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ANTIGENIC ANALYSIS OF DEVELOPMENTAL

STAGES OF *ASCARIS SUUM*

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Although the antigens of helminth parasites have been studied since the early part of this century, relatively little is known about how the host responds to them or which antigens, if any, induce an effective or functional immune response. The complexity of the antigens prevented various workers from making precise analyses of the nature of each antigenic component and its fate in the host.

In general, methods developed for the isolation and characterization of microbial and other antigens have been employed in studies of helminth antigens. Among others, techniques such as immunoelectrophoresis, agar-gel diffusion, fractionation, and chemical extraction have been used successfully. Helminth antigens which have been studied by these methods include those prepared from various stages, such as undeveloped and infective eggs, larvae, adults, and certain metabolic antigens (excretions and secretions) obtained from larvae. From such studies the complexity of the antigens, and to a limited degree their similarities and differences have been demonstrated.

The adult stages have received more attention than the others because of their abundance and availability. Studies have shown that they are antigenically more complex than immature stages and, thus, probably more difficult to analyze. This has been the case particularly in serological studies utilizing antigens of adult worms. Although immature stages are less complex and lend themselves to easier analyses, it is difficult to obtain sufficient quantities for definitive studies.

Helminth antigens have been investigated extensively for their potential value in serological tests and to a limited degree for their ability to induce protective immunity. Although some antigens show value in diagnostic serological tests, the lack of specificity has limited their use. Attempts to induce protective immunity in the host by artificial immunization have been essentially unsuccessful. This has been due partially to a lack of information about the interaction between host and parasite and to the difficulties of obtaining the appropriate functional antigens.

The lack of specificity, the failure to induce protective immunity, and the complex nature of adult stages emphasize the need for continued studies on antigens of adult and immature stages. Included among the components which have been studied very little for their antigenicity are enzymes, metabolites such as exsheathing and hatching fluid, and certain developmental stages.

Some helminth parasites have been shown to elaborate enzymes such as proteinases and lipases during their entrance into, and subsequent infection of the host. Because enzymes exhibit a high degree of substrate specificity and can be quite antigenic, they may be valuable as test antigens and as vaccines. In general, studies have shown that

enzymes hydrolyzing high molecular weight substrates can be completely inhibited by antibody (Cinader, 1963).

Earlier studies with metabolic antigens, suggested strongly that they played a major role in inducing functional immunity and to a limited degree were useful in serological tests. Hatching fluid, a physiologically functional substance, is produced upon appropriate stimulation of infective eggs and aids the larvae in attempts to escape from the eggs. Ascaris hatching fluid has been used experimentally to detect precipitating antibodies in rabbits after only two weeks of infection (Soulsby, 1963). This suggested that the substance could be used in serological tests to detect recently acquired infections. Among the properties which make hatching fluid an excellent subject for immunological studies is its content of enzymes. Roger (1958) found that it contains a chitinase, a lipase, and possibly a proteinase. All three enzymes are apparently involved in the hatching process. At present, none of these enzymes has been studied for its immunological properties. Before critical, well-controlled studies can be performed, they must be isolated in relatively pure form and more must be learned about their kinetics.

Although most of the developmental stages have been studied individually for their antigenic components, essentially no attempt has been made to identify stage specific antigens nor those transitional or embryonal antigens occuring during development from one stage to the next. Such studies are needed not only to characterize the antigens but also to provide a better understanding of the immunological aspects of the host-parasite relationship. A clear knowledge of the latter would also provide the basis for evaluation of antigens for use in serological tests

as well as for their use in vaccines.

The major objectives of the present research were to:

1. demonstrate and characterize antigens associated with certain developmental stages of Ascaris suum and study their relationship to "metabolic" antigens (hatching fluid and larval secretions - excretions).
2. demonstrate the presence of antibody to chitinase and to identify hatching fluid chitinase by immunoelectrophoresis.

Literature Review

Antigenic characterization of Ascaris lumbricoides began when Canning (1929), employing a precipitin ring test, demonstrated the antigenicity of certain tissue fractions. Campbell (1936, 1937) isolated and detected specific antigens from polysaccharide fractions of human and swine strains of Ascaris lumbricoides. Oliver-Gonzalez (1943) isolated various tissue and body fluids from Ascaris and attempted to determine which of them were involved in the stimulation of antiparasitic factors. Among antisera prepared to cuticle, egg, intestine, muscle, sperm, and coelomic fluid, he obtained highest titers, by precipitin ring test, with the anti-cuticle and anti-egg serums. He also demonstrated, by in vitro tests, that only the anti-egg serum would immobilize and kill third stage larvae. In a later study (1944a), he isolated a polysaccharide fraction from adults, found that it would inhibit agglutination of human erythrocytes, and suggested that it was immunologically related to human isoantigens. Subsequently (1944b) he reported that the same fraction actively inhibited A1 and B2 serum agglutinins and the hemolysis of sheep

cells. He concluded from these studies that the Ascaris polysaccharide fraction was related to the Forssman antigen.

In a preliminary investigation of the antigenic structure of Ascaris adults, Soulsby (1957), using agar-gel diffusion, found that saline extracts of whole worms, intestine, cuticle, and a polysaccharide fraction contained 9, 14, 9, and 4 antigenic components, respectively, when reacted with homologous rabbit antisera. He also demonstrated a Forssman-like antibody by absorption of the anti-intestine serum with sheep blood cells. Employing the same technique, Kagan (1957) reported similar results in his studies of Ascaris antigens. Using rabbit antisera against whole worm extracts only, he found that the whole worm extract contained 8 to 11 antigens; muscle, 5 to 6; enteric fluid, 10 to 14; cuticle, 8; undeveloped egg, 6; and infective eggs, 2. It should be pointed out that the number of antigenic components found in each extract was determined by using antisera prepared against the whole worm extract. If homologous antisera to each extract had been used, it is possible that a larger number of antigenic components would have been demonstrated. Subsequently, Kagan (1958) reported that a purified polysaccharide fraction from adults contained five components and that four of them were heat stable.

Employing anti-Toxocara sera only, Huntly et al. (1963) demonstrated differences and similarities between human Ascaris and Toxocara canis adults by agar-gel diffusion tests.

Kent (1960) isolated several fractions from Ascaris adults. Extraction of homogenized and delipidized worms with triple distilled buffered water, followed by column fractionation, yielded five fractions.

All of the fractions but one were found to be glycoprotein. The fractions were examined by agar double diffusion and immunoelectrophoresis for their sensitivity and specificity in sero-diagnosis. The greatest sensitivity and specificity were obtained with fractions number four and five which, interestingly, contained a high per cent of carbohydrate and a low per cent of protein.

Recently, Tormo and Chordi (1965) analyzed protein and polysaccharide extracts of Ascaris adults by immunoelectrophoresis. They found a total of twenty antigenic components with the homologous antisera from artificially immunized rabbits and seven with antisera from infected rabbits and humans.

Relatively few studies have been done with metabolic antigens of Ascaris. Using agar-gel diffusion techniques, Soulsby (1963) found that antisera from infected rabbits produced two precipitin bands when they were reacted with hatching fluid. Because the bands appeared with antisera collected fifteen days post-infection, he suggested that hatching fluid antigens could be used to differentiate active from previous infections.

Studies of helminth antigens occuring in developmental stages have not been reported. However, studies concerned with biochemical and physiological changes associated with certain developmental stages have been reported. Fairbairn (1955) investigated embryonic and post-embryonic changes in the lipids of Ascaris eggs and found, among other things, certain changes in saponifiable lipids during embryonation. In a related study, Passey and Fairbairn (1957) examined the conversion of fat to carbohydrates during embryonation of Ascaris eggs. They concluded that the

carbon of partially oxidized fragments of fatty acids was incorporated into glycogen and trehalose. Of interest was the fact that protein and non-protein nitrogen remained essentially constant during embryonation. Other biochemical changes associated with development have been reported by Costello (1964) who made a comparative biochemistry study of developing eggs. Rogers (1958), in an investigation of the physiological process of hatching of embryonated Ascaris eggs, found that a lipase, chitinase, and possibly a proteinase were involved. Fairbairn (1961) made a more detailed study of the conditions necessary for artificially stimulating eggs to hatch. Among other things, he found that the eggs had to be at least 16 days old before they could be stimulated to hatch.

