IMMUNODIFFUSION STUDIES WITH *ASCARIS SUUM* GOEZE, 1782 AND *TOXOCARA CANIS* (WERNER), 1782

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ABSTRACT

IMMUNODIFFUSION STUDIES WITH *ASCARIS SUUM* GOEZE, 1782 AND *TOXOCARA CANIS* (WERNER), 1782

by Reginald George Nash

The antigens of *Ascaris suum* and *Toxocara canis* were studied by a slide microtechnique of double diffusion. Diffusion of antigens against homologous antisera produced in rabbits indicated the following numbers of antigenic components in various stages of the life cycles of the two species: six in the unembryonated eggs of both species, four in the embryonated eggs of both species, eight in the adult stage of *T. canis*. No antiserum was used to the antigens of the adult stage of *A. suum*.

Cross reactions were demonstrated by diffusion of each antiserum against the heterologous antigens. The results indicated that most antigenic components cross reacted with the heterologous species.

Two specific antigens were demonstrated in the adult stage of *T. canis* when compared to the adult stage of *A. suum*. Absorption of the antiserum to adult *T. canis* with the antigen to

adult A. suum and diffusion of the absorbed antiserum against the antigen of adult T. canis produced two precipitin lines. These two precipitin lines represent specific antigens of T. canis.

Absence of mutual absorption of antisera with antigens indicated a greater complexity of antigenic components in unembryonated eggs, embryonated eggs and whole worms of A. suum and T. canis than was revealed by diffusion of antisera to each of the stages in the life cycles of these roundworms against their homologous antigens.

Precipitins were indicated in the serum of mice infected with A. suum or T. canis when the serum of infected mice was diffused against microcultures of living larvae of the species with which the mice were infected.

The effects on the serum of albino mice infected with A. suum or T. canis were investigated by immunoelectrophoresis. Twenty serum fractions, including two alpha-twos not reported by previous investigators, were demonstrated in normal mouse serum with this technique. Comparison of the immunoelectrophoretic patterns of infected and normal mice revealed that there was an increase in α_2II globulin in mice infected with A. suum and an increase in gamma globulin in the serum of mice infected with T. canis. Dilutions of the sera

of infected mice prior to electrophoresis compared with similar dilutions of normal mouse serum proved that the apparent increases in globulin fractions in infected mice were not due to maintenance of the levels of α_2 II and gamma globulins while other components of the serum decreased in amount.

The increase in the α_2 II globulin in mice infected with A. suum and the increase in gamma globulin in mice infected with T. canis were not due to production of antibodies to antigens in the unembryonated eggs, embryonated eggs or adults of the species with which the mice were infected. Absorption of the sera of infected mice with these antigens had no effect on the increased fractions in the sera of infected mice.

The changes in serum globulin fractions of mice infected with A. suum or T. canis are not specific to the roundworms. The increase of α_2 II globulin in mice infected with A. suum may be the result of inflammation and tissue damage in the liver since the larvae of A. suum remain in the liver much longer than those of T. canis. The increase in gamma globulin in the serum of mice infected with T. canis may be due to bacterial invasion of the tissues of the intestine following penetration of the larvae.

IMMUNODIFFUSION STUDIES WITH *ASCARIS SUUM* GOEZE, 1782 AND *TOXOCARA CANIS* (WERNER), 1782

By

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INTRODUCTION

A comparative study of the antigens of Ascaris suum Goeze, 1782 and Toxocara canis (Werner), 1782 was completed during the course of this investigation. The double diffusion slide microtechnique of Wadsworth (1957) was used.

Characterization of the antigens of parasitic helminths has been a subject of frequent investigation due to their involvement in most immunological responses of the host including immunity and hypersensitivity. Antigens which are specific to helminths and others which are common to many organisms have been demonstrated. Kent (1960) isolated water soluble antigens from A. suum, four of which were shown to be quite specific by precipitation with homologous antibodies from experimentally infected rabbits and by lack of cross reactivity with sera from other helminth infections such as trichinosis, echinococcosis and schistosomiasis. The Forssman antigen, present in widely separated species, was demonstrated in Trichinella spiralis by Mauss (1941). Marked elevation in heterophile antibodies against the Forssman antigen was demonstrated in

animals infected with A. suum by Soulsby (1958). Oliver-Gonzales (1946) reported that rabbits infected with A. suum showed an increase in the A_1 and A_2 blood isoagglutinins.

Antigens that stimulate the production of antibodies which do not induce an immunity that prevents re-infection are called nonfunctional antigens; those which stimulate the production of antibodies active in immunity are called the functional antigens (Soulsby 1963). The isolation of specific antigens is of importance in relation to diagnostic tests but specific antigens can not be considered as synonymous with functional antigens (Soulsby 1963).

Analyses of the antigens of A. suum by gel diffusion methods have been reported by Soulsby (1957) and Kagan (1957). Huntley and Moreland (1963) demonstrated the similarity between the antigens of Toxocara canis and A. lumbricoides by use of gel diffusion.

Recent investigations by means of fluorescent antibody techniques indicate that the functional antigens are elaborated by larvae of the parasitic nematodes (Taffs and Voller 1963; Crandall et al. 1963).

A study of the effects on the serum of albino mice infected with A. suum or T. canis was undertaken in this study by use of the micromethod of immunoelectrophoresis developed by

Scheidigger (1955).

The alterations in serum proteins during infection with several nematode parasites have been investigated. Leland et al. (1959) demonstrated that in calves infected with Trichostrongylus axei there was a decrease in total serum proteins with an increase or maintenance of the alpha-two globulin level. Andersen et al. (1960) reported that calves infected with the medium stomach worm, Ostertagia ostertagi, showed a marked decrease in amount of albumin and an increase in one or more of the globulins. Leland et al. (1955) found that in rats infected with Nippostrongylus muris the total serum protein was slightly increased and the beta globulin was distinctly increased. Weber (1957) found the gamma globulin was increased in cattle exposed and re-exposed to the lungworm Dictyocaulus viviparus. There was an increase in beta and gamma globulins in lambs infected with Strongyloides papillosus as reported by Turner (1959). Sadun (1957) demonstrated that rabbits infected with Toxocara canis developed hypergammaglobulinemia and there was an increase in the alpha-one fraction of the serum.

RELATED LITERATURE

There are several methods of gel diffusion: single diffusion described by Oudin (1952), double diffusion devised by Ouchterlony (1953), and immunoelectrophoresis developed by Grabar and Williams (1953).

Oudin (1952), of the Pasteur Institute, developed the first method of gel diffusion which consisted of capillary tubes filled with agar in which complex antigen-antibody systems could be analyzed by allowing reactions to occur. The antibodies were mixed with the agar which was then allowed to harden. Antigens were overlaid and layers of precipitates formed at different levels depending upon the rates at which the antigens diffused into the gel. This method is known as simple or single diffusion.

Ouchterlony (1953), at the Karolinska Institute, developed a similar method in which both the antigen and antibody were allowed to diffuse into an agar plate which initially contained neither reactant. Because of this, the method was called double

diffusion. Drops of antigen and antibody were placed in separate wells cut in an agar plate. Movement of the reactants in the agar depended upon their concentrations and diffusion coefficients. Precipitin lines formed when the diffusing antigen met its specific antibody or antibody with which it could cross react. Identification of these precipitin lines was possible if either a known antigen or antibody was placed in a third well. When the diffusing reactants met, the line of precipitate formed by this reaction joined the line formed by the previous reaction to form a continuous precipitin line. Naming of various precipitin lines as reference points in a complex mixture of proteins was possible by this method.

Immunoelectrophoresis, the most recent of the methods in gel diffusion to be developed, is based upon the principle of electrophoresis which has long been known. Electrophoresis may be defined as the migration of particles under the influence of an electrical field (Lewis, 1960). Porret (1816) observed the passage of water through an animal membrane under the influence of an electrical current. Hardy (1899) found that negatively charged particles of egg albumin could be made positive by addition of acid and with the change from alkalinity to acidity the particles migrated in the opposite direction in the electrical field. The electrical properties of proteins were

determined by the nature of the reaction, either acid or alkaline, in the field. If the field was alkaline the particles became electronegative or if the field was acid the particles became electropositive. The particles moved at different speeds depending upon the net charges of the particles - i.e., the greater the number of charges, the faster the migration (Lewis 1960).

The microscopic method of electrophoresis was first used to study blood cells, protozoans and bacteria in a liquid medium in which ionizing compounds were incorporated to render it conductive (Abramson 1934). The medium containing the electrolytes and the cells to be studied were put into a glass tube and placed horizontally on the microscope stage. A current was then passed through the tube and the movements of the blood cells, protozoans and bacteria in the electrical field could be observed.

The microscopic method was replaced by the moving boundary method of electrophoresis perfected by Tiselius (1937). Movement of the boundary between two solutions such as a protein and a buffer could be observed if an electrical current was passed through the two solutions after they were placed in a U-shaped tube so a sharp boundary existed between the two. A series of moving boundaries representing different protein ions

was obtained upon passage of an electrical current through the tube when the protein solution contained ions of different charges or sizes. By this method Tiselius (1937) discovered that the serum globulin of the blood contained three fractions which he named alpha, beta and gamma globulin.

The late nineteen forties marked the development of a third technique called zone electrophoresis (Kunkel and Tiselius 1951). In this method a solid or semi-solid medium was used through which the electrically charged particles were moved by an electrical current. The separated particles aggregated in zones in the medium. Thus it was known as zone electrophoresis. By 1950 this method was being used in diagnosis of nephrosis, liver malfunction and multiple myeloma.

Grabar and Williams (1953) reasoned that it might be possible to combine the refinement of separation of protein mixtures by electrophoresis with the double diffusion technique of Ouchterlony. If a glass plate were covered with buffered agar gel and the serum were electrophoresed in the agar gel, then the various proteins in the serum should be strung out in a line of spots or zones as if they had been placed there in the first place. After electrophoresis the antigens would diffuse radially from each of the spots as they would if on an Ouchterlony plate. In order to locate these antigens visually, antibody

solution could be placed in a longitudinal trough on the plate and allowed to diffuse laterally toward the radially diffusing spots of antigen. When the specific antibody and antigen encounter one another a precipitin line should form in an arc. This technique is now known as immunoelectrophoresis.

