitinase was present in the egg fluid which bathes the larva within the egg. However, the amount of chitinase present in this fluid was probably insufficient to permit hatching. Upon proper stimulation, the larva apparently

responded by releasing esterase and proteinase along with sufficient chitinase to enable it to hatch. Either the esterase or the proteinase might act by attacking the inner membrane, rendering it permeable to chitinase and proteinase, which could then reach and attack the chitin and protein layers of the shell. After hatching *in vitro*, the second-stage larva continued to produce chitinase and proteinase. If the larva continues to produce these enzymes after hatching *in vivo*, they might serve it also as penetration-migration enzymes which could attack constituents of the tissues through which the larva must pass. The proteinase especially seems a likely candidate for such a dual role. The fact that antisera from *Ascaris*-infected rabbits partially inhibited the activity of hatching fluid chitinase and proteinase when tested *in vitro* strongly suggests that these enzymes were released in the tissues of the host. The detection of hyaluronidase activity in infective eggs and hatched larvae (and to some extent in second-stage larval E.S.) indicates that hyaluronidase might also be produced *in vivo* by migrating larvae and function as another migration-aiding enzyme. Antibodies produced in the host during infection possibly could inhibit the action of these enzymes in the tissues as they have been shown to do *in vitro* against chitinase and proteinase and perhaps give the host a means of defense.

Preliminary attempts to isolate the enzymes in hatching fluid were generally met with little success. The technique of adsorption onto colloidal chitin produced the best results since a greater than three-fold increase in chitinase activity was observed while no esterase or proteinase was detected in the material recovered. When the chitinase-active material was subjected to polyacrylamide gel disc electrophoresis,

two dark-staining protein bands (Figure 5F) were observed, suggesting that the chitinase activity might be a product of a two-component enzyme system. A more complete fractionation of hatching fluid would be required to test this possibility. Identical results were observed with chitinase recovered by the same method from an extract of sonicated infective larvae.

Column chromatography with Sephadex G-100 separated hatching fluid into five protein fractions (Figure 4). Enzyme analysis of the fractions showed fractions I, II, and III possessed proteinase activity (Table 19) while chitinase activity was restricted to only fraction II. Possibly the presence of proteinase in these three fractions denotes the occurrence of more than one proteinase in hatching fluid. On the other hand, this might simply be the result of an incomplete separation with some of the same enzyme being found in all three fractions. Analysis of these fractions by disc electrophoresis might have given a clue as to whether more than one proteinase existed. However, the presence of Sephadex beads in some fractions interfered with satisfactory analysis by this procedure. Since all the chitinase activity and most of the proteinase activity were found in fraction II, an attempt at separation of these two enzymes was made with DEAE Sephadex A-50. This attempt was completely unsuccessful as no trace of chitinase or proteinase was detected in either of the two fractions recovered.

Disc electrophoresis revealed a number of similarities between several enzyme preparations. Seven protein bands were seen in hatching fluid collected by the Fairbairn method (Figure 5A). Bands 3 and 6 were wide and dark-staining. Justus (1968) had described five precipitin

bands in hatching fluid when examined by immunoelectrophoresis. Two of these bands were reported as very heavy and thick in appearance. It is probable that bands 3 and 6 in the current study correspond to the two heavy bands described by Justus. Hatching fluid collected by the Jaskoski method (Figure 5B) appeared to have had at least four protein bands (1, 3, 6, and 7) in common with the hatching fluid recovered by Fairbairn's method while three bands (2, 4, and 5) were missing. Also, two additional bands (8 and 9) not seen in the Fairbairn method hatching fluid were observed. In view of the fact that both types of hatching fluid were similar in their possessing strong chitinase, esterase, and proteinase activity, it is surprising to find so much apparent difference in protein composition as seen by disc electrophoresis. These results would lead one to suspect that the three enzymes in question might be included in the four shared bands.

When hatching fluid chitinase purified by adsorption onto chitin was electrophoresed, only two bands (3 and 6) appeared (Figure 5F). These looked identical to the two predominart bands seen in hatching fluid. Since no proteinase or esterase activity was detected in the preparation, one would conclude that one or both of these bands was responsible for chitinase activity. Bands 3 and 6 were also found after electrophoresing larval bathing fluid collected from infective eggs by the xylene method (Figure 5C). This material had strong chitinase activity but no esterase or proteinase activity. Bathing fluid collected by the needle method (gel not shown) gave identical results. The electrophoretic analysis supports the conclusion that larval bathing fluid

contains very little except chitinase. It also strengthens the case for a chitinase role for bands 3 and/or 6.

Disc electrophoresis performed on the second stage larval E.S. indicated this material was somewhat more complex than hatching fluid. Justus (1968) reported ten precipitin bands upon immunoelectrophoretic analysis. All the bands present in hatching fluid were also found in the E.S. In the current analyses eight protein bands were found in the E.S. collected from larvae hatched by the Fairbairn method (Figure 5D) and nine in the E.S. from larvae hatched by the Jaskoski method (Figure 5E). With the exception of band 5, all the bands present in hatching fluid were found in both E.S. samples. Since these larvae were separated from unhatched eggs prior to incubation and collection of E.S., it is safe to conclude that the E.S. was not contaminated with hatching fluid and that the proteins present in the E.S. were actually produced by the hatched larvae. It can also be seen that the larval E.S. was nearly the same in protein composition whether the larvae were hatched by the Fairbairn method or the Jaskoski method.