Rhodes and co-workers (1965) demonstrated, by enzyme inhibition and immunodiffusion, the presence of specific antibodies in sera obtained from swine and guinea pigs artificially immunized with purified malic dehydrogenase (MDH) of Ascaris. The swine serum contained both precipitating and inhibiting antibodies which could be separated by cellulose ion exchange fractionation. Malic dehydrogenases obtained from closely related parasites, such as Toxocara canis and Ascaridia galli, were inhibited by the antiserum and gave cross reactions, as shown by immunodiffusion. Some success was achieved in inducing protective immunity in guinea pigs by immunizing them with MDH. However, the protection was not as good as that in animals given infective eggs. It is of interest to note that antibodies to malic dehydrogenose could not be detected in sera from animals immunized with crude extracts.

CHAPTER II

MATERIALS AND METHODS

Preparation of Antigens

Undeveloped Egg

Eggs were collected from the terminal portion of the uterus of mature Ascaris collected from a local abattoir. Following three washes with distilled water and centrifugation at 1,720 X G for 5 minutes, the eggs were treated with 3 volumes of 2 per cent sodium hydroxide for approximately 16 hours at room temperature to remove the outer protein layer. After washing 3 or 4 times with distilled water (approximately 10 volumes of water per wash) to remove the sodium hydroxide, approximately 10 ml of eggs were suspended in 25-30 ml of 0.85 per cent sodium chloride and subjected to sonic vibration in a Raytheon sonic oscillator set at 1.25 amperes. Ice water was circulated through the oscillator jacket to minimize heat effects. Sonication was continued until all of the eggs were disrupted, as indicated by microscopic examination. This usually required 2 to 2.5 hours. They were then removed, placed in an erlenmeyer flask containing 100 ml of sterile 0.85 per cent sodium chloride, and extracted for 24 hours at $4^{o}C$. The egg extract was removed and centrifuged at 10,000 X G for thirty minutes at $4^{o}C$. The supernatant fluid was then removed and dialysed against four changes of distilled water (2 liters) over

a period of 24 hours at 4°C. The dialysate was collected, lyophilized, and stored at 4°C.

Infective Egg

Eggs were collected as described above and processed in the same manner as the undeveloped eggs. Following treatment with sodium hydroxide and subsequent washings, they were suspended in a 0.1N sulfuric acid solution containing aqueous merthiolate (1:10,000). One-half ml of eggs, suspended in 10 ml of acid-merthiolate solution was placed in each of several plastic petri dishes. The use of petri dishes for embryonation of eggs was suggested by Hinck (L.W. Hinck, personal communication). The petri dishes were incubated in a humidity chamber for 5 weeks at room temperature. At the end of this time the eggs were examined microscopically and the per cent of embryonation determined by counting 100 eggs. Only those dishes with at least 90 per cent of the eggs fully embryonated were used. Generally, a 90 to 95 per cent embryonation rate was obtained using this method. The eggs were pooled and washed 5 or 6 times with distilled water (in a total of 60 volumes of water) to remove the sulfuric acid. A rubber spatula was used to aid removal of eggs from the petri dishes. Following the final wash and centrifugation, the eggs were disrupted, extracted, dialysed, lyophilized, and stored as described above for undeveloped eggs.

Hatching Fluid

The method of Fairbairn (1961), slightly modified, was employed to obtain hatching fluid. Approximately 10 to 12 ml of infective eggs were deshelled by treatment with fresh 6 per cent sodium hyperchlorite

solution (2 ml hyperchlorite per ml eggs) and agitation in a water bath shaker maintained at 35°C. The degree of deshelling was determined by microscopic examination. Complete deshelling usually required from 2.5 to 3 hours. Following removal of the shells, the eggs were washed at least 8 times with distilled water, using 20 ml of water per wash, to remove the sodium hyperchlorite. They were then transferred to a 250 ml round bottom flask containing 20 ml of a solution consisting of 0.25 M sodium chloride and 0.1 M sodium bisulfite and allowed to incubate at 39°C for 2 or 3 minutes. An equal volume of gassed 0.1 M sodium bicarbonate was then added (The sodium bicarbonate was gassed by bubbling it with a 95 per cent nitrogen - 5 per cent carbon dioxide gas mixture until a pH of 8 was obtained). The container was then stoppered, placed in a 39°C water bath, and gassed (95% N, 5% CO_2) by bubbling for 15 minutes. At the end of this time the container was placed in a water bath shaker, maintained at 39°C, and agitated until hatching was complete. Usually 3 hours were required for hatching, as determined by microscopic examination. Hatching fluid was collected only if a 85 to 90 per cent hatch rate was achieved. To collect the hatching fluid, the larvae and the medium containing the hatching fluid were transferred to 50 ml centrifuge tubes and centrifuged at 1,720 X G for 10 minutes. The supernatant fluid containing the hatching fluid was removed and centrifuged at 10,000 X G for 30 minutes at 4°C. The supernatant fluid was removed and dialysed against four changes of distilled water (2 liters) at 4°C over a period of 24 hours. The dialysate was then lyophilized and 1 mg was subsequently assayed for chitinase activity (See Chitinase Assay Procedure). The lyophilized hatching fluid was stored at 4°C.

Second Stage Larvae Antigen

Larvae were collected from the hatched eggs and washed 5 times with 0.85 per cent NaCl maintained at 38°C. A total of approximately 200 ml of 0.85 per cent NaCl was used to wash 6 ml of larvae. Following the last washing they were suspended in sterile saline and disrupted by sonic vibration using the same procedure described above. This was followed by extraction with saline, dialysis, and lyophilization as described above.

Second Stage Larvae Excretion and Secretions (E.S.)

Larval E.S. antigen was obtained by incubating washed larvae from hatched eggs in a lactated Ringers solution (Eli Lilly) containing 300 units of penicillin and 300 mcg of streptomycin. The larvae were incubated for 24 hours at 38°C in a water bath shaker. At the end of incubation, a sample was examined microscopically to determine per cent viability of the larvae. In all preparations, at least 85 to 90 per cent of the larvae were found to be alive and moving about. The solution was transfered to 50 ml centrifuge tubes and centrifuged at 1,720 X G for 10 minutes. The supernatant fluid containing the excretions and secretions was recovered and recentrifuged at 10,000 X G for 30 minutes at 4°C. The final supernatant fluid was collected, dialysed, and lyophilized.

First Stage Larvae (Egg)

Undeveloped eggs were collected, washed, treated with sodium hydroxide, and washed again as described above. They were then incubated for 14 days using the method described for the preparation of infective eggs. At the end of this time, approximately 90 to 95 per cent of the eggs had developed to the first stage. The eggs were collected and pro-

cessed as described for infective eggs.

Adult Antigen

Male and female adults were obtained from a local abattoir. Twenty of each sex were selected and washed several times in 0.85% sodium chloride at 38°C to remove all visible debris. Following the final wash, the worms were placed in a pre-cooled (4°C) Waring blender and homogenized in a small amount of cold 0.85% sodium chloride until only small pieces remained. This material was then sonicated using the method already described. The sonicate was extracted and the extract was processed as described for preparation of the other antigens.

Preparation of Antisera

Antigens were suspended in 0.5 ml of 0.85% sodium chloride, mixed with an equal volume of incomplete Freund's adjuvant and injected subcutaneously every ten days into each of two albino rabbits weighing approximately three kg. Four different injection sites (left and right shoulders and flank regions) were used per immunization. The immunizing dose was based upon dry weight (lyophilized) or protein content. The latter was determined using Folin-Ciocaltean Phenol reagent according to the method of Kabat and Mayer (1964). Either 1.0-1.5 mg (dry weight) or 0.5 mg protein was used for the primary immunization. With each suceeding injection, the dosage was increased 1 mg. Immunizations were continued until the desired quantity of precipitating antibody was obtained. This was determined by Arthus reactions and by periodic bleedings and examination of the sera by immunoelectrophoresis. When Arthus reactions were obtained and when no additional precipitin bands were seen, the animals were

given an intravenous challenge consisting of 1 mg of antigen. Intracardial bleedings were begun 10 to 12 days later. The sera collected from each rabbit were stored separately at -20°C.