The method of immunoelectrophoresis offers great refinement in the separation of protein mixtures (Crowle 1961). Older methods such as chemical analysis of groups of proteins yielded the separation of albumin and globulins. The globulin fraction can be separated into alpha, beta and gamma globulin by zone electrophoresis as stated above. These fractions are also made up of groups of proteins as revealed by immunoelectrophoretic studies of serum (Williams and Grabar 1955). The technique of immunoelectrophoresis as described by Grabar and Williams (1953) required rather large amounts of reactants and a long period of electrophoresis. Scheidigger (1955) adapted the procedure to a miniature method which made it possible to perform the entire procedure on a microscope slide covered with a very thin layer of buffered agar gel. This greatly shortened all steps of the procedure and also reduced the amounts of reactants needed.

Clausen and Heremans (1960) introduced a further modification of the procedure by which it was possible to demonstrate

identical reactions in two samples electrophoresed on the same slide, very much in the same way these reactions can be identified on an Ouchterlony plate. Agar was aspirated from an agar covered slide to produce two circular holes used as wells for the antigens, eight millimeters distance being left between the two wells. A discontinuous or interrupted trough was placed between the two antigen wells and a continuous trough was placed laterally to each of the antigen wells. Following electrophoresis of the sera the lateral troughs were filled with the respective homologous antisera and the central discontinuous trough was filled with a mixture of the two antisera. Identical precipitins from both of the electrophoresed sera formed continuous arcs.

Previous to 1955 only three papers existed relating to the use of electrophoresis as a tool to elucidate the effect of helminthic infections on the plasma proteins of the host. These are by Wright and Oliver-Gonzales (1943) and Schwonzen (1951) concerning Trichinella spiralis and Evans et al. (1955) on Schistosoma mansoni.

Since 1955 numerous papers have appeared in the literature concerning changes in plasma proteins as the result of helminthic infection. Leland, et al. (1955) reported that in

rats infected with Nippostrongylus muris the total serum protein was slightly increased and the beta globulin was greatly increased. In rats heavily infected with Cysticercus fasciolaris, Kraut (1956) found there was an increase in total globulin due to increases in the alpha-two, beta and gamma globulins. He concluded that these changes were not specific and that the degree of increase in globulins was correlated with the degree of liver involvement. Ground worm material was injected into rats and an immunity to subsequent infection developed without increases in any globulin fraction. Sadun et al. (1957) demonstrated that rabbits infected with Toxocara canis developed hypergammaglobulinemia and there was an increase in the alpha-one fraction of the serum. Weber (1957) found that gamma globulin was increased in cattle exposed and re-exposed to the lungworm Dictyocaulus viviparus. An increase in the beta and gamma globulins in lambs infected with Strongyloides papillosus was reported by Turner (1959). Leland et al. (1959), studying Trichostrongylus axei infections in calves, observed hypoproteinemia and an increase or maintenance of the alpha-two globulin level. Kruidenier and Katoh (1959) observed an increase in the beta and gamma globulins in the serum of cats and rats infected with the lung fluke Paragonimus kellikoti. Andersen et al. (1960) demonstrated that calves infected with

the medium stomach worm, Ostertagia ostertagi, showed a marked decrease in the amount of albumin and an increase in amount of one or more of the serum globulins.

Stauber (1954), basing his statement largely on chemical analyses, reported that albuminemia and hyperglobulinemia are characteristic of many conditions including parasitic infections. Loss of plasma protein resulting in albuminemia could be due to lesions in the alimentary tract through which leakage of albumin could occur (Leland 1961). Perlmann et al. (1943) found that with lesions caused by burns there was a decrease in total plasma volume. Changes in the relative amounts of various serum proteins may be the result of rate of synthesis or loss of some fractions by internal destruction or external leakage (Leland 1961). There is a possibility that a decrease in albumin which usually maintains serum osmotic pressure may effect an increase in some other serum fraction to compensate for the loss (Leland 1961).

The liver was indicated as the site of production of serum albumin by Peters and Anfinsen (1950). The alpha and beta globulins are also thought to be synthesized in the liver (Leland 1961). Miller and Bale (1954) found that the alpha globulins were produced and turned over at a more rapid rate than other

protein components of the serum. Raffel (1961) believes that plasma cells are the site of production of gamma globulin.

Kuttler and Marble (1960) produced an experimental anemia in sheep by bleeding them in order to determine if the changes observed in serum fractions during parasitic infections could be due to the anemia which frequently accompanies such infections. The results indicated that the changes in serum proteins observed during infection were not due to anemia.

Shedlovsky and Scudder (1942) found that an increase in alpha globulin occurred when inflammation or tissue damage was involved. The changes in serum proteins of hosts infected with parasitic helminths are associated with inflammation or tissue damage and therefore the changes can not be considered as specific to the parasite (Leland 1961). Further evidence to support the view that changes in serum globulin fractions during infection with parasites are non-specific was found in the work of Atchley et al. (1961) in regard to experimental infection of guinea pigs with the protozoan Entamoeba histolytica. Heavily infected animals developed a change in the alpha-one region as revealed by immunoelectrophoretic technique. Since tissue damage should be correlated with the degree of infection, the change in the alpha-one region could very well be associated

with the extensive tissue damage present in heavy infections with E. histolytica.

Kent (1960) isolated antigens from adult female A. suum. The worms were homogenized and defatted with ethanol and ether and extracted with buffered distilled water. After centrifugation the decanted supernatant was used as a source of antigens. The proteins were separated by paper electrophoresis and agar gel electrophoresis. Five major fractions were recovered. Ouchterlony double diffusion tests and immunoelectrophoresis indicated at least four specific antigens precipitated by the homologous antibodies from infected rabbits. Sera from suspected cases of visceral larva migrans, presumably of Toxocara canis origin, did not react with the antigens in most cases but the sera of the two human patients with A. lumbricoides infections reacted with the antigens twelve days before eggs were found in the stools.

The immunoelectrophoretic characteristics of normal mouse serum was presented by Heremans et al. (1959). Antimouse serum containing the largest number of antibodies was produced by giving rabbits four intramuscular injections of 0.5 milliliter of mouse serum at intervals of six weeks. Freund's adjuvant was used with the first of these injections. Immunoelectrophoresis was done by the method of Scheidigger (1955).

After development of the precipitin arcs, the bows were labeled according to their relative electrical mobilities within the alpha, beta and gamma regions, starting with those most anodically placed and progressing to those most cathodically placed. Heremans et al. (1959) observed at least twenty different fractions in mouse serum. These were a pre-albumin, albumin, four bows in the alpha-one region, six in the alpha-two region, one in the beta-one region, four in the beta-two region, three in the beta-three region and one in the gamma region.

Later, in the immunoelectrophoretic studies of sera from mice carrying transplantable plasma cell leukemias, Clausen and Heremans (1959) found that increases in amounts of various fractions of the serum could be ascertained by a dilution of serum previous to electrophoresis. Comparisons of the dilutions of serum from normal animals with the serum of animals bearing the transplantable plasma cell leukemias revealed changes which were not readily detectable without dilutions of the sera.

The similarities of human and mouse serum proteins were demonstrated by Clausen and Heremans (1960) by use of the immunoelectrophoretic method. Two antigen wells were placed on an agar covered slide. Mouse serum and

human serum were placed in the two respective wells. A trough parallel to the path of electrophoretic migration was cut laterally to each of the antigen wells into which the homologous antisera were placed following electrophoresis. Another discontinuous or interrupted trough was cut between the antigen wells into which a mixture of antihuman and anti-mouse antisera was placed. This way of cutting the agar left several areas through which proteins of both mouse and human sera could diffuse toward one another. The method allowed cross reactions to occur between homologous mouse and human proteins. Mouse serum proteins which cross reacted with horse antihuman antiserum were prealbumin, albumin, four alpha globulins, three beta globulins and gamma globulin. Among the fractions of mouse serum which were found to share antigenic determinants with human serum were gamma globulin and α_2II globulin, found to be related to human haptoglobin.

There has been considerable temptation to interpret any changes in serum protein fractions of hosts infected with helminth parasites as being associated with immunity but there has been little justification for this except when the gamma globulin fraction was involved (Leland 1961). Immunity of a host to helminth parasites is the result of response of the host to antigens of the parasite. Consequently, the antigens

of helminths have been investigated to determine which antigens are involved in the production of immunity of the host (Soulsby 1963).

The antigens of ascarids have been investigated frequently since Canning (1929) demonstrated that various tissue fractions of A. suum were antigenic. He injected rabbits with various tissues of A. suum to obtain antisera. Higher titres of antibody were secured with homologous tissue antigens than with heterologous tissue antigens. The antigenicity of a polysaccharide fraction from A. suum was demonstrated by Campbell (1936). In 1937 he found that the polysaccharide fraction of ascarids of humans and pigs were serologically specific. Oliver-Gonzales (1943) reported that the egg of A. suum contained an antigen responsible for precipitins in the serum of immune rabbits which killed the larvae of A. suum in vitro. In 1944 he found that the polysaccharide fraction of A. suum inhibited agglutination of human erythrocytes and that the polysaccharide fraction was related immunologically to human erythrocyte antigens in type A and type B blood. The polysaccharide fraction inhibited the hemolysis of sheep cells. He concluded that the polysaccharides of A. suum and other helminths were antigenic and related to human isoagglutinins and Forssman antigen. In 1946 he found that infection in

rabbits with A. suum larvae caused an increase in A 1 and A 2 isoagglutinin titre in the hosts serum. Serum absorbed with cuticle of A. suum gave no reduction in titre. In 1946 he showed that helminth polysaccharides inhibited the action of A 1 and A 2 isoagglutinins in human serum. In 1953 he reported that the erythrocytes in type O and type B human blood could absorb the polysaccharide fraction of A. suum. The A 2 isoagglutinin titre of serum was reduced to zero with these antigens.