CHAPTER V

SUMMARY

Hatching fluid, second-stage larval excretions and secretions (E.S.), and various larval extracts of Ascaris summ were studied with respect to enzymes that might aid in hatching or intrahost migration. Proteinase, as well as chitinase and esterase, were demonstrated in hatching fluid, second-stage larval E.S. and several larval extracts. Proteinase and esterase were found in all extracts from eggs developed for 0 to 28 days while chitinase was not found in extracts of eggs incubated less than 10 days. Hyaluronidase activity was detected in both the extracts and E.S. of hatched second-stage larvae incubated in vitro for 24 hours but not in the fluid released during the hatching process.

In vitro hatching was carried out by the Fairbairn method which requires a medium containing a reducing agent as well as dissolved CO_2 and by the Jaskoski method which employs a medium saturated with CO_2 but containing no reducing agent. Very little difference was detected in the esterase or the proteinase activity when the hatching fluids from the two methods were compared. Chitinase activity was considerably greater in the hatching fluid collected by the Fairbairn method.

The egg fluid which bathes the unhatched second-stage larva was collected and analyzed. The fluid showed strong chitinase activity but

no esterase or proteinase. This suggests that the latter two enzymes were secreted in response to hatching stimuli.

Hatching fluid chitinase was found to give the highest activity readings when a lyophilized colloid of purified Ascaris egg shell chitin was employed as the substrate. Studies on the effects of various conditions on the assay of this enzyme showed that activity increased with the incubation temperature to a peak at 50 C, beyond which the activity declined rapidly. Activity was also influenced markedly by the pH and type of buffer employed. When tested over a pH range of 4.0 to 9.0, activity increased with the pH to a maximum at pH 5.8, with additional rise in pH accompanied by a drop in enzyme activity. At the optimal pH, higher activity was recorded in a phosphate buffer than in a phthalate buffer.

A stability study conducted with hatching fluid chitinase showed no loss of activity from freezing and thawing at least three times and only a slight loss of activity after lyophilization and relyophilization. Only a 26.8 per cent loss of activity was recorded after subjecting the hatching fluid to a temperature of 25 C for 72 hours; however, a temperature of 95 C for 5 minutes resulted in a 95.6 per cent loss.

Proteinase was demonstrated in hatching fluid by both the Azocoll method, which employs a collagen substrate, and the Anson method using either casein or denatured hemoglobin as the substrate. Enzyme activity increased with the incubation temperature to 55 C followed by a sharp decline in activity at higher temperatures. Increasing pH also brought an increase in enzyme activity with the highest readings recorded at pH 8.8 in a borate buffer when assayed by the Azocoll method. However,

the peak activity was produced in a phosphate buffer at pH 8.0 when the Anson method was employed.

The assay system used for measuring esterase could not be adapted to determine the influence of pH on enzyme activity. It was observed, however, that activity was greatly affected by the incubation temperature. Activity increased with the temperature to a peak value at 40 C. Instability of the substrate prevented testing at higher temperatures.

Studies were conducted to test for inhibition of Ascaris hatching fluid chitinase and proteinase activity by reaction with antisera. Anti-hatching fluid antisera gave virtually complete inhibition of both enzymes. A partial inhibition of each was observed when Ascaris infection antisera was employed. Similar results were achieved with infection antisera prepared against Toxocara canis. The degree of inhibition, however, was less with the heterologous infection antisera.

Attempts to isolate the components of hatching fluid by fractionation procedures were generally unsuccessful. Column chromatography with Sephadex G-100 produced five protein fractions with proteinase activity in fractions I, II, and III and chitinase in only fraction II. No esterase activity was found in any of the fractions. Efforts to separate the two enzymes in fraction II by the use of Sephadex A-50 yielded two protein fractions, neither of which possessed chitinase or proteinase activity.

Hatching fluid chitinase was purified by adsorption of the enzyme on colloidal chitin from which it was subsequently eluted and recovered. Esterase and proteinase were not detected in the recovered

material which did have strong chitinase activity. An increase in chitinase activity of more than three fold was recorded.

Polyacrylamide gel disc electrophoresis was used to analyze the protein composition of hatching fluid and excretions and secretions (E.S.) collected by both the Fairbairn and Jaskoski methods; larval bathing fluid from infective eggs; and hatching fluid chitinase purified by adsorption on colloidal chitin. Seven protein bands were found in the Fairbairn method hatching fluid while six were found in the hatching fluid collected by the Jaskoski method. Eight bands appeared in the E.S. collected by the former method, with nine bands being noted in the latter E.S. The electrophoresis performed on the purified chitinase showed only two bands, both of which stained very dark. These bands also appeared in both hatching fluid samples and both E.S. samples as well as in the larval bathing fluid. The bathing fluid also contained one other band.

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