Immunoelectrophoretic Analysis (IEA)

Immunoelectrophoresis was done according to the method described by Scheidegger (1955) as outlined in the LKB (6800A) Immunoelectrophoresis Operational Manual. A 10 mg per ml concentration of the antigen was prepared. Approximately 0.005 ml was then placed in a well cut in the agar and electrophoresed for one hour at a setting of 250 volts and 50 MA. Precooled (4°C) 0.1M vernonal (Barbital) buffer, pH 8.6, was used in all experiments. Following electrophoresis, a trough was cut on each side of the well, 2 mm from the well. Homologous antiserum was placed in one trough and heterologous antiserum was placed in the other. This permitted comparisons of antigens. After the application of antisera to the troughs, the slides were incubated in a humid chamber at room temperature for a period of 24 hours. They were then processed and stained with amido Schwartz according to the procedure given in the LKB IE. Operational Manual.

Composite Drawing of Immunoelectrophoretic Patterns

An overhead projector (3M) was used to project the image of each slide onto a piece of paper. The images projected onto the screen were 4 times the size of the original slide. The projected precipitin bands were traced onto the sheet of paper. The drawings were then retraced on a large piece of acetate paper and photographically reduced.

Absorption of Antisera

A one milligram quantity of lyophilized extract was mixed with 1 ml of the antiserum to be absorbed. Precipitates were allowed to form for 1 hour at 37°C in a water bath, and then at 4°C overnight. Following centrifugation for 20 minutes at 1,720 X G at 4°C, the absorbed antiserum was recovered and tested by IEA. In some instances, only two absorptions were needed while in others three or four were required.

Chitinase Assay

The method of Reissig et al. (1955), with slight modification, was employed to assay for chitinase activity. One mg of lyophilized antigen, 2 mg purified lobster chitin colloid, and 1 ml of 0.1 M phosphate buffer, pH 6, were placed in a 15 ml conical centrifuge tube. The method of Reynold (1954) was used to purify the chitin. The colloid was prepared from the purified chitin according to the method of Skujens and co-workers (1965). The tube was tightly stoppered and agitated for 1½ to 3 hours in a 38°C water bath shaker. Following incubation, the tube was centrifuged at 1,720 X G for 2 minutes and a 0.5 ml sample of the supernate was assayed for end product (N-acetylglucosamine). A Bausch and Lomb Spectrophotometer (Model 20) was employed to measure optical densities using a wave length of 585 mu. The amount of end product in the test sample was estimated by extrapolation from a curve obtained from similar tubes containing known amounts of N-acetylglucosamine.

Protein and Carbohydrate Assays

A 1 mg sample of each antigen was analysed for carbohydrate and protein content. Lang's (1958) method for determination of Kjeldahl

nitrogen was used for protein estimations. The method outlined by Kabat and Mayer (1964), reaction for total hexoses, was used for carbohydrate determinations.

Antibody Inhibition of Chitinase

One mg of each antigen was assayed for the presence of chitinase according to the method described previously (Chitinase Assay). Concentrations of 1 mg, 500 mcg, 250 mcg, and 125 mcg of antigen per 0.1 ml of 0.85 per cent sodium chloride were placed in test tubes containing 1 ml of either antiserum or normal serum (control) diluted 1 to 2. All sera were first cleared by centrifugation at 25,000 X G for 45 minutes at 4°C. (Campbell, et al., 1964). Prior to testing, the antiserum and the normal serum control were matched, as closely as possible, as to optical density at a wave length of 625 mu. A control containing 0.1 M phosphate buffer (pH 6) and antigen only was also included for each antigen concentration. All tubes were stoppered and placed in a 37°C water bath for one hour. They were then stored for 2 hours at 4°C., after which time the pH of each tube containing antiserum and normal serum was adjusted to 6 by the addition of one drop of 1 N HCl. One drop of phosphate buffer was added to buffer control tubes to maintain equal volumes. The total contents of each tube was then transferred to a 15 ml conical centrifuge tube containing 1 mg of lyophilized chitin colloid. A 0.1 ml volume of phosphate buffer was then added to each tube. All tubes were tightly stoppered, placed in a 38°C water bath shaker, and agitated for 3 hours. The tubes were centrifuged for 3 minutes at 1,720 X G and a 0.5 ml sample was assayed for end product using an additional modification of Reissig's method to test for N-acetylglucosamine. After the addition of 0.1 ml of

potassium tetraborate, each tube was tightly stoppered to prevent evaporation and placed in a 70°C water bath for 45 minutes. (In the original method, the tubes are placed in a boiling water bath for 3 minutes after addition of tetraborate.) Although the degree of color development was slightly reduced, the prolonged heating at a reduced temperature prevented undesirable coagulation of serum proteins which might interfere with optical density measurements of the end product. The small amount of serum precipitate that formed in some cases was removed by centrifugation (1,720 X G for 2 minutes) before optical density measurements were made. Control blanks of antisera and normal sera were processed as described above.

Preliminary Attempts to Identify Hatching Fluid Chitinase by Immunoelectrophoresis

Hatching fluid was obtained by the method described previously (Hatching Fluid) and assayed for the presence of chitinase. Only preparations exhibiting chitinase activity were used. Five mg of lyophilized hatching fluid, 15 mg of purified lobster chitin, and 1 ml of 0.1 M phosphate buffer (pH 6) were placed in a 15 ml conical centrifuge tube. Five mg of hatching fluid and 1 ml of buffer were placed in a second tube to serve as a heat stability control. The tubes were tightly stoppered, placed in a 38°C water bath shaker, and agitated for 24 hours. They were then centrifuged at 1,720 X G for 3 minutes. The supernate was recovered and saved. The enzyme-substrate complex was washed five times with phosphate buffer, using 5 ml of buffer per wash, by centrifuging for three minutes at 1,720 X G. The first four washings were pooled and kept separate from the fifth washing. The absorbed hatching fluid and washings

were maintained at 4°C until they were lyophilized. After the last washing, the enzyme-substrate complex was suspended in 5 ml of a 15 per cent sodium chloride-0.1 M phosphate buffer solution (pH 6.0) and allowed to elute at 4°C for 14 hours. The mixture was then centrifuged for 4 minutes at 1,720 X G and the salt eluent was recovered. The salt eluent, absorbed hatching fluid, and washings were then dialyzed against four 2,000 ml changes of distilled de-ionized water at 4°C over a period of 24 hours. The dialysates were lyophilized, reconstituted in 0.1 ml of 0.85 per cent NaCl, and analyzed by immunoelectrophoresis. To determine if incubation at 38°C for 24 hours had an effect upon the hatching fluid, it was compared with non-incubated hatching fluid using immunoelectrophoretic analysis.

CHAPTER III

RESULTS

Protein and Carbohydrate Assay

Lyophilized antigens were analyzed for their carbohydrate and protein content and the results are given in Table 1. All but two of the antigens, undeveloped egg and first stage larva (egg), contained a higher per cent of protein than carbohydrate.

Immunoelectrophoretic Analysis of Antigens

The immunoelectrophoretic patterns obtained when the various antigen preparations (undeveloped egg, first stage larva (egg), infective egg, hatching fluid, second stage larva, second stage larva E.S., female and male adult) were tested by immunoelectrophoresis (IE), with homologous and heterologous antisera are summarized by the composite drawings in Figure 1. These drawings were made from appropriate slides following electrophoresis of the antigen, reaction with antibody, and staining of the resulting precipitates. A summary of these results is also presented in Table 2. Prior to making critical analysis of the antigen preparations, various concentrations were tested with homologous antisera to determine which concentration gave the largest number of bands and which, if any, of the components was in antigen excess. In all cases, the maximum number of bands could be demonstrated by using an antigen concentration

TABLE 1

RESULTS OF PROTEIN AND CARBOHYDRATE ASSAYS OF ANTIGENS

Antigen Preparation	µg Protein/Mg[a]	µg Carbohydrate/Mg[a]
Undeveloped Egg	411.4	533.2
First Stage Larva (Egg)	390.0	533.2
Infective Egg	497.9	206.6
Hatching Fluid	455.0	126.6
2nd Stage Larva	572.0	333.2
2nd Stage Larva (E.S.)	572.0	203.3
Female Adult	715.0	293.2
Male Adult	455.0	400.0

[a]Values represent a single determination.