Sprent and Chen (1949) found that active or passive immunity could not be induced in mice with whole worms or various tissue antigens of A. suum.

Sprent (1949) found that repeated intravenous injections of antigens of A. suum in humans, rabbits, guinea pigs and mice produced skin sensitivity in the absence of precipitins in man, anaphylactic sensitivity in rabbits and precipitins in the absence of skin sensitivity in mice. In 1950 he found that guinea pigs infected with A. suum could be anaphylactically shocked with other nematode antigens but not with pneumococcus polysaccharide, hemolyzed group A erythrocytes, normal guinea pig kidney and lung, acanthocephala, cestode, annelid or molluscan antigens. The substance causing shock was both protein and polysaccharide in nature. In 1951 he

reported that guinea pigs infected with A. suum could be anaphylactically shocked with extracts of the larvae of A. suum and their metabolic products.

In reporting on the behavior of the larvae of A. suum in mice, Sprent (1952) stated that the larvae that penetrated the intestine went to the liver, lungs and intestine from which they were expelled with the fecal material. On the way to the lungs the larvae grew from an average of 0.2 mm. to 1.05 mm. in length in a period of eight days. In mice that were reinfected few larvae migrated to the lungs and those that did were much smaller than those in the first infection. Many larvae were encapsulated by accumulations of inflammatory cells. Sprent (1952) reported that in mice infected with A. suum the larvae stayed in the liver for four days following infection. Congestion of the lungs appeared from the fifth through the eighth day. No larvae were found in the mice after two weeks. The larvae of T. canis in mice were found to migrate through the somatic tissues without reaching the intestine. The kidneys were badly infected from the second to the seventh day. The larvae left the liver much sooner in mice infected with T. canis than in mice infected with A. suum.

Nichols (1956b) stated that the behavior of the larvae of T. canis and A. suum was strikingly similar in mice and man.

Beaver (1952) called this condition visceral larva migrans. Heiner and Kevy (1956) found that if T. canis was the causative agent the condition persisted for as long as sixteen months and with massive infections death resulted. Nichols (1956a) reported that the only means of diagnosing this syndrome was to find the larvae in the tissues of man. Differential serological diagnosis may be possible in the future by isolation of specific antigens from the larvae of each of the species (Kagan et al. 1959). Cross reactions between the antigens of A. suum and T. canis preclude serological diagnosis at present (Huntley and Moreland 1963). The analysis of antigens of A. suum by double diffusion was reported by Soulsby (1957). Antiserum in 0.4 percent agar was placed in a serological tube, overlaid by 0.5 milliliter of 0.4 percent agar in saline and superimposed by 0.5 milliliter of antigen homologous to the antiserum. The antigens used were saline extracts of the tissues of A. suum. Double diffusion was allowed to procede for fourteen days. Whole worm antigen produced a maximum of nine bands of precipitate with its homologous antiserum; intestine produced fourteen bands; cuticle produced nine bands. Dense rings indicated protein antigens whereas fine rings indicated polysaccharide antigens.

Kagan (1957) reported the results of double diffusion

studies with antigens of A. suum. Antisera against whole worm antigen was produced in rabbits. Antigens were prepared from unembryonated eggs, embryonated eggs, enteric fluid, muscle, cuticle and whole worms of A. suum. Whole worm antigens were prepared from T. canis, T. cati and A. lumbricoides, ascarids from the dog, cat and man respectively. Double diffusion tests indicated there were ten antigenic components when whole worm antigen of A. suum was tested against full strength antiserum. Full strength antiserum to whole worm antigen of A. suum indicated the following number of antigenic components when tested against various tissues: muscle, five; cuticle, six; embryonated eggs, two; unembryonated eggs, six. When the full strength antiserum to whole worms of A. suum was diffused against whole worm antigen of T. canis, four bands of precipitin were observed; with T. cati, five; with A. lumbricoides, seven.

In all of the absorption studies done by Kagan (1957), the antiserum to whole worms of A. suum was diluted one to four which greatly simplified the results since many precipitin lines did not appear with this dilution of the antiserum. This procedure does not reveal whether the precipitin lines lost by dilution of the antiserum would have been absorbed. When the diluted antiserum to whole worms of A. suum was diffused against the homologous antigen four precipitin bands were observed. When the

diluted antiserum was absorbed with the antigen of unembryonated eggs of A. suum, no precipitin lines were observed. Absorption of the diluted antiserum with antigen of embryonated eggs and subsequent diffusion against whole worm antigen yielded four precipitin lines. These results indicated that the unembryonated egg contained all of the antigens present in the adult stage but the embryonated egg contained none of the antigens present in the adult stage.

Huntley and Moreland (1963) demonstrated the antigenic similarity between T. canis and A. lumbricoides, ascarids from the dog and man respectively, by use of a gel diffusion technique. An antiserum produced in rabbits to antigens in whole worms of T. canis was used throughout the study. Fresh antigens from adult whole worms of T. canis and A. lumbricoides were diffused against the antiserum. At least nine antigenic components were demonstrated in the extract from whole worms of T. canis and at least five cross reacting antigens were observed in the extract from the whole worms of A. lumbricoides.

Cross reactions between closely related ascarids have been noted by many: Kagan et al. (1959), using the bentonite flocculation test; Olson (1960), using the in vitro larval precipitate test; Huntley and Moreland (1963), using gel diffusion.

Recent investigations by means of fluorescent antibody techniques indicate the antigens functional in immunity are elaborated by larvae. Taffs and Voller (1963) reported that the embryonated eggs of A. suum were antigenic in the sense that they stimulated the production of protective antibodies by the host. The third stage larvae had antigenic sites at the natural orifices and on the cuticle. Crandall et al. (1963), working with A. suum, found by means of fluorescent techniques that the cuticle of second and third stage larvae was antigenic but that the cuticle of the adult stage was not.

A method of detection of metabolic antigens from nematode larvae by a microculture agar gel technique was reported by Olson et al. (1960). Excretions and secretions of living larvae of Trichinella spiralis were allowed to diffuse into the agar where reactions with sera of rabbits infected with the nematode could be observed.

In all analyses by gel diffusion methods, results indicate that molecules may carry determinant groups in exposed, reactive positions or in hidden, non-reactive positions (Ouchterlony 1962). He reported that most biological materials carry similar molecules with determinants of more than one kind in various combinations.

When two antigens are being compared by double diffusion methods, precipitin lines in the patterns are characterized by size, density, stability, whether stationary or migratory, broad or narrow, sharp or diffuse, splitting or coalesced (Ouchterlony 1962). Precipitin lines form when the reactants meet at equivalence. Diffusion coefficients and initial concentrations of reactants as well as solubility of the precipitate in antigen and/or antibody will determine the site of precipitation. If the precipitin line is stationary at the site of its first appearance, there is equivalence of antigen and antibody at this site and at the source of origin.

Duplication of lines does not occur unless one reactant is in excess of the other (Ouchterlony 1962). He stated that under such conditions the selective barrier of the precipitate is diminished and the reactant that is in excess may diffuse through the barrier and cause a duplicate precipitin line to be formed. Another possibility to account for duplicate lines is that different determinants may be attached to the same antigen molecule as suggested by Jennings et al. (1960).

Ouchterlony (1962) stated that in comparative studies of antigens it was necessary to consider the possibility that the antigens are bispecific or multispecific, that is, the

reactive particle may carry determinants of two or more separate specificities. Antibodies may also be bispecific or multispecific.

Korngold (1956) stated that the concept of cross reaction applied to serological relationships between different single antigens which possessed similar structural groupings within their molecules. An antiserum containing antibodies against structural groupings or determinants that two different proteins have in common will react with both proteins to the same extent. If the antiserum contains antibody against a grouping in the homologous antigen but absent from the cross reacting antigen the latter will react with less antibody than the former. Such an antiserum will still react with the homologous antigen after having been absorbed by the heterologous antigen.

Two different mixtures of proteins are often compared on an Ouchterlony double diffusion plate by diffusion of the protein antigens against an antiserum that is homologous to one of the protein mixtures (Korngold 1956). The wells to contain the reactants are usually placed so they form the corners of an equilateral triangle. Precipitin lines which completely coalesce will form if the antiserum contains antibodies against structural

groupings which the two different proteins have in common. Precipitin lines bend toward one another at the point of coalescence. Sometimes an antiserum contains antibody against determinants that the homologous and cross reacting antigens have in common as well as against determinants that are unique to the homologous antigen. The precipitin lines formed by reaction of the antiserum with the homologous and heterologous antigen will not coalesce perfectly but the precipitin line formed by the homologous antigen will continue beyond the point of union of the two precipitin lines to form a "spur".

Two different antigens are sometimes compared by double diffusion against an antiserum that is homologous to neither of the antigens (Ouchterlony 1962). Double spurs can form when an antiserum reacts with the two heterologous but cross reacting antigens if these antigens have at least one determinant that is common and one that is different. The density of double spurs diminishes abruptly in relation to the main precipitin lines. The two precipitin lines coalesce before the double spurs appear and remain fused after the spurs develop. Spurs can be used to determine the degree of cross reactivity (Korngold 1956). The antigens which are not closely related give dense and greatly extended spurs. Closely related antigens

give short spurs which deviate from the direction an uninfluenced line would have taken.

When two antigens are diffused against an antiserum on an Ouchterlony plate precipitin lines often intersect without coalescing in a reaction of non-identity (Ouchterlony 1962). Reactions of non-identity can be distinguished from double spurs because the density of precipitin lines does not diminish rapidly beyond the point of intersection. Precipitin lines do not bend toward one another at the point of intersection in reactions of non-identity (Ouchterlony 1962).

The wide application of diffusion-in-gel methods for immunological analysis is reflected in a bibliography published by Ouchterlony (1962) in which there are 1185 references cited in the field during the period from 1956 to 1960.

