ANTIGEN HETEROGENEITY OF THE ADULT *ANCYLOSTOMA CANINUM*

By

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors.

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ABSTRACT

Canine ancylostomiasis is a widespread nematode disease. It has been shown that in endemic areas, more than 50% of all dogs are infected and over 8% of infected puppies die, while many more suffer growth retardation. A live larval vaccine has been developed but it has given minimal protection to the vaccinated animals. Therefore a search for purified protective antigens was deemed necessary in view of developing a protective vaccine. This work was thus aimed at studying the antigenic fractions of the adult A. caninum as well as the host immune response to the whole worm extract and various fraction products.

Adult A. caninum were collected from the intestinal tract of sacrificed dogs, washed thoroughly in phosphate buffered saline (PBS) to get rid of the faecal material and were homogenised using sterile frozen mortar and pestle. The homogenate was suspended in PBS, clarified by centrifugation and standardized to 2 mg of protein per millilitre of extract. The above extract was combined with complete Freund's adjuvant and used to produce hyperimmune sera in dogs which had no prior exposure to ancylostoma infection, and also in rabbits.

To enable a study of the relationship between the faecal egg count and immune response a large

number of sera, together with corresponding faecal samples were collected from free roaming dogs caught within the City of Nairobi. Also in an effort to find out whether antibodies to A. caninum were excreted in the intestinal tract of infected dogs, faecal material from infected dogs was suspended in PBS. The suspension was clarified by centrifugation and then filtered through a 0.45 micron millipore membrane filter and reduced to 1/100 of original volume. Also the mucosal lining of the infected dog's intestinal tract was ground and suspended in PBS. The mixture was treated similarly to the faeces, with the final filtrate being 100 times more concentrated. Both the faecal and mucosal extracts were tested for the presence of antibodies with the whole worm extract.

Eight mg of PBS worm extract was passed through Sephadex G - 200 and 6.7 ml fractions collected. Using the elution profile, the fractions were pooled into six composite groups. The extract was also fractionated in both analytical and preparative isoelectric focusing. Analytical isoelectrofocusing was done with 1.6 mg of extract applied on an ampholine thin layer polyacrylamide gel with a pH range of 3.5 to 9.5. The isoelectric points of the various fraction bands were determined by overlaying the plate over a predetermined pH curve. Preparative isoelectric focusing was carried out with 10 mg of

extract in a 110 ml ampholine column stabilised with sucrose and glycerol at 9°C. One hundred and thirty 1 ml fractions were collected and the corresponding pH values recorded. Seven composite fractions were prepared from the 130 (1 ml) fractions.

The whole worm extract was also fractionated on polyacrylamide precast gel with a density gradient of 4 to 30 per cent. After staining with amido black the protein bands were clearly visible and were compared to the migration profile of a standard map of known proteins, thus making it possible to determine the molecular weights of the various proteins. The gel was sliced into 12 respective slabs from which the proteins were extracted for serological tests.

The fractions thus obtained from the various analytical systems were tested for antigenic activity with sera raised in rabbits, dogs, as well as sera obtained from free roaming dogs. They were also tested with the faecal and mucosal extracts. Double diffusion precipitation together with various electrophoretic tests were applied to the extracts and the sera. The fractions were also tested for their ability to elicit active cutaneous anaphylaxis in the experimental dogs and rabbits. They were also tested for their ability to induce indirect haemagglutination with the various sera.

In order to determine the location of the antigenic component in the adult A. caninum, hyperimmune sera, raised in rabbits and dogs were fractionated to obtain IgG fractions. The latter was tagged with fluorescein isothiocyanate and overlaid onto the dissected A. caninum and examined in the fluorescent microscope.

The various fractionation methods applied to A. caninum revealed a multiplicity of antigenic components. Sephadex G - 200 showed that the major component of the A. caninum extract comprised of small molecular weight substances of less than 4.0×10^4 Daltons which did not react with the hyperimmune sera raised in rabbits or dogs. However the substances induced active cutaneous anaphylaxis in infected and sensitised dogs and rabbits. A smaller fraction of high molecular weight (2.0×10^5 Daltons) showed immune precipitation with rabbit sera in various electrophoretic test systems.