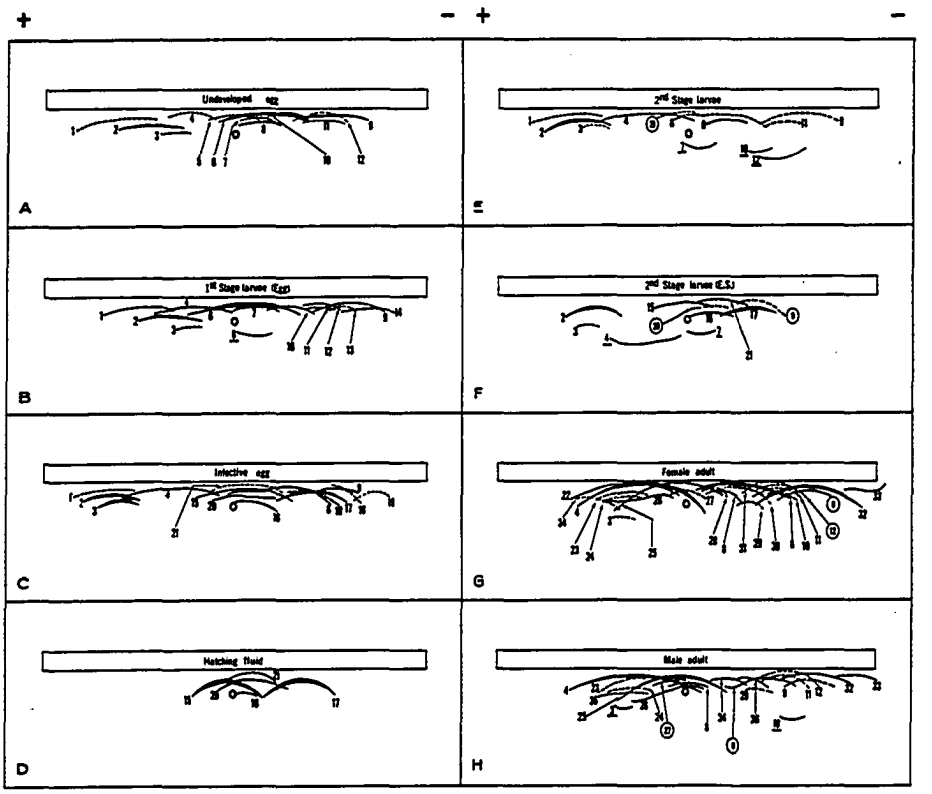

Figure 1. Immunoelectrophoretic analysis (micro-method) of undeveloped eggs, first stage larvae (eggs), infective eggs, hatching fluid, 2nd stage larvae, 2nd stage larvae (E.S.) and male and female adult antigen preparations as revealed by reaction with homologous or heterologous rabbit antisera. Antisera was placed in troughs and antigens in wells.

Dashed lines - weak precipitin bands
Circled numbers - designation is questionable
Underlined numbers - precipitin bands obtained with heterologous antisera

TABLE 2

RESULTS OF IMMUNOELECTROPHORETIC ANALYSIS

	Precipitin Band Number Designation																					
Antigen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22 through 36
Undeveloped Egg	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	
First Stage Larva (Egg)	+	+	+	+	-	+	+	(+)	+	+	+	+	+	+	-	-	-	-	-	-	-	
Infective Egg	+	+	+	+	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	
Hatching Fluid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	
2nd Stage Larva	+	+	+	+	-	+	(+)	+	+	(+)	+	(+)	-	-	-	-	-	-	-	-	+?	
2nd Stage Larva (E.S.)	-	+	+	(+)	-	-	(+)	-	+?	-	-	-	-	-	+	+	+	-	-	+?	+	
Female Adult	-	-	+	+	-	+	-	+	+	+	+	+?	-	-	-	-	-	-	-	-	-	22 through 34
Male Adult	-	-	(+)	+	-	+	-	+	+	(+)	+	+	-	-	-	-	-	-	-	-	-	23-27, 29, 32-36

\+ Present
\- Absent
() Obtained with heterologous antisera
? Number designation is questionable

of 10 mg of protein per ml. Any concentration below this resulted in a loss of certain bands. However, in three instances a previously undetected precipitin band appeared when lower antigen concentrations were used. Precipitin band number 16 appeared only when a 5 mg/ml concentration of infective egg or second stage larva E.S. antigen was used, while a 2.5 mg/ml concentration of hatching fluid was required to demonstrate the presence of this band when it was reacted with homologous antiserum. Each antigen was tested with homologous antiserum collected from each of the two rabbits immunized with each antigen. Each pair of rabbits produced antisera that gave the same number of bands when reacted against the homologous antigen. However, in some cases the precipitin bands were sharper and much easier to see with antiserum collected from one of the two rabbits. No bands were obtained when antigens were tested with preimmune serum.

When it was tested with its homologous antiserum, the undeveloped egg extract was found to contain a minimum of 12 antigenic components (Slide A, Figure 1 and Table 2). Precipitin bands 2, 6, and 8 were more intense than the others and were considered to be major bands. No additional bands were demonstrated when the material was reacted with antisera prepared to the other antigens. The undeveloped egg extract contained one stage specific antigen (band 5) which was not present in any of the other antigen preparations.

The first stage larva (egg) extract was found to contain 13 antigenic components (Slide B, Figure 1 and Table 2). All of the precipitin bands were demonstrated with homologous antisera except for band 8 which was demonstrated with antiserum to undeveloped egg. Precipitin bands 2

and 6 were considerably more intense than the others. Precipitin bands 13 and 14 were considered stage specific since they were not demonstrated in the other antigen preparations.

The immunoelectrophoretic pattern obtained with the infective egg extract and its homologous antiserum is presented in Slide C, Figure 1 and Table 2. Of the 14 precipitin bands shown, numbers 2, 3, and 17 were of the greatest intensity. Band number 16 could be demonstrated only with a 1:2 dilution of the usual 10 mg/ml antigen concentration. No additional precipitin bands could be seen by testing the antigen with heterologous antisera. Seven antigenic components (precipitin bands 15 through 21) were found in the extract of infective eggs which were not present in antigens prepared from the earlier stages of development. Conversely, the infective egg extract lacked 7 antigenic components (bands 5, 7, 8, 11, 12, 13, and 14) that were present in either the undeveloped egg or first stage larva (egg) antigen extract.

When hatching fluid was analysed by I.E., it was shown to have at least 5 antigenic components (Slide D, Figure 1 and Table 2). One of these, band 16, could be demonstrated only by diluting the hatching fluid extract as described previously. Precipitin bands 15 and 17 were very heavy and thick in appearance. No additional bands were found when the antigen was tested with heterologous antisera. Bands similar to those demonstrated when hatching fluid was reacted with its homologous antiserum were also seen when the antigen was reacted with antiserum to infective egg and to second stage larval E.S. antigen preparations. The preparation of second larval stage (collected after hatching of eggs) contained only 1 component that appeared to be similar to those found in

hatching fluid.

Immunoelectrophoretic analysis showed 4 bands of identity between the infective egg and hatching fluid. Reciprocal comparisons were made and the same results were obtained. The bands that were identical were 15, 17, 20, and 21.

Immunoelectrophoretic patterns produced with the second stage larvae and second stage larvae (E.S.) are presented in Slides E and F, Figure 1 and Table 2. Twelve and 10 precipitin bands were demonstrated in the second stage larvae and larval E.S. antigen preparations, respectively. Three antigens of the second stage larvae (numbers 7, 10, and 12) were found with heterologous anti-undeveloped egg serum. Of those obtained with homologous antiserum, bands 2 and 3 were major bands while band 21 was especially weak and difficult to demonstrate. Bands 4 and 7 in the E.S. antigen were demonstrated by reacting the antigen with heterologous antisera. Band 4 appeared with second stage larval antiserum while band 7 was detected with undeveloped egg antiserum. Precipitin band 16 could be demonstrated only with a 1:2 dilution of the usual 10 mg per ml concentration of the antigen. Two E.S. antigenic components (bands 2 and 17) appeared to be in highest concentration, while bands 9 and 20 were very weak, making it very difficult to compare them with components present in other antigen preparations.