MATERIALS AND METHODS

A. Gel diffusion studies.

The adults of Toxocara canis were secured from eight to twelve week old dogs obtained from various Humane Societies. Piperazine citrate was used as an anthelmintic. The adult females of T. canis were slit longitudinally and the distal one-third of the bifurcated uterus was removed. Removal of the eggs was accomplished by placing each uterus between two microscope slides and applying pressure. The eggs were placed in 0.5 percent formalin to inhibit bacterial and mold growth during embryonation. The eggs were aerated by stirring daily for a period of a month when it was assumed that all eggs would have completed embryonation.

Because of the large numbers of embryonated eggs required for these studies, round, covered plastic containers eight inches in diameter were used so that the eggs from at least fifteen adult female worms could be embryonated in one container. After embryonation, eggs were stored at room temperature.

The eggs in the proximal two thirds of the uteri were removed by pressure and frozen for future use in preparation of unembryonated egg antigen.

Occasionally, a few dogs were secured from private sources. These dogs were euthanized after which the intestines were stripped to remove the adults of T. canis. Adult female worms were placed in saline and incubated at 37° C. for seventy two hours during which oviposition continued. The female worms were then removed and the eggs were separated from the saline by centrifugation. The eggs were embryonated in 0.5 percent formalin. Large numbers of eggs were obtained by this method but many did not embryonate. A higher percentage of embryonation was secured with eggs taken from the uteri of worms removed from their hosts by anthelmintics.

The adults of A. suum were obtained by stripping the intestines of slaughtered pigs. Eggs were removed from the uteri of the female worms by pressure and the eggs were embryonated in the 0.5 percent formalin as described above for T. canis. Unembryonated eggs were collected as described above for T. canis and frozen for future use in preparing an antigen.

Water soluble antigens were prepared from unembryonated eggs, embryonated eggs and adult whole worms of each of the

nematode species. Unembryonated egg antigen was prepared by placing equal volumes of unembryonated eggs and saline in a homogenizer and by means of a Sargent's cone driven stirring motor homogenization was attained in ten minutes. This material was centrifuged at 4000 r.p.m. for ten minutes and the supernatant was divided into two milliliter amounts and frozen for future use as unembryonated egg antigen. Embryonated egg antigen was prepared by removal of the eggs by centrifugation from the formalin solution in which they had been embryonated. The eggs were washed five times in distilled water to remove the residue of formalin. Equal volumes of embryonated eggs and saline were homogenized by the method described for the unembryonated eggs. After centrifugation the supernatant material was divided into two milliliter amounts and frozen for future use as embryonated egg antigen. Whole worm antigen was prepared by homogenizing equal volumes of whole worms and saline. This material was centrifuged for ten minutes at 4000 r.p.m. The supernatant was divided into two milliliter amounts and frozen for future use as a whole worm antigen.

An antiserum to each of the six antigens was produced. Each of six rabbits was injected intraperitoneally twice weekly

for a period of two months with two milliliters of one of the six respective antigens. After the initial series of injections of antigen each of the rabbits was injected with three milliliters of the appropriate antigen every month to maintain antibody production. The rabbits were bled by intracardial puncture nine days after the last injection of antigen. Rabbits could be bled without anesthesia if they were placed on their backs with the eyes covered. Fifteen milliliters of blood could be removed each month from each rabbit without apparent harm. Antisera were merthiolated 1 part to 10,000, divided into two milliliter amounts and frozen until used.

A modification of the slide microtechnique of Wadsworth (1957) was used throughout the gel diffusion studies. Two by three inch glass slides were thoroughly cleaned in glacial acetic acid, transferred by forceps through three washes of distilled water and stored in absolute ethyl alcohol until needed. The slides were air dried just prior to use.

A 0.7 percent agar solution was prepared in 0.85 percent saline. Sodium azide was added to 0.01 molar concentration to inhibit growth of bacteria and molds. Three milliliters of the agar solution were pipetted onto each slide. The agar covered slides were placed in the refrigerator for five minutes to allow the agar to gel. Holes were cut in the agar with a number

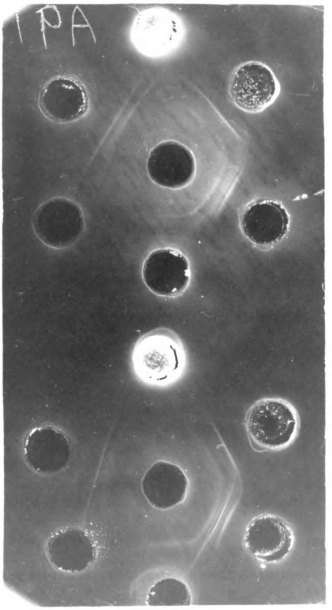

Figure 1. Gel Diffusion Slide Illustrating Configuration of Wells for Reactants.

three cork borer and aspirated to form wells to contain the reactants. The configuration of the wells is evident by examination of Figure 1, the central well being for the antiserum and the surrounding six wells for the antigens.

Each of the six antisera was diffused against the six antigens to determine the number of precipitin reactions taking place between the antiserum and the homologous and heterologous antigens.

Portions of each antiserum were absorbed by each of the six antigens by addition of an equal volume of antigen. The absorbed antiserum was diffused against each of the antigens. Normal rabbit serum was diffused against all of the antigens as a control. Precipitin lines developed by an antiserum to heterologous antigens represent cross reactions. These precipitin lines should not appear following absorption of the antiserum with the heterologous antigens.

In an effort to obtain antigens of excretion and secretion, embryonated eggs of A. suum and T. canis were hatched mechanically by the method of Cleeland and Laurence (1962). Approximately 100,000 embryonated eggs were washed five times with distilled water to remove the formalin in which they had been embryonated. The eggs were placed in a 125 milliliter Erlenmeyer flask containing 20 milliliters of medium 199 and a one

inch teflon bar. The flask containing the embryonated eggs was placed on a magnetic stirrer and run at a speed just under that which caused frothing of the medium. The eggs of A. suum were hatched in twenty four hours. The eggs of T. canis hatched much more quickly, four hours usually being sufficient for hatching of almost 100 percent of the embryonated eggs.

After hatching, the larvae were centrifuged for two minutes at 1,000 r.p.m. and the supernatant fluid was discarded. The sediment containing the larvae was transferred to 20 milliliter screw capped tissue culture tubes which contained 7 milliliters of medium 199, 3 milliliters of beef serum and penicillin and streptomycin in concentrations of 250 units each per milliliter of culture medium. Aseptic techniques were used throughout the culturing procedure. Cultures were incubated at 37° C. .

Gel diffusion studies were done on slides prepared as described previously (see page 30). Freshly hatched T. canis or A. suum were placed in the center well and tested against each of the five antisera used throughout this study. The antigens of secretion and excretion of the larvae of T. canis and A. suum were also diffused against the sera of mice infected for three weeks with T. canis or A. suum. Each of five mice was infected with 1500 embryonated eggs of A. suum. Similarly, each of five

mice was infected with 1500 embryonated eggs of T. canis by stomach intubation. The sera from the five mice infected with each species were pooled and diffused against micro-cultures of larvae of each of the species of nematodes. More media and some larvae were added periodically to the center well to prevent dehydration and to aid in building up a suitable concentration of the antigens of secretion and excretion in the agar so reactions could occur with antibodies.

After charging the wells with reactants, in all of the gel diffusion studies, the slides were placed in a moist chamber which consisted of a dissecting tray with wet paper towels on the bottom and a cover of aluminum foil. Slides were incubated at 37° C. for forty eight hours. Reactant wells were recharged twelve hours after incubation began.

All slides were read while still wet by means of oblique lighting and use of a ten power hand lens. After the first reading, slides were placed in 0.85 percent saline for three hours to remove some of the unreacted protein, washed in distilled water, covered with filter paper and placed in an incubator at 37° C. to dry. The short period of washing was necessary as further washing obliterated some of the precipitin lines. After thorough drying, the slides were stained

with Ponceau S for one half hour. No destaining was done since this made the weak precipitin lines invisible. Stained slides could best be viewed by placing them in a photographic enlarger where the amount of light and the magnification could be quickly changed so all of the precipitin lines could be seen. Hazy precipitin lines were not recorded in this study.

B. Immunoelectrophoretic studies.

Three rabbits were used in the preparation of anti-mouse serum. Two of these rabbits were given intramuscular injections of 0.5 milliliter of mouse serum twice weekly for a period of eight weeks. Following this series of injections an intraperitoneal injection of 0.5 milliliter of mouse serum was given every two weeks to maintain antibody production. Blood was removed by decapitation of mice for the first of these injections. Later, it was discovered that blood could be obtained from mice more quickly by intracardial puncture with a 19 gauge hypodermic needle and syringe.

One of the two rabbits injected with mouse serum by the above method developed a strong antibody titre to twenty fractions of mouse serum whereas the other rabbit developed only a weak titre to three fractions of mouse serum. Continued

injections of mouse serum did not stimulate further antibody production in this rabbit.

The third rabbit was injected by a modification of the method of Proom (1943). The following materials were mixed.

5 milliliters of mouse serum

16 milliliters of distilled water

18 milliliters of 10 percent $KAl(SO_4)_2 \cdot 12 H_2O$

Then 5 N NaOH was added by the drop method until no further precipitation occurred. Following centrifugation the sediment was washed twice with saline after which the sediment was made up to a volume of 20 milliliters in saline and frozen until used.

The rabbit was injected according to the following schedule. On the first and fourteenth days five milliliters were injected intramuscularly in each buttock. On the twenty fourth day one milliliter of untreated serum was injected intraperitoneally. Thereafter, one milliliter of untreated serum was injected intraperitoneally every two weeks to maintain antibody production.

Blood was withdrawn from the three rabbits used for production of antimouse serum by intracardial puncture and

the serum was divided into two milliliter amounts and frozen until used.

Two by three inch glass slides used in the immuno-electrophoretic studies were cleaned in the same way as those used for the gel diffusion studies (see page 30). Removal of all materials which might prevent the agar gel from adhering to the slides was necessary.

The two following buffer solutions were prepared using the method of Hirschfeld (1960). The buffer to be used in the electrode vessel contained the following materials.