In the second stage larvae no antigens were found which were unique to that stage. It is of interest to note that it did not contain any antigenic components similar to those in the hatching fluid with the possible exception of one minor component (band 21), but it did share components with the second stage larval E.S. antigen preparation as well as

components similar to those in infective egg, first stage larva (egg), undeveloped egg, and adult antigens. The E.S. antigen appeared to have no unique components. It contained 4 antigenic components (15, 17, 20, and 21) that were similar to those in hatching fluid. The latter observation was confirmed by reciprocal antigen-antibody comparisons. Indirect evidence indicated that antigenic component 16 was also similar.

The patterns of the female and male adult antigenic preparations are shown in Slides G and H, Figure 1 and Table 2. A minimum of 21 antigenic components were found in the female adult while 19 were found in the male adult. All of the female components were demonstrated with homologous antisera. Two (bands 3 and 10) of the precipitin bands present in the male were demonstrated with heterologous female antiserum. The female adult contained 12 antigenic components which were not present in any of the immature stages while the male contained 10 such antigens. Results of comparisons between females and males showed that the male had 2 antigens not shared with the female while the female had 4 antigens not shared with the male.

Antibody Absorption

To further demonstrate the stage specificity of certain antigens and the relationships of the metabolic antigens (hatching fluid and second larvae E.S.) to them, antibody absorption studies were conducted. Only antisera to those materials showing antigenic similarity were used. Also, only heterologous absorptions were done. Following absorption, the absorbed antisera and unabsorbed antisera was allowed to react with the test antigen. The results are presented in Table 3. They will be described and discussed in another section.

TABLE 3

RESULTS OF ANTIBODY ABSORPTION STUDIES

Antiserum Absorbed	Absorption Antigen	Bands Remaining After Absorption
Undeveloped Egg	1st. Stage Larva (Egg)	4
	Infective Egg	5, 8
	2nd Stage Larva (E.S.)	2, 3, 5, 8, 12
	Female Adult	2, 3, 5,
1st. Stage Larva (Egg)	Undeveloped Egg	4, 14
	Infective Egg	0
	2nd Stage Larva (E.S.)	2, 4, 14
	Female Adult	2
Infective Egg	Undeveloped Egg	2, 15, 17, 20, 21
	1st. Stage Larva (Egg)	2, 15, 17, 20, 21
	Hatching Fluid	2, 3, 4, 6, 9
	2nd Stage Larva (E.S.)	3
	Female Adult	2, 3, 15, 17, 20, 21
Hatching Fluid	Undeveloped Egg	15, 17, 20, 21
	Infective Egg	0
	1st. Stage Larva (Egg)	15, 17, 20
	2nd Stage Larva (E.S.)	15, 17, 20
2nd Stage Larva (E.S.)	Undeveloped Egg	2, 15, 17, 20, 21
	1st. Stage Larva (Egg)	2, 17
	Infective Egg	0
	Hatching Fluid	2
	Female Adult	All
Female Adult	Undeveloped Egg	All (except 4, 8, and 9)
	1st. Stage Larva (Egg)	All (except 4, 8, and 9)
	Infective Egg	All (except 4, 6, 8 and 9)

Preliminary Attempts to Identify Hatching Fluid Chitinase

Experiments were conducted to further characterize hatching fluid. Attempts were made to identify the band(s) associated with the chitinase known to be present in hatching fluid. Following absorption of the hatching fluid with purified, particulate, commercial lobster chitin, attempts were made to elute the enzyme from the thoroughly washed substrate by treatment with sodium chloride. Subsequently, the absorbed hatching fluid and eluted component were analysed by IEA. The results are given in Figure 2. Following absorption, band number 15 was removed (Slide A, Figure 2). After the chitin was washed with buffer and eluted with sodium chloride, band 16 was recovered (Slide B, Figure 2). A precipitin band having the same immunoelectrophoretic pattern was demonstrated in hatching fluid diluted 1:4 (Slide D, Figure 2). No precipitin bands were obtained with the last (5th) wash (Slide C, Figure 2). The salt eluted component was also assayed for chitinase activity, but none was detected.

Assay of Antigen Preparations for Presence of Chitinase Activity

Antigens were assayed for chitinase activity and the results are summarized in Table 4. All of the preparations contained chitinase except the undeveloped egg and female adult extracts. One mg of each preparation was also tested for the presence of free N-acetylglucosamine prior to assay for chitinase activity. None was detected in any of the preparations. The highest concentration of chitinase was found in the infective egg extract while the smallest amount was found in the second stage larval extract.

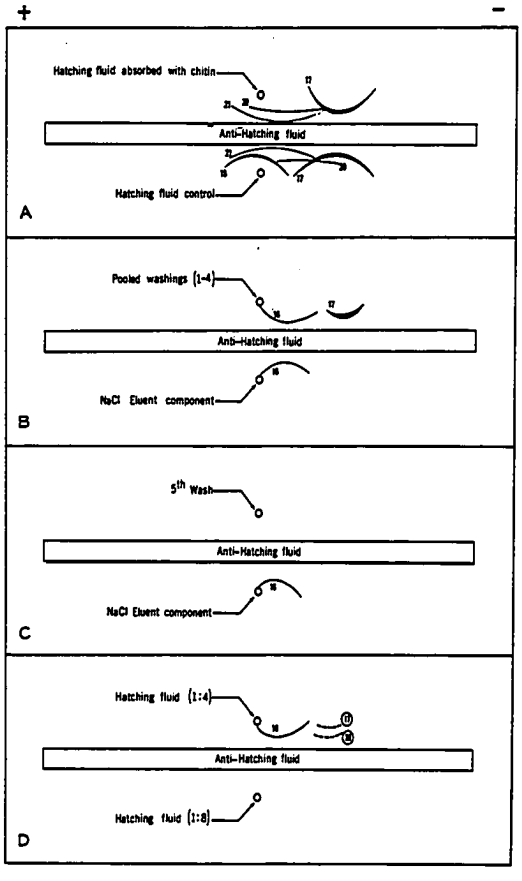

Figure 2. Immunoelectrophoretic analyses of hatching fluid following absorption with chitin.

Dashed lines - weak precipitin bands
Circled numbers - designation is questionable

TABLE 4

ASSAY FOR PRESENCE OF CHITINASE
IN ANTIGEN PREPARATIONS

Antigen Preparation	N-Acetylglucosamine (µg/mg)[a]
Undeveloped Egg	-
1st. Stage Larva (Egg)	6.0
Infective Egg	18.3
Hatching Fluid	15.0
2nd Stage Larva	4.1
2nd Stage Larva (E.S.)	11.2
Female Adult	-

[a] Mean values of two assays.

Antibody Inhibition of Chitinase

Preliminary experiments were conducted to determine if hatching fluid chitinase could be inhibited with homologous antiserum or antiserum prepared to either infective egg, undeveloped egg, first stage larva (egg), second stage larva, commercial (fungal) chitinase, or Toxocara canis hatching fluid. Also included was a control of bovine serum albumin (BSA) and anti-BSA to determine if antibody-antigen complexes non-specifically interfere with hydrolysis of chitin by chitinase. Substrate and commercial chitinase were also reacted in the presence of hatching fluid antiserum to determine if the serum nonspecifically inhibits enzyme hydrolysis. Normal serum and buffer controls were included. Following the incubation of 1 mg of HF with serum, chitin was added and after 3 hours incubation at 38°C the supernatant fluid was assayed for end product (N-acetylglucosamine). One step in this method requires that the supernatant fluid be boiled for 3 minutes. This coagulated the serum and prevented optical density readings (O.D.). However, color did develop in the coagulated serum and could be easily seen. Visual observation showed that inhibition was obtained only in the hatching fluid antiserum-hatching fluid chitinase system and to a lesser degree with the infective egg antiserum-hatching fluid chitinase system. However, because of lack of standardization and because of the coagulation problem the results were not considered definitive or conclusive. For these reasons, a modified procedure, described in Materials and Methods, was developed which prevented coagulation of the serum and permitted O.D. readings to be made. However, due to the limited supply of hatching fluid, the previous experiment was not repeated or elaborated upon. Instead, the modified procedure

was used to determine if the chitinase(s) of first stage larva (egg), infective egg, and hatching fluid could be inhibited by homologous antiserum under more standardized conditions as described in Materials and Methods (p. 14). The results are presented in Table 5. The chitinases of the infective egg and hatching fluid, but not the first stage larva (egg), were inhibited by their homologous antiserum.

Subsequent experiments were done to determine if the chitinases of these three materials were antigenically related. Five hundred µg and 250 µg quantities of infective egg and hatching fluid and 1.0 mg of first stage egg extract were allowed to react with homologous and heterologous antisera. This was followed by assays for enzyme activity by the procedure described above. The results are presented in Table 6. The hatching fluid antiserum inhibited the infective egg chitinase and its own chitinase but not that of the first stage larva (egg). The infective egg antiserum inhibited the chitinase of the hatching fluid and first stage larva (egg), as well as its own. The first stage larva (egg) antiserum was not reacted against hatching fluid or infective egg chitinase since earlier experiments had shown that it failed to inhibit homologous chitinase. The antigen preparation used were the same as that used in the previous experiment (See Table 5). The values obtained in both experiments with the homologous system were quite similar.

TABLE 5

INHIBITION OF CHITINASE IN FIRST STAGE LARVA (EGG), INFECTIVE EGG, AND HATCHING FLUID ANTIGENS BY HOMOLOGOUS ANTISERA

Antigen Preparation	Concentration µg	Optical Density[a] Homologous Antiserum	Normal Serum Control	Buffer Control
First Stage Larva (Egg)	1000	.110	.162	.060
	500	.090	.080	.050
	250	.025	.030	.020
	125	.015	.000	.010
Infective Egg	1000	.185	.750	.255
	500	.080	.280	.130
	250	.000	.150	.080
	125	.000	.090	.040
Hatching Fluid	1000	.000	.290	.160
	500	.000	.240	.115
	250	.000	.120	.060
	125	.000	.100	.020

[a]Values are from a single determination.

TABLE 6

INHIBITION OF CHITINASE IN HATCHING FLUID, INFECTIVE EGG, AND FIRST STAGE LARVA (EGG) BY HOMOLOGOUS AND HETEROLOGOUS ANTISERA

Antigen Preparation	Concentration µg	Optical Density[a] Anti-HF	Anti-Inf. Egg	Normal Serum
Hatching Fluid (HF)	500	.000	.020	.230
	250	.000	.000	.165
Infective Egg (Inf.E.)	500	.050	.000	.200
	250	.000	.000	.120
First Stage Larva (Egg)	1	.120	.030	.190

[a]Values represent a single determination.

CHAPTER IV

DISCUSSION

Results of the protein and carbohydrate assays showed that most of the extracts studied contained a higher per cent of protein than carbohydrate. As one would expect, as the eggs developed from the undeveloped to the infective stage, there was an increase in protein and a reduction in carbohydrate content. The values obtained with the hatching fluid are difficult to understand, in that some 40 per cent of its composition was not accounted for. The amount of protein present in the hatching fluid was comparable to that detected in the other stages. It is possible that some of the material for which we could not account is lipid, but it is doubtful that this could account for all of it.

The antigenic complexity of the various developmental stages examined was demonstrated by immunoelectrophoresis. As anticipated, the adult stages appeared to be considerably more complex than the other stages. The undeveloped egg, first stage larva (egg), infective egg, second stage larva, male adult, and female adult extracts contained 12, 13, 14, 12, 19, and 21 antigenic components, respectively. From these results, the increase in complexity, as well as changes in antigenic composition, during the course of development were clearly shown. The number of antigenic components found in the female adult preparation was comparable to that reported by Tormo and Chordi (1965). More antigenic components were demonstrated

in the undeveloped and infective eggs than had been reported previously (Kagan, 1957). Perhaps additional antigenic components could have been demonstrated by using gamma globulin as the antibody rather than serum. However, analysis of the antigens used in this study would have been greatly complicated if immunization or serological procedures that showed a significant number of additional antigenic components had been used.

The results of the present studies revealed antigenic similarities as well as distinct differences (stage specific antigens) between developmental stages. The immunoelectrophoretic patterns obtained with the undeveloped egg and first stage larva (egg) extracts were very similar. Nearly all of the antigenic components present in the undeveloped egg had similar counterparts in the first stage larva (egg). The results of antibody absorption studies further demonstrated similarity of the antigenic components (Table 3). Only precipitin band number 4 remained following absorption of the undeveloped egg antiserum with first stage larva (egg) antigen, while bands number 4 and 14 remained following absorption of the first stage larval antiserum with undeveloped egg antigen. It thus appeared that precipitin band number 14 was specific for the first stage larva (egg) and that absorption was incomplete for precipitin band number 4. However, precipitin bands number 5 and 13 had also been shown to be stage specific for undeveloped egg and first stage larval extract, respectively. The unexpected absorption of antibodies to these components may be accounted for in two ways. The first stage larva (egg) extract preparation was definitely contaminated with extracts from undeveloped eggs, in that 2 to 5 per cent of the eggs failed to reach the first stage of development. This concentration of undeveloped egg in the first stage

larval extract may have been sufficient to absorb antibody associated with precipitin band 5 which had been shown to occur only with undeveloped egg extract. A possible explanation for the absorption of antibody involved in band number 13 by the undeveloped egg extract may be that the undeveloped egg preparation contained this component in amounts insufficient for producing a visible antigen-antibody reaction but in large enough amounts to absorb antibody.

The infective egg preparation was found to contain, possibly, 7 stage specific antigens (Table 2). Antibody absorption studies (Table 3) confirmed the specificity of 4 of these 7 components. The specificity of components number 18 and 19 was questionable because antibodies to them were removed by absorption with undeveloped egg, first stage larva (egg), and female adult extracts. It is possible that in these three preparations these antigenic components were present in concentrations high enough to absorb antibody but not high enough to produce a visible precipitin reaction in the homologous systems. The antibody absorption data (Table 3) indicated that antibody to component number 16 was removed by heterologous absorption. However, this might be misleading since the absorbed sera were not tested with the appropriate antigen concentration (5 mg/ml) to demonstrate the component. The low supply of antigens prevented the testing of absorbed serum routinely with diluted antigen as well as with the usual 10 mg/ml concentration. Further studies are necessary to clarify the status of this component. The continued presence of antibody associated with band number 2 following absorption with heterologous undeveloped egg and first stage larva (egg) was probably due to incomplete absorption. As will be seen in Table 2, the

first stage larva (egg) extract contained 6 antigenic components which were not similar to those present in the infective egg (bands 5, 7, 8, 11, 12, and 13), while the undeveloped egg contained 5 unrelated antigenic components (bands 7, 8, 11, 12, and 13). However, this difference was not confirmed by antibody absorption (Table 3). Again, the lack of antigens containing organisms all of the same developmental stage probably accounted for the difficulties encountered in interpretation, i.e., the infective eggs used to prepare antigens contained a small percentage of first stage larvae (egg) and undeveloped eggs. Thus, the presence of antigens of these "contaminants" could account for the unexpected complete absorption that occurred when first stage antisera was absorbed with infective egg extract. Although antiserum absorption studies can confirm the interpretation of immunoelectrophoretic results and, in some instances, suggest relationships not discernible by immunoelectrophoresis alone, such studies may confuse the picture if the antigens used for absorption contain mixtures of several antigens. Unfortunately this was the case in the present study.

Results of the immunoelectrophoretic analysis of hatching fluid showed that it contained a minimum of 5 antigenic components (Table 2). Of these, 4 were similar to antigens present in the infective egg stage, when 10 mg per ml antigen concentration was used for analysis, a fifth band (number 16) was found to be similar in analyses using diluted antigen preparations. Thus, these antigenic components (bands 15, 16, 17, 20, and 21) were first demonstrated in the infective egg and were recovered from the material (hatching fluid) released during the hatching process. The fact that none of the 5 antigenic components of hatching fluid were

present in the earlier stages, other than the infective egg, indicated their specificity for that stage. With one exception, this specificity was further demonstrated by antibody absorption studies, i.e., precipitin band number 21 failed to appear following absorption of hatching fluid antiserum with first stage larva (egg) antigen. The reason for this is uncertain. However, it is possible that some of the eggs used to prepare the first stage larval antigen were close to full development, even though they were harvested after only 14 days of incubation. Again, the component involved in band 21 may have been present in first stage extract but not in high enough concentration to form a visible precipitin band with antibody.