Diethylbarbituric acid	13.80 grams
Sodium veronal	87.60 grams
Calcium lactate	3.84 grams
Distilled water to	10 l.

The buffer to be used in the agar layer was prepared as follows.

Diethylbarbituric acid	16.6 grams
Sodium veronal	105.10 grams
Calcium lactate	15.36 grams
Distilled water to	10 l.

A buffered agar gel was prepared to serve as the supporting medium for the electrophoretic studies. A 1.4 percent

agar solution was prepared using ion agar produced by Consolidated Laboratory, Inc. in distilled water. Two parts of the buffer intended for the agar gel were mixed with one part of distilled water. The agar and diluted buffer were heated for five minutes in an autoclave at 20 pounds pressure after which both were filtered. Equal parts of the diluted buffer and the 1.4 percent agar solutions were mixed resulting in a 0.7 percent buffered agar solution. The agar renders the supporting medium semi-solid and the buffer renders the medium conductive.

Three milliliters of the buffered agar were pipetted onto the surface of each slide. A perfectly flat surface was employed in order to insure even distribution of the agar solution on the slides. The slides were then placed on 6 by 8 inch strips of plexiglass and refrigerated for five minutes to allow the buffered agar to harden.

After the agar gel had hardened, holes were aspirated to form wells to contain the material to be electrophoresed. Pasteur pipettes with an inside diameter of approximately two millimeters were selected for use in aspiration of the holes in the agar gel. Longitudinal slits approximately three millimeters wide to be used to hold the antiserum following electrophoresis were cut with a scalpal prior to electrophoresis

but they were not removed until electrophoresis had been completed. Plexiglass templates with holes and slits cut in the desired locations were used to process the slides more quickly.

Albino mice of the Rockland strain, each weighing 25-30 grams, were infected with 1500 embryonated eggs of T. canis or A. suum. The embryonated eggs were washed five times in distilled water to remove the formal in which embryonation had occurred. Five counts of 0.1 milliliter each were taken of a suspension of embryonated eggs. The average of these five counts was used as a basis for infection of the mice. Mice were lightly etherized and the eggs were introduced by stomach intubation with a Pasteur pipette. Each of five mice was infected with 1500 embryonated eggs of T. canis. The same procedure was used in infecting each of five mice with 1500 embryonated eggs of A. suum.

Samples of blood were drawn from the mice prior to infection and every three days thereafter for a period of three weeks following which the infected mice were exsanguinated and the serum was frozen for future use. Blood was withdrawn from each of the infected mice by clipping off the tip of the tail and filling heparinized capillary tubes with blood. One end of the tube was plugged with clay. The tubes were

centrifuged for five minutes at 4000 r.p.m. . The portion of the capillary tube containing the serum was broken off and the serum was transferred to a tuberculin syringe using a 27 gauge needle. A small drop of this serum was placed in a hole aspirated from the agar covered slide just prior to electrophoresis.

The slides were prepared so that the sera of mice infected with A. suum, normal mice and mice infected with T. canis could be directly compared on the same slide. The configuration of the slides can be seen in Figure 4.

After the serum from the mice infected with A. suum, normal mice and mice infected with T. canis was placed in the wells, a constant current of 110 volts was passed through the buffered agar gel for one and one-half hours. A voltage meter was used to test the actual voltage through the buffered agar on the slides.

The longitudinal slits which had been cut in the agar before electrophoresis were now removed by aspiration to form troughs. These troughs were filled with antimouse serum which had been developed in rabbits. Slides were then placed in a moist chamber consisting of a covered dissecting tray containing some water and were incubated at 37° C. for twenty

four hours to allow development of precipitin lines.

In order to observe more clearly the precipitin lines which seemed to represent increases in amounts of certain globulin fractions during the course of infection, dilutions of the sera of infected mice were run by electrophoresis and precipitin lines were developed by undiluted antimouse serum. The diluent was 0.85 percent saline which did not react with antimouse serum when run as a control.

Five mice were infected with 1500 embryonated eggs of A. suum and five mice were infected with 1500 embryonated eggs of T. canis by the method previously described (see page 39). The mice were bled by a tail vein and 1 to 16 and 1 to 32 dilutions of the sera were electrophoresed previous to infection and every week for a period of three weeks following infection.

Infectivity of the eggs was checked by autopsy of a mouse presumably infected with each of the roundworms seven days after the eggs were given to them. Larvae of each of the species were observed.

In an effort to determine if changes in the globulin fractions of the serum during the course of infection were due to antibody production, sera from mice with a three week infection

with these roundworms were absorbed with antigens prepared from the various stages in the life cycles of the roundworms. The antigens used in these absorption studies were prepared as described above for the gel diffusion studies (see page 28). Antigens of unembryonated eggs, embryonated eggs and whole worms of each of the species were used to absorb serum specimens from mice infected with A. suum, uninfected control mice and mice infected with T. canis. These three serum specimens, absorbed by an equal amount of one of the antigens, were electrophoresed on the same slide and precipitin lines were developed by diffusion against full strength antimouse serum. Comparisons could then be readily made. The antigens were run by electrophoresis as controls and they did not react with antimouse serum.

RESULTS

A. Gel diffusion studies.

A series of two-fold dilutions of the antigens in 0.85 percent saline was diffused against homologous antisera to determine the dilution of each antigen which produced the maximum number of precipitin lines. One to two dilutions of the antigens of adult whole worms of A. suum and T. canis produced the maximum number of precipitin lines when the antigens were diffused against homologous antisera. One to four dilutions of the remaining antigens produced the maximum number of precipitin lines when diffused against their homologous antisera. Dilutions of the antigens producing the greatest number of precipitin lines were used throughout the gel diffusion studies.

The antiserum to adult whole worms of A. suum was very weak, producing only four distinct precipitin lines with its homologous antigen. The antiserum to adult whole worms of T. canis was strong, producing eight precipitin lines with the

homologous antigen. Since the antiserum to adult whole worms of T. canis would disclose all of the cross reactions to the antigen from adult whole worms of A. suum, only the former antiserum was used throughout this study. No precipitin lines developed when normal rabbit serum was diffused against all of the antigens as a control. The following numbers of precipitin lines were developed when the remaining antigens were diffused against their homologous antisera: six with the unembryonated egg antigen of A. suum, six with the unembryonated egg antigen of T. canis, four with the embryonated egg antigen of A. suum and four with the embryonated egg antigen of T. canis.

Precipitin lines developed by an antiserum to heterologous antigens represent cross reactions. When the antiserum to adult whole worms of T. canis was used to develop precipitin lines with the five heterologous antigens, the following numbers of precipitin lines were observed: eight with the antigen of adult whole worms of A. suum, five with the antigen of unembryonated eggs of T. canis, one with the antigen of unembryonated eggs of A. suum and none with the antigens of the embryonated eggs of either species.

Table 1 summarizes the numbers of precipitin lines developed when the antigens to different stages of the life cycles

of the two species of roundworms are diffused against homologous and heterologous antisera.

If the antigen from the adult whole worms of T. canis was diffused against the heterologous antisera, a corresponding number of precipitin lines should appear as in the reciprocal procedure described above. Table 1 shows the following numbers of precipitin lines developed when the antigen of adult whole worms of T. canis was diffused against the various heterologous antisera: five with the antiserum to the unembryonated eggs of T. canis, one with the antiserum to the unembryonated eggs of A. suum, none with the antiserum to the embryonated eggs of A. suum and three with the antiserum to the embryonated eggs of T. canis. When the antiserum to embryonated eggs of T. canis was diffused against antigen of adult T. canis, three precipitin lines formed whereas the reciprocal procedure produced no precipitin lines.

Precipitin lines demonstrated by diffusion of an antiserum against the various heterologous antigens should not appear following absorption of the antiserum with the heterologous antigens. When the antiserum to adult whole worms of T. canis was absorbed by the heterologous antigens and subsequently diffused against these antigens, none of the precipitin lines representing

Table 1. Numbers of precipitin lines developed when antisera are diffused against homologous and heterologous antigens.

	Antigens					
Antiserum	A	B	C	D	E	F
A	8	8	5	1	0	0
C	5	2	6	2	1	0
D	1	4	2	6	0	2
E	3	1	4	0	4	0
F	0	3	0	3	0	4

A - adult whole worms of T. canis

B - adult whole worms of A. suum

C - unembryonated eggs of T. canis

D - unembryonated eggs of A. suum

E - embryonated eggs of T. canis

F - embryonated eggs of A. suum

cross reactions to these antigens developed as shown in Table 2.

Comparison of the data in Tables 1 and 2 reveals the number of precipitin lines which developed when the antigen from adult whole worms of T. canis was diffused against its homologous antiserum as compared to the number of precipitin lines which developed following absorption of the antiserum with the heterologous antigens. Absorption of the antiserum with its homologous antigen acted as a control. The number of precipitin lines removed by absorption of the antiserum with heterologous antigens and subsequent development of the absorbed antiserum with the heterologous antigens represents the number of cross reactions.

If the reactions of the antigens with each of the antisera are analyzed in the same way as was done for the antiserum to adult whole worms of T. canis, several unexpected results are revealed. Examination of Table 1 shows that when the antigen of unembryonated eggs of T. canis was diffused against antiserum to embryonated eggs of the same species four precipitin lines developed whereas only one developed with the reciprocal procedure. Also, when the antiserum to the unembryonated eggs of A. suum was diffused against the

A - adult whole worms of T. canis

B - adult whole worms of A. suum

C - unembryonated eggs of T. canis

D - unembryonated eggs of A. suum

E - embryonated eggs of T. canis

F - embryonated eggs of A. suum

Table 2. Numbers of precipitin lines detected when antigens are diffused against antisera A and C absorbed with each antigen.

Antiserum plus absorbing antigen	Antigens A	B	C	D	E	F
A + A	0	0	0	0	0	0
A + B	2	0	0	0	0	0
A + C	2	2	0	0	0	0
A + D	5	2	1	0	0	0
A + E	3	3	1	0	0	0
A + F	4	2	2	1	0	0
C + A	0	2	0	0	0	0
C + B	2	0	3	0	0	0
C + C	0	0	0	0	0	0
C + D	3	0	1	0	0	0
C + E	4	2	5	0	0	0
C + F	4	2	4	1	0	0

embryonated eggs of the same species two precipitin lines were observed but with the reciprocal procedure three precipitin lines appeared.

The numbers of precipitin lines developed after absorption of the antiserum to the unembryonated eggs of T. canis and A. suum by the various antigens are shown in Table 2 and Table 3 respectively. The data in relation to the antiserum to the embryonated eggs of A. suum are presented in Table 4.

Five precipitin lines developed when the antiserum to adult whole worms of T. canis was diffused against the antigen of unembryonated eggs of the same species. The reciprocal procedure also produced five precipitin lines. These five precipitin lines represented cross reactions. An examination of double diffusion slides demonstrated the identity of three antigens in the adult whole worms of T. canis with three antigens in the unembryonated eggs of the homologous species. Reciprocal absorption of the antisera with antigens indicated that there were cross reactions other than the reactions of identity.

Three reactions of identity developed between antigens of whole worms of T. canis and embryonated eggs of the same species when these antigens were diffused against the antiserum to embryonated eggs of T. canis.

A - adult whole worms of T. canis

B - adult whole worms of A. suum

C - unembryonated eggs of T. canis

D - unembryonated eggs of A. suum

E - embryonated eggs of T. canis

F - embryonated eggs of A. suum

Table 3. Numbers of precipitin lines observed when antigens are diffused against antisera D and E absorbed with each antigen.

Antiserum plus	Antigens					
absorbing antigen	A	B	C	D	E	F
D + A	0	4	0	3	0	0
D + B	0	0	0	1	0	0
D + C	0	3	0	3	0	0
D + D	0	0	0	0	0	0
D + E	0	3	1	3	0	0
D + F	0	4	2	4	0	0
E + A	0	0	0	0	2	0
E + B	0	0	0	0	2	0
E + C	0	0	0	0	2	0
E + D	2	0	3	0	2	0
E + E	0	0	0	0	0	0
E + F	3	0	3	0	3	0

Table 4. Numbers of precipitin lines demonstrated by diffusion of antigens against antiserum F absorbed with each antigen.

Antiserum plus	Antigens					
absorbing antigen	A	B	C	D	E	F
F + A	0	2	0	2	0	1
F + B	0	0	0	0	0	0
F + C	0	0	0	0	0	0
F + D	0	0	0	0	0	1
F + E	0	2	0	2	0	2
F + F	0	0	0	0	0	0

A - adult whole worms of T. canis

B - adult whole worms of A. suum

C - unembryonated eggs of T. canis

D - unembryonated eggs of A. suum

E - embryonated eggs of T. canis

F - embryonated eggs of A. suum

Only one reaction of identity was observed among the antigens of the unembryonated eggs, embryonated eggs and adult whole worms of T. canis. The reaction of identity was demonstrated by diffusion of the above antigens against the antiserum to the unembryonated eggs of T. canis. Absorption studies showed that there were cross reactions other than those revealed by reactions of identity.

Two precipitin lines developed when the antiserum to unembryonated eggs of A. suum was diffused against the antigen from unembryonated eggs of T. canis. The reciprocal procedure yielded two precipitin lines as shown in Table 1. These two precipitin lines represented cross reactions. In order to determine if these were identical antigens, double diffusion slides were examined for reactions of identity. There were two reactions of identity when the antiserum to unembryonated eggs of A. suum was diffused against the antigens of unembryonated eggs of A. suum and T. canis. The reciprocal procedure also resulted in two reactions of identity. The two cross reacting antigens were removed by absorption of the antiserum to unembryonated eggs of A. suum with the antigen to unembryonated eggs of T. canis as shown in Table 3. The results of mutual absorption studies of the antisera show

that there were reactions other than those revealed by the reactions of identity.

When microcultures of living larvae were diffused against the antisera used throughout this study, one precipitin line developed with the antiserum to embryonated eggs of the homologous species. No cross reaction was observed.

When microcultures of living larvae were diffused against the sera of mice infected with each of the species of nematodes no clearly defined precipitin lines were discernible A hazy area appeared in the agar when larvae of each species was diffused against the sera of mice infected with that species indicating that precipitins were present.

B. Immunoelectrophoretic studies.

When normal mouse serum was run by electrophoresis and precipitin lines were developed with antimouse serum, twenty clearly distinguishable precipitin lines developed. Not all of these precipitin lines were visible on the same slide. From the most anodic to the most cathodic in position, these precipitin lines represent the following serum fractions: 1 albumin, 2 alpha-one globulins, 8 alpha-two globulins, 1 beta-one globulin, 4 beta-two globulins, 3 beta-three globulins and 1 gamma globulin. The relative positions of these precipitin lines are

shown in Figure 2. The precipitin lines were named according to the nomenclature of Heremans et al. (1959) who worked out the immunoelectrophoretic characteristics of normal mouse serum.

Heremans et al. (1959) observed precipitin lines representing a pre-albumin and 2 alpha-one globulins which were never observed during the course of this study. However, this study revealed two alpha-two globulins that were not recorded by the previous investigators. If one draws a perpendicular from the widest point of the precipitin arc to the path of electrophoretic migration, the center of concentration of the antigen responsible for the development of the precipitin line can be located. The centers of concentration for two of the antigens called alpha-twos by Heremans et al. (1959) lie much farther anodically than do the centers of concentration for the remaining antigens called alpha-twos. In the present study the centers of concentration for six of the alpha antigens lie close together and much farther cathodically than the remaining two alpha antigens as seen in Figure 3.

The immunoelectrophoretic pattern of normal mouse serum as developed by each antimouse serum must be ascertained before the antimouse serum can be used to determine the effects

Figure 2. Composite of Immunoelectrophoretic Patterns of Normal Mouse Serum.

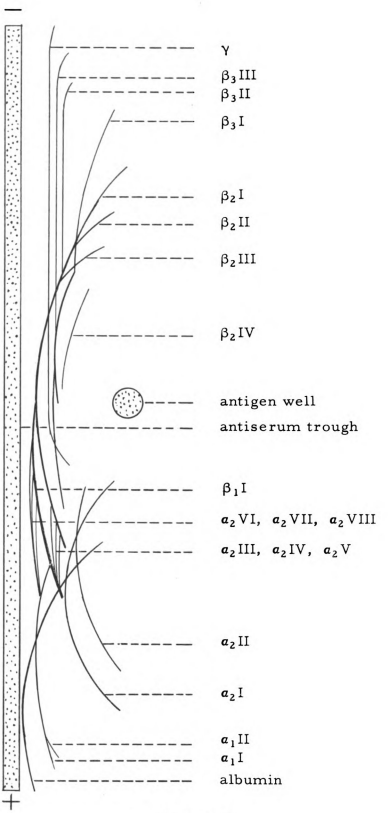
−
+
γ
β3III
β3II
β3I
β2I
β2II
β2III
β2IV
antigen well
antiserum trough
β1I
α2VI, α2VII, α2VIII
α2III, α2IV, α2V
α2II
α2I
α1II
α1I
albumin

Figure 2.

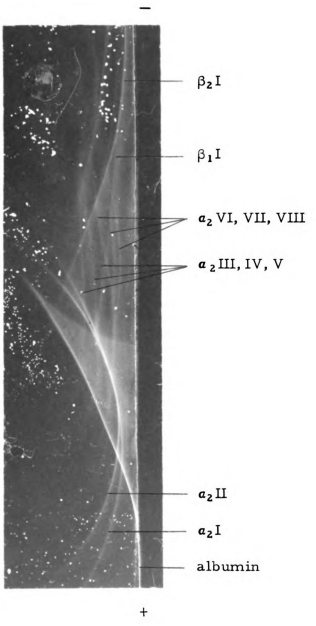

Figure 3. Alpha-two Components in Normal Mouse Serum.

of parasitic infection on the serum proteins of mice. After the electrophoretic patterns of normal mouse serum had been studied, an effort was made to secure comparative data on the effects of infection with T. canis and A. suum on the serum of individual mice. Five mice were infected with 1500 embryonated eggs of T. canis and five mice were infected with 1500 embryonated eggs of A. suum. A series of slides was prepared every three days for three weeks. Although three series of slides were prepared in an effort to secure comparative data on the effects of infection on individual mice, the results of immunoelectrophoresis were found to be too inconsistent to justify such comparisons. However, the large number of slides accumulated as the result of this effort became very useful in establishing changes which occurred in the serum globulins of mice during the course of infection with these two species of roundworms.

An apparent increase in the α_2II serum globulin of mice infected with A. suum was revealed by comparison of the electrophoretic pattern of serum from a normal mouse with that of a mouse infected for eighteen days with A. suum as shown in Figure 4. In order to determine whether this was an apparent or real increase in the amount of this globulin

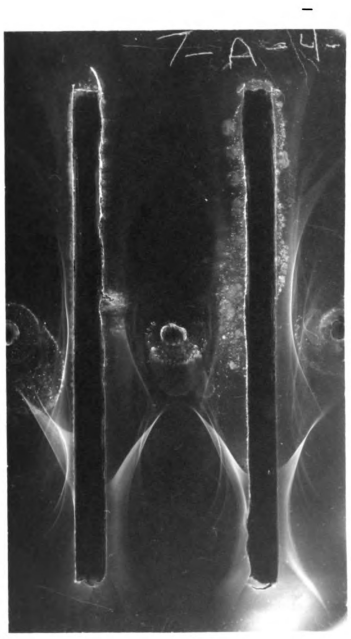

Figure 4. Immunoelectrophoretic Patterns of Undiluted Mouse Serum: from left to right--A. suum infected, normal, T. canis infected.

fraction, serum dilutions of 1 to 16 and 1 to 32 were prepared each week for a period of three weeks from five mice infected with A. suum. These serum dilutions were run by electrophoresis and developed by undiluted antimouse serum. By comparison of the diluted serum from the infected mice with the serum from the uninfected control mice which was similarly diluted, the increase in the α_2II serum globulin became evident on the seventh day following infection as shown in Figure 5.