From the above analyses it is obvious that the antigenic components of hatching fluid are present in extracts of infective eggs but not in undeveloped egg or first stage extracts. This finding suggests that considerable antigenic change takes place between the first and second larval stage of development. This change may have occurred during molting of the larva from the first to the second stage. This would suggest that molting and hatching fluid are closely related physiological substances. Perhaps the only differences between the two is that hatching fluid contains enzymes which assist the larvae to emerge from the eggs. One must, of course, realize that the antigens associated with subsequent molts may have stage specific components not detected in the present study. Comparative studies would be desirable if antigens of these stages could be collected.

Hatching fluid contained those antigenic components released from the egg during the first one or two hours of hatching. Since these same

components were found in the infective egg, it is possible that they bathed or surrounded the larva in the infective egg before the hatching stimuli were applied. The release of components unique to the hatching process was not demonstrated. If unique components were present, either (1) the immunoelectrophoretic procedure was not sensitive enough to detect them, (2) they remained attached to substrates associated with the egg, or (3) they were not present in high enough concentration either to provoke significant antibody or elicit a subsequent precipitin reaction.

The results obtained with the second stage larvae (E.S.) preparation showed that at least 4, and possibly 5 (bands 15, 16, 17, 20, and 21) components were similar to those of hatching fluid and infective egg. It is possible that part of the hatching fluid was E.S., or that antigenic substances were elaborated by the larvae which were antigenically similar to those present in the infective egg and hatching fluid. Apparently these components were produced in the egg prior to hatching, as was suggested from the results of the comparison made between the hatching fluid, E.S., and infective egg. The results of the absorption study were somewhat difficult to interpret in that not all of the hatching fluid antibodies were absorbed with E.S. extract. The fact that reciprocal absorption of the E.S. antiserum with infective egg or hatching fluid antigens resulted in removal of antibodies of this antigen suggested that the absorption was incomplete. The E.S. preparation also contained antigenic components which were similar to those present in undeveloped egg, first stage larva (egg), and infective egg. This was shown both by immunoelectrophoresis and by absorption. These particular antigens probably represented contaminations that arose from the overnight incubation

of the hatched larvae (which unavoidably included considerable hatched and unhatched egg debris) and as such are not part of the E.S. antigenic mosaic.

The fact that all of the second stage larval antigenic components were similar to those present in undeveloped egg and first stage larva (egg), with the exception of the component involved in band 21, suggests that they were all somatic in nature. Additional evidence for this conclusion was provided by the results of the comparisons made between this extract, hatching fluid, and E.S. preparations. That is, second stage larval extract lacked the majority of antigens associated with E.S. and hatching fluid (bands 15, 16, 17, and 20).

Comparisons made between the second stage larvae and adult stages revealed that they shared at least 8 similar antigens. The second stage larval extract also contained 3 antigenic components (bands number 1, 2, and 7) which were not found in female or male adults. Because these 3 antigens were not present in the adult stages they are probably unique to the immature stages and disappear during the course of development from the second stage to mature adults.

Although antibody absorption studies were done using the second stage larval extract, the results were not interpretable. Evidence indicated that a high degree of contamination with other stages was responsible for the confusing results. This was perhaps to be expected since the extract of sonicated larvae also contained undeveloped eggs, infective eggs that failed to hatch, and egg shell debris. Unfortunately there was no way of separating the larvae from these contaminants.

The much larger number of antigenic components present in the

adult stages, as opposed to those present in the immature stages, might be a quantitative reflection of the higher degree of differentiation of the adult stage. None of the antigens in the adult was found to be similar to those of the hatching fluid, thus demonstrating further the specificity of the latter antigens for the infective egg and larval excretions and secretions. It appeared also, from the comparisons made with the immature stages, that antigenic components associated with bands 1, 2, 5, and 7 are transient or embryonic in nature in that they were demonstrated in the immature but not the adult stages. However, the results of the antibody absorption study showed that only the antigens of bands 2 and 5 were embryonic. It is not known why antibodies involved in bands 1 and 7 were absorbed out of antisera against the immature stages. Especially since these antigens could not be demonstrated by reacting immature stage antigen against adult antisera or by reacting adult antigen against immature stage antisera. It is possible that the rabbits used to prepare antisera to the adults failed to respond to these components. Obviously, antigenic components 1 and 7 were present in immature and adult stages. No absorption studies were conducted with the male adult antigen except in regard to its relationship to the adult female.

Attempts to identify the antigen band(s) associated with chitinase by absorbing the enzyme onto substrate, followed by salt elution and subsequent immunoelectrophoresis, were not successful. Following the absorption of hatching fluid with chitin, one major antigenic component (band 15) was missing. Immunoelectrophoretic analysis showed that the absorbed component possessed a total net negative charge, at a pH of 8.6. However, the absorption was done at a pH of 6.0. The possibility that the

absorption of this substance by chitin was nonspecific can not be excluded. Attempts were made to isolate the absorbed component from the chitin by salt elution and to identify it by immunoelectrophoresis. The eluted component obtained was not the anodic band number 15 which had been initially absorbed out, since the results of immunoelectrophoresis showed that the eluted component migrated toward the cathode rather than the anode.

The salt-eluted component showed the same immunoelectrophoretic position as antigenic component number 16 of diluted hatching fluid, second larvae E.S., and infective egg preparations. However, no chitinase activity could be demonstrated with the salt eluted component. It may be that the salt treatment was too harsh, resulting in a total loss of activity, or possibly the amount of eluted material was insufficient for enzyme detection by the assay method employed. Thus, no direct evidence was obtained as to which component contains chitinase. If band 15 involved such a component, then salt treatment failed to remove it from the substrate or else destroyed it completely. More critical studies are needed before definite conclusions can be made as to whether component 15 or 16, or some other component, represents chitinase.

Each antigen preparation was assayed for the presence of chitinase. The finding of chitinase in the first stage larvae (eggs) infective eggs, second stage larvae, and second stage larval E.S. preparations was totally unexpected. Results of studies conducted by other investigators had indicated that chitinase was an induced enzyme (Roger, 1958; Fairbairn, 1961). The present findings strongly suggest that this is not the case, especially in view of the fact that chitinase was found in 14 day old eggs. It has been shown that eggs less than 16 days old can

not be stimulated to hatch (Fairbairn, 1961). There was a possibility, as mentioned previously, that some of the eggs used in the present study to prepare the first stage larva (egg) extract had developed much more rapidly than others and therefore were nearly, or fully mature. In any case, neither the 14 day old eggs nor the infective eggs were stimulated to hatch prior to disruption by sonic vibration. It seems highly unlikely that disruption at 4°C by sonic oscillation would have provided adequate and proper stimulation for enzyme inducement. Thus, chitinase production was not dependent on the usual stimuli used to induce hatching. The presence of chitinase activity in the second larvae E.S. suggested that chitinase was secreted by larvae. Although excretions and secretions were collected from hatched larvae only if an acceptable percentage of eggs (90 to 95 per cent) had hatched, some unhatched embryonated eggs were always present. Some of these unhatched eggs might have hatched and released their chitinase during the incubation of the larvae E.S. Indirect evidence against this possibility was the relatively high chitinase activity in the E.S. antigen. Considerably more activity was shown than could be attributed to the possible hatching of the small percentage of unhatched eggs that were present. Also, it was noted that if infective eggs did not hatch within 3 hours of stimulation very few, if any, would hatch with further stimulation.

The presence of chitinase in the second stage larvae extracts would suggest that the enzyme was present in significant amounts in somatic tissue. However, the presence of unhatched eggs, whose contents would be released during sonic disruption of the larvae, was probably responsible for the presence of enzyme, especially in view of the

relatively small amount of chitinase detected.

Chitinase-inhibiting antibodies were measured by determining their ability to inhibit the enzyme from hydrolyzing its substrate. Of the 3 systems adequately studied (hatching fluid, infective egg, and first stage larva (egg)), only the chitinase(s) of the first two were inhibited by their homologous antisera. Apparently the first stage larva antiserum contained no inhibiting antibodies (Table 5). The relatively small amount of chitinase present in the first stage larva (egg) antigen (Table 4) might have been insufficient to induce the production of antibodies. It is also possible that the rabbits failed to produce antibody even though chitinase was present.