There was an increase in the amount of gamma globulin in mice infected with T. canis as shown in Figure 5. This increase became apparent on the seventh day following infection and persisted through the third week of infection as revealed by serum dilutions which were electrophoresed and diffused against full strength antimouse serum.

Dilution of the serum prior to electrophoresis proved to be a very satisfactory method of detecting increased amounts of various serum components. Without dilution the increased components were often masked by other components.

The sera of infected mice were absorbed with antigens in order to determine if the alterations in the serum globulin fractions during infection were due to antibody production. The pooled sera of five mice infected for three weeks with

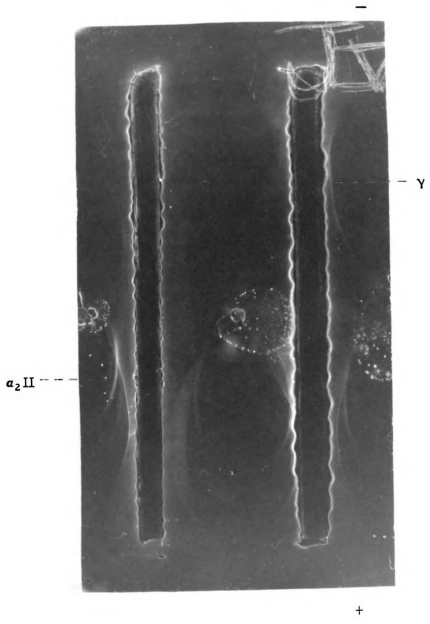

Figure 5. Immunoelectrophoretic Patterns of Diluted Mouse Serum: from left to right --A. suum infected, normal, T. canis infected.

A. suum, uninfected control mice and five mice infected for three weeks with T. canis were absorbed with the antigens prepared from unembryonated eggs, embryonated eggs and adult whole worms of A. suum and T. canis. This procedure would reveal if antigens in any of these stages of the life cycles of these roundworms would absorb the material in the increased fractions of globulin in the infected mice. Following electrophoresis of the absorbed sera they were diffused against full strength antimouse serum.

Neither the α_2II fraction in the serum of mice infected with A. suum nor the gamma globulin in the serum of mice infected with T. canis was affected by absorption of sera with the various antigens. Figure 6 shows the results of absorption of the serum of mice infected with A. suum with the antigen prepared from the adult whole worm of A. suum. Figure 7 shows the results of absorption of the serum of mice infected with T. canis with the antigen prepared from the adult whole worms of T. canis.

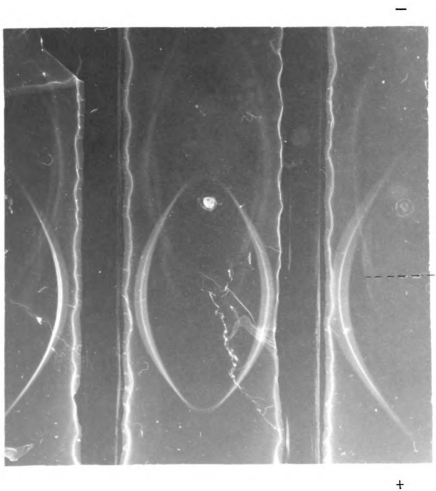

Figure 6. Immunoelectrophoretic Patterns of Mouse Serum: from left to right--normal, absorbed with antigen of adult A. suum; A. suum infected, absorbed with antigen of adult A. suum; A. suum infected, unabsorbed.

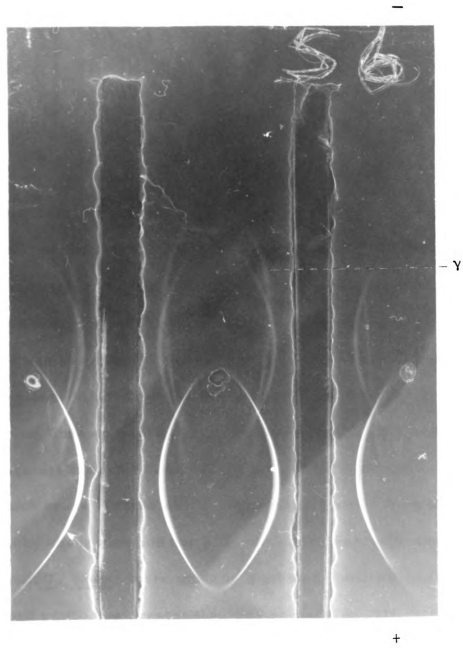

Figure 7. Immunoelectrophoretic Patterns of Mouse Serum: from left to right -- T. canis infected, unabsorbed; T. canis infected, absorbed with antigen of adult T. canis; normal, absorbed with antigen of adult T. canis.

DISCUSSION

A. Gel diffusion studies.

When the antiserum to the adult whole worms of T. canis was diffused against its homologous antigen, eight precipitin lines developed. Two precipitin lines developed when the antiserum was absorbed with the antigen of adult whole worms of A. suum and diffused against the antigen of adult T. canis. No precipitin lines appeared when the absorbed antiserum was diffused against antigen of adult A. suum. If the eight antibodies which cross reacted with A. suum are removed from the antiserum by absorption and two still remain to react with antigens of adult T. canis, there must be a greater complexity of antigens and antibodies present than was revealed by diffusion of the antiserum against its homologous antigen.

The two precipitin lines which developed when the antiserum to whole worms of T. canis was absorbed by whole worm antigen of A. suum and diffused against antigen of whole worms of T. canis represent specific antigens of adults of T. canis when

compared to the same stage of development in the life cycle of A. suum. Even though these are specific antigens present in the adult stage of T. canis, they are probably not functional antigens since there is considerable evidence that the larval stages rather than the adult stages of parasitic nematodes elaborate the functional antigens.

As previously stated, eight precipitin lines developed when full strength antiserum to whole worms of T. canis was diffused against the homologous antigen. Eight precipitin lines were also observed when this antiserum was diffused against the antigen of whole worms of A. suum as shown in Table 1, indicating eight cross reactions.

Kagan (1957), using the reciprocal procedure, found that full strength antiserum to A. suum adults developed ten precipitin lines when diffused against the homologous antigen. Four precipitin lines appeared when the antiserum to adult A. suum was diffused against the antigen to whole worms of T. canis. If the antiserum to A. suum adults produced ten precipitin lines with homologous antigen and four with the antigen of adult T. canis then six specific antigens are indicated in the adult stage of A. suum when compared to the adult stage of T. canis.

If there are six specific antigenic components present in the antigen of whole worms of A. suum as indicated by the work of Kagan (1957) and in addition eight antigenic components which cross react with the antiserum to whole worms of T. canis as indicated in the present study, then at least fourteen antigenic components must exist in the whole worms of A. suum. Kagan (1957) concluded by different reasoning that a minimum of fourteen antigenic components were present in the antigen of whole worms of A. suum.

Full strength antisera were used in all absorption studies during this investigation whereas Kagan (1957) used a single antiserum diluted one to four. Comparisons are therefore difficult.

In the present study when the antiserum to whole worms of T. canis was absorbed with antigen of unembryonated eggs of A. suum and diffused against the antigen of whole worms of A. suum, two precipitin lines were observed whereas this absorbed antiserum developed five precipitin lines when diffused against antigen of whole worms of T. canis. This indicated that there were three antigens present in the unembryonated eggs of A. suum which cross reacted with antibodies in the antiserum to whole worms of T. canis.

The work of Kagan (1957) indicated that the unembryonated eggs of A. suum contained all the antigens present in the adult stage of this species. In the present study absorption of antiserum to unembryonated eggs of T. canis with the antigen of whole worms of the same species indicated the adult worms contained all antigens present in the eggs. The reciprocal procedure demonstrated that the unembryonated eggs did not contain all of the antigens in the adult worm.

The development of precipitin lines when the antiserum to embryonated eggs of T. canis was diffused against antigen of the adult worms of the same species and the lack of development of precipitin lines with the reciprocal procedure may be due to the great differences that exist in the ability of rabbits to produce antibodies. Another possible explanation is that the two antigens used were not of equal potency.

Hyperimmune animals develop antibodies which display great cross reactivity (Ouchterlony 1962). The antiserum to the embryonated eggs of T. canis used in this study may be from a hyperimmune rabbit since great cross reactivity was displayed by this antiserum.

The development of precipitin lines when microcultures of living larvae of T. canis and A. suum were diffused

against the antiserum of embryonated eggs of the homologous species is not surprising since the larvae were in the same stage of development as they were in the embryonated eggs.

Lack of development of clearly defined precipitin lines when microcultures of living larvae of each species were diffused against the serum of mice infected with these species may be due to the unavoidable bacterial contamination in the well containing the larvae.

The antigens in the two nematodes studied are potent allergins. After almost daily exposure to these antigens for eighteen months, a differential white blood count of the present investigator revealed an eosinophilia of 45 percent. Skin contact resulted in an immediate hypersensitive response of edema and a delayed response of dermatitis. Inhalation resulted in symptoms of hayfever and asthma. Serum tested by double diffusion against all of the antigens used in the present study revealed no precipitins. Sprent (1949) found that the antigens of A. suum caused skin sensitivity in humans without the development of precipitins.

B. Immunoelectrophoretic studies.

Immunoelectrophoresis brings about resolution of the

globulins into many individual components where minute qualitative and structural abnormalities as well as quantitative changes of each component may be studied.

The increase in the α_2II globulin fraction in mice infected with A. suum may be due to inflammation or tissue damage. Shedlovsky and Scudder (1942) found that there was an increase in alpha globulin under these conditions regardless of their cause.

The liver has been indicated as the site of production of the alpha and beta globulins (Leland 1961). Peters and Anfinsen (1950) found that the liver was also the site of production of the serum albumin. Sprent (1952) found that in mice infected with A. suum the larvae remained in the liver for four days whereas in mice infected with T. canis the larvae left the liver much sooner. Perhaps the presence of A. suum larvae in the liver for this extended period of time results in considerable tissue damage and inflammation which effects an increase in the rate of synthesis of α_2II fraction of the serum.