A subsequent study was undertaken to learn if the chitinases of the hatching fluid, infective eggs, and first stage larvae (eggs) were antigenically similar as determined by inhibition by homologous and heterologous antibody. The results (Table 6) indicated that infective egg antiserum inhibited the chitinases present in hatching fluid and first stage larval extract. On the other hand, hatching fluid antiserum inhibited infective egg chitinase but had slight, if any, effect on chitinase present in first stage larva (egg) antigen. This suggested that the chitinase of the first stage larva was antigenically different from that of the hatching fluid and may reflect a developmental change in the enzyme. The lack of inhibition by the hatching fluid antiserum is unexplainable at present unless one presumes that the chitinase present in the first stage larva (egg) and infective eggs disappeared during the hatching process. Further work would be required to clarify the significance of these findings.

The specificity of hatching fluid chitinase deserves further study. The results of preliminary experiments indicated that antiserum to chitinase does not react with heterologous chitinases. These results need to be evaluated more critically. If they are confirmed, it is possible that chitinase would be a useful serological antigen or an effective antigen for immunization.

CHAPTER V

SUMMARY

Various developmental stages (undeveloped egg, first stage larva-egg, infective egg, and male and female adults) and certain metabolic substances (hatching fluid and second stage larval secretions and excretions) of Ascaris suum were analysed by immunoelectrophoresis for their antigenic components. A total of 12, 13, 14, 12, 19, and 21 antigenic components were found in soluble extracts of undeveloped eggs, first stage larvae (eggs), infective eggs, hatched second stage larvae, male adult and female adults, respectively.

The immunoelectrophoretic patterns obtained with extracts of undeveloped eggs and first stage larvae (eggs) were very similar. The former contained one antigen which was not present in any of the other antigen preparations, while the latter contained 1, and possibly 2, stage-specific antigens.

The infective egg soluble extract contained 4 (possibly 5) stage-specific antigens. Hatching fluid contained the same 5 components. None of these 5 components was present in earlier development stages, indicating the specificity of these antigens for the infective egg stage. These and other findings suggested that considerable antigenic changes take place between the first and second larval stages of development. No unique antigens were found in the hatching fluid.

The second stage larval E.S. antigen preparation contained 10 antigenic constituents. Of these, at least 4, and possibly five, were similar to those present in the hatching fluid and infective egg. This finding suggested that these 5 components are secreted or excreted by the larva and accumulate in the fluid surrounding the larva prior to hatching.

No unique antigens were found in the second stage larval extract. The results indicated that this preparation was grossly contaminated with antigens of earlier stages of development, making antibody absorption results difficult to interpret.

Relatively little antigenic differences were found between the female and male adults. However, they contained considerably more antigenic components than did the immature stages.

Attempts to identify hatching fluid chitinase using immunoelectrophoresis met with very limited success. Two antigenic components were suspected of being chitinase. One was revealed by absorption of hatching fluid with chitin while the other was demonstrated following salt elution of the substrate. No enzyme activity could be detected in the salt eluted fraction.

All of the antigen preparations contained chitinase, as shown by assay, with the exception of the undeveloped egg and adult extracts. The presence of relatively small amounts of chitinase in the second stage larval extract was attributed to unhatched infective eggs present during preparation of the larval extract. The results indicated that chitinase is not an induced enzyme, as had been reported previously, since it was detected in 14 day old eggs as well as in unstimulated, infective eggs.

Chitinase was also detected in the second stage larval (E.S.) preparation which suggested that the enzyme was elaborated by larvae.

From a study conducted to determine the presence of chitinase inhibiting antibodies in first stage larva (egg), infective egg, and hatching fluid antisera, it was found that only the latter two contained such antibodies. Subsequent experiments revealed that the hatching fluid antisera inhibited the chitinase of hatching fluid and infective egg, but inhibited only slightly, if at all, that of the first stage larva (egg). Infective egg antisera markedly inhibited the chitinase(s) of all three preparations.

BIBLIOGRAPHY

CAMPBELL, D. H. 1936. An antigenic polysaccharide fraction of Ascaris lumbricoides (from hog). J. Infect. Dis. 59: 266-280.

CAMPBELL, D. H. 1937. The immunological specificity of a polysaccharide fraction from some common parasitic helminths. J. Parasit. 23: 348-353.

CAMPBELL, D. H., GARVEY, J. S., CREMER, N. E., and SUSSDORF, D. H. Methods in Immunology, W. H. Benjamin, Inc., New York, 1964. pp. 43-44.

CANNING, G. A. 1929. Precipitin reactions with various tissues of Ascaris lumbricoides and related helminths. Am. J. Hyg. 9: 207-266.

CINADER, B. 1963. Antibody to enzymes - A three-component system. Ann. N. Y. Acad. Sci. 103: 494-548.

COSTELLO, L. C. 1964. The comparative biochemistry of developing Ascaris eggs. III. Flavin adenine dinucleatide extracted from unembryonated eggs. Exp. Parasit. 15: 1-16.

FAIRBAIRN, D. 1955. Embryonic and postembryonic changes in the lipids of Ascaris lumbricoides eggs. Can. J. Biochem. Physiol. 35: 122-129.

_____. 1961. The in vitro hatching of Ascaris lumbricoides eggs. Can. J. of Zool. 39: 153-162.

HUNTLY, C. C. and MORELAND, ALVIN. 1963. Gel-diffusion studies with Toxocara and Ascaris extracts. Am. J. Trop. Med. & Hyg. 12: 204-208.

KAGAN, I. G. 1957. Serum agar double diffusion studies with Ascaris antigens. J. Infect. Dis. 101: 11-19.

KAGAN, I. J., JESKA, E. L. & GENTZKOW, C. J. 1958. Serum-agar double diffusion studies with Ascaris antigens. II. Assay of whole worm and tissue antigen complexes. J. Immunol. 83: 400-406.

KABAT, E. A. Kabat and Mayer's Experimental Immunochemistry, 2nd ed. Charles C. Thomas, Springfield, Ill., 1964.

KENT, N. H. 1960. Isolation of specific antigens from Ascaris lumbricoides (var. suum). Exp. Parasit. 10: 313-322.

LANG, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. 30: 1692-1694.

OLIVER-GONZALEZ, JOSE. 1943. Antigenic analysis of the isolated tissue and body fluids of the roundworm, Ascaris lumbricoides var. suum. J. Infect. Diseases 72: 202-212.

_____. 1944a. The inhibition of human isoagglutins by a polysaccharide from Ascaris suum. J. Infect. Dis. 74: 81-84.

OLIVER-GONZALES, J. and TORREGROSA, M. V. 1944b. A substance in animal parasites related to the human isoagglutinogens. J. Infect. Dis. 74: 173-177.

PASSEY, R. F. and FAIRBAIRN, D. 1957. The conversion of fat to carbohydrate during embryonation of Ascaris lumbricoides eggs. Can. J. Biochem. Physiol. 35: 511-525.

REISSIG, J. L., STROMINGER, J. L., and LEIOIR, L. F. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217: 959-966.

REYNOLDS, D. M. 1954. Exocellular chitinase from a Streptomyces sp. J. Gen. Microbiol. 11: 150.

RHODES, M. B., KELLY, G. W. and MARSH, C. L. 1965. Studies in helminth enzymology. IV. Immune responses to malic dehydrogenase from Ascaris suum. Exp. Parasit. 16: 373-381.

ROGERS, W. P. 1958. Physiology of the hatching of eggs of Ascaris lumbricoides. Nature. 181: 1410-1411.

SCHEIDEGGER, J. J. 1955. Uno micro methode de 1' immunoelectrophorese. Int. Arch. Allergy 7: 103.

SKUJINS, J. J., POTGEITER, H. J., and ALEXANDER, M. 1965. Dissolution of fungal cell walls by a Streptomycete Chitinase and B-(1-3) gluconase. Arch. Biochem. Biophys. 111: 358.

SOULSBY, E. J. L. 1957. Antigenic analysis of Ascaris tissues by the double diffusion precipitin test. Trans. Royal Soc. of Trop. Med. and Hyg. 51: 9-10.

_____. 1963. The relative value of differences in antigens from various stages of helminth parasites as used in diagnostic tests. The American J. of Hyg. Monographic Series #22. July. pp. 47-56.

TORMO, J. and CHORDI, A. 1965. Immunoelectrophoretic analysis of *Ascaris suum* antigens. *Nature*. 205: 983-985.