The possibility that there was a decrease in albumin during infection was considered although the immunoelectrophoretic technique would not reveal such a reduction. Leland (1961)

stated that osmotic pressure was maintained largely through the albumin fraction so that any loss of albumin might result in a compensatory reaction to restore serum osmotic pressure. Miller and Bale (1954) found evidence that the alpha globulins are produced and turned over at a more rapid rate than other serum protein fractions. Therefore, the alpha globulins might very well take over the function of lost albumin.

Plasma proteins may be lost in severely affected animals through the damaged mucosa of the alimentary tract. A loss of plasma proteins in this way in mice infected with A. suum could result in synthesis of increased amounts of alpha globulin to compensate for this loss.

The α_2II globulin in mouse serum was shown to be related to human haptoglobin by Clausen and Heremans (1960). This was done by the immunoelectrophoretic method using a specific antihaptoglobin antiserum obtained by complete absorption of an antihuman antiserum with the serum from a patient with pernicious anemia. In this disease there is a startling decrease in circulating serum haptoglobin. The antihaptoglobin antiserum developed only one precipitin line with human serum and only one line with mouse serum which corresponded to the α_2II fraction of mouse serum.

The discovery that the increase in the α_2II fraction in the serum of mice infected with A. suum is not due to antibody production to the antigens tested is not surprising since most antibodies are known to be associated with the gamma globulin in the serum. That the increase in gamma globulin in the serum of mice infected with T. canis is apparently not due to antibody production, as demonstrated by the absorption studies performed, is somewhat surprising. However, these tests show only that if there are antibodies present to A. suum or T. canis antigens, that these antibodies were not produced by the mice as a result of exposure to the antigens used for absorption. Excretion and secretion of antigens by the larvae of A. suum or T. canis in the infected mice could cause antibody production. These antibodies might not be removed by absorption of sera with the antigens used.

The increased amount of gamma globulin in the serum of mice infected with T. canis may be due to secondary bacterial invasion following larval penetration of the intestine. This is a rather remote probability since the larvae of A. suum also penetrate the intestine and no increase in the amount of gamma globulin is observed in the serum of mice

infected with this roundworm. However, the newly hatched larvae of T. canis were observed to be much larger than those of A. suum. The larvae of T. canis may damage the tissues of the intestine to a greater extent than those of A. suum thus paving the way for secondary bacterial invasion.

The possibility that the increase in gamma globulin was only relative to the other serum proteins due to maintenance of the gamma globulin level while other fractions decreased in amount was considered. Comparison of diluted serum of uninfected mice with the diluted serum of mice infected with T. canis, as shown in Figure 7 revealed that there was a real increase in amount of gamma globulin in mice infected with T. canis.

The six antigens used in gel diffusion studies were electrophoresed in an attempt to secure precipitin lines by development of the electrophoresed antigens by serum from mice infected with A. suum or T. canis. Although this was done every three days for a period of three weeks after infection, no precipitin lines were observed which indicated lack of development of precipitating antibodies by the mice or that if precipitating antibodies were produced they never reached equivalence with antigens in this experimental procedure.

Kent (1960) found that glycoprotein complexes isolated from the water soluble portion of adult A. suum could be separated into five major components by agar gel electrophoresis. Four were active immunologically and they seemed to be somewhat specific since sera from suspected cases of visceral larva migrans, presumably of T. canis origin, did not react with the antigens of two human patients twelve days before the eggs were found in the stools.

Huntley and Moreland (1963) demonstrated precipitins to water soluble antigens of adult whole worms of T. canis in two children with visceral larva migrans, however, one of these children also had precipitins which reacted with the water soluble antigens of A. lumbricoides indicating a cross reaction. Cross reactions between closely related ascarids have been noted by Kagan, et al. (1959), using the bentonite flocculation test; Olson (1960), using the in vitro larval precipitate test; Huntley and Moreland (1963), using gel diffusion techniques.

There is evidence that a decrease in serum albumin and an increase in globulin components of the serum occurs in animals infected with certain parasites (Stauber 1954). Many parasitic infections cause an anemia in the host. Changes

in serum proteins in animals infected with parasites could be directly due to the anemia and not to the parasite. Kuttler and Marble (1960) found that in producing an experimental anemia by bleeding sheep and comparing their serum proteins to those of heavily parasitized sheep that the heavily parasitized sheep had a relatively lower albumin and higher alpha-two globulin fraction than the sheep with the induced anemia.

The changes noted in the present study in the globulin fractions of mice infected with A. suum and T. canis are not specific to the infections.

SUMMARY AND CONCLUSIONS

A. Summary.

A comparative antigenic analysis of the unembryonated eggs, embryonated eggs and adults of T. canis and A. suum was completed by the double diffusion slide microtechnique of Wadsworth (1957). Diffusion of antisera to adult whole worms of T. canis, unembryonated eggs and embryonated eggs of T. canis and A. suum against homologous antigens revealed the number of antigenic components in each of the antigens. Cross reactions were demonstrated by diffusion of each antiserum against heterologous antigens.

Diffusion of the sera of mice infected with A. suum or T. canis against the antigens of excretion and secretion from microcultures of living larvae of these roundworms demonstrated the presence of weak precipitins in the serum of the infected mice. Further research is indicated to clarify the role of antigens of excretion and secretion from larval stages of A. suum and T. canis in the development of antibodies which provide immunity to subsequent infection.

The effects of infection with T. canis and A. suum on the serum proteins of mice were investigated by immunoelectrophoresis. There was an increase in α_2II serum globulin in mice infected with A. suum. The serum of mice infected with T. canis showed an increase in gamma globulin. These alterations in serum globulin fractions during infection are not due to production of antibodies to the antigens found in the unembryonated eggs, embryonated eggs or adult stages of the species with which the mice were infected. Absorption of the sera of infected mice with these antigens had no effect on the increased globulin fractions.

The increase in the amount of α_2II serum globulin in mice infected with A. suum was not specific to the roundworm but was apparently the result of inflammation and tissue damage, particularly in the liver. The larvae of A. suum remain in the liver much longer than those of T. canis. The increase in gamma globulin in mice infected with T. canis was indicated to be non-specific to the helminth. Secondary invasion of the tissues of the intestine by bacteria following entrance of the larvae of T. canis could account for an elevation in the amount of gamma globulin. Since the larvae of T. canis are much larger than those of A. suum their penetration of the intestine would effect easier access by bacteria.

The results of diffusion of antigens of excretion and secretion from microcultures of living larvae against antisera to embryonated eggs and sera of infected mice revealed no cross reactions between species. Cross reactions were indicated in all other experimental procedures. Because of this, further research is indicated to determine if the embryonated eggs or cultures of larvae could be used as a source of antigen in the serological diagnosis of visceral larva migrans.

B. Conclusions.

1. Diffusion of antigens against homologous antisera indicated the following numbers of antigenic components in various stages of the life cycles of T. canis and A. suum: six in the unembryonated eggs of both species, four in the embryonated eggs of both species, eight in the adult stage of T. canis. No antiserum to adult A. suum was used in this study.

2. Cross reactions were demonstrated by diffusion of each antiserum against the heterologous antigens. The results indicated that most antigenic components cross reacted with the heterologous species.

3. Diffusion of the antigens of adult worms of A. suum against the antiserum to adult T. canis demonstrated eight

cross reacting antigenic components in the adults of A. suum.

4. Absorption of the antiserum to adult T. canis with the antigen to adult A. suum and diffusion of the absorbed antiserum against the antigen of adult T. canis produced two precipitin lines. These two precipitin lines represent specific antigens in the adult stage of T. canis when compared to the adult stage of A. suum.

5. Lack of mutual absorption of antisera with antigens indicated a greater complexity of antigenic components in unembryonated eggs, embryonated eggs and adult worms of A. suum and T. canis than was revealed by diffusion of antisera to each of the stages in the life cycles of these roundworms against their homologous antigens.

6. Antigens of excretion and secretion from microcultures of newly hatched larvae of T. canis and A. suum developed precipitin lines when diffused against antisera to embryonated eggs of the homologous species.

7. Precipitins in the serum of mice infected with A. suum or T. canis were indicated by hazy areas of precipitation in the agar when the serum of infected mice was diffused against microcultures of living larvae of the species with which the mice were infected.

8. Normal mouse serum contained twenty fractions as revealed by the technique of immunoelectrophoresis.

9. The alpha-two fraction of mouse serum was found to contain two components not reported by previous investigators.

10. Antimouse sera produced in rabbits varied tremendously in the number of fractions to which antibodies were present and in the amount of antibody present to the various components of mouse serum.

11. There was an apparent increase in the α_2II fraction of the serum of mice infected for one week with 1500 larvae of A. suum. Electrophoretic patterns of dilutions of serum from the infected mice proved that the apparent increase in the α_2II fraction was not due to maintenance of the α_2II level while other serum components decreased in amount.

12. The increase in amount of the α_2II in the serum of mice infected with A. suum was not due to production of antibodies to antigens in the unembryonated eggs, embryonated eggs or adult worms of A. suum as indicated by the absence of absorption of the increased fraction of serum with these antigens.

13. The increase in amount of the α_2II fraction of the

serum of mice infected with A. suum was not specific to the roundworm.

14. An increase in the amount of gamma globulin in the serum of mice infected with 1500 larvae of T. canis became apparent one week following infection.

15. Electrophoretic patterns of dilutions of the serum of infected mice revealed that there was an actual increase in amount of gamma globulin rather than a maintenance of the level of gamma globulin while other components decreased in amount.

16. The increase in amount of gamma globulin in mice infected with T. canis was not due to production of antibodies to antigens in the unembryonated eggs, embryonated eggs or the adult of T. canis because the serum of infected mice was not affected by absorption with these antigens.

17. The change in the serum globulin of mice infected with T. canis was apparently not specific to the helminth.

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