In the analytical isoelectrofocusing of the whole worm extract, proteins focused at between pH 4.0 and 6.4 with a particular concentration at pH 5.6. This material focusing at pH 5.6 was very reactive in both double diffusion and agar gel electrophoresis systems when tested with rabbit sera. When fraction III of Sephadex G - 200 (the most reactive fraction with rabbit sera), was isoelectrofocused on the

analytical gel it had an isoelectric point (pI) of 5.6 as well. When the whole worm extract was applied on the preparative isoelectrofocusing column a heavy protein concentration occurred at pH 5.3, which complemented the analytical profile. In both systems it was shown that the material with pI range of 5.3 to 5.6 had the highest antigenic activity in the electrophoretic system. The material was shown to have an approximate molecular weight of 1.7×10^5 Daltons as determined by the polyacrylamide gel electrophoresis. This material elicited low active cutaneous activity in immunised dogs and rabbits.

While the rabbit antisera were very reactive with the A. caninum whole worm extract and various fractions, the sera of both the infected and vaccinated dogs reacted poorly in many of the immunologic precipitation systems, using either whole or fractionation antigens. This was so even after the sera were concentrated by ammonium sulphate precipitation or lyophilisation methods. It was also shown that the faecal and mucosal extracts did not have appreciable reactivity with the whole or fractionated antigens in either double diffusion precipitation or in immune electrophoresis, although they reacted in indirect haemagglutination test leading one to conclude that the faecal or mucosal extracts did not have appreciable amounts of precipitating antibodies detectable by the test systems used here.

The canine sera were however shown to be similar to rabbit sera in inducing passive cutaneous anaphylaxis in rabbits. Both types of sera were also active in the indirect haemagglutination test using the whole worm extract and various fractionation products.

IgG from both dog and rabbit sera showed a weak immunofluorescent reaction on the cuticular layer of the dissected A. caninum. The reaction was stronger with canine IgG. Fluorescence was also observed on the oesophageal, gut and secretory gland cavities of the adult worm using both canine and rabbit sera. Thus it was concluded that the active antigens in the worm originated from both structural components as well as secretory materials of the worm.

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I. INTRODUCTION

The worms of the family Ancylostomatidae, commonly called hookworms are cosmopolitan especially in the tropical countries (Dunn, 1959). Most of the species in the family are voracious blood suckers. The dog hookworm Ancylostoma caninum is capable of sucking up-to 0.8 ml of blood in 24 hours (Wells, 1931). Thus a puppy or a debilatated dog can suffer severe hypo-chromic anemia and in the young mortalities of 8% have been recorded (Ndiritu and Al Sadi, 1977). The adults on the other hand do not suffer severe mortalities but instead show reduced performance. In addition to these effects the irritation on the intestinal mucosa may result in haemmorrhagic enteritis from which bacterial infections may ensue (Hoskins, 1962; Miller, 1971). Ancylostoma caninum has also been observed to cause a severe pruritic and erythematous dermatitis in the dog, whose clinical history resembles that of generalised demodectic mange (Buelke, 1971). In children, A. caninum has been known to cause cutaneous larval migrans (Fuller, 1966).

So far the control of ancylostomiasis is by chemotheraphy, often initiated after the appearance of the clinical signs, a time which is usually too late for some of the animals to recover. In an attempt to control ancylostomiasis chemoprophylactic methods similar to those developed for canine heart-worm have been attempted (Soulsby, 1968). To many

dog owners chemoprophylaxis is inconvenient and expensive, in addition to the possible adverse drug effects especially in paediatric, gravid and geriatric animals (Ndiritu and Enos, 1977; Spinelli and Enos, 1979. Due to these problems an immunoprophylactic approach has long been sought for. Attempts to vaccinate dogs with live infective larvae date back to 1931 and 1939 when McCoy, Otto and Kerr, respectively, injected dogs with a low dose of infective larvae. This unfortunately led to active disease in most of the vaccinates and for quite some time vaccination with live larvae was not attempted. It was not until 1966 when Miller vaccinated dogs subcutaneously with live larvae but this time attenuated by x-irradiation. Vieira and Rombert (1974) used ultra violet radiation for attenuating the A. caninum larvae and thereafter vaccinated dogs intramuscularly and subcutaneously, respectively. Bezubik et al. (1977) used UV-radiation to attenuate the larvae of O. circumcincta with which he vaccinated sheep. The general conclusion from the above work was that the irradiated larvae conferred a low level protective immunity. The use of the live attenuated larvae vaccine has met with limited success which has led to an intensified search for alternative approaches in immunoprophylactic methods, especially the purified antigens (Cox, 1978).

This work is aimed at studying the antigenic composition of the adult A. caninum using various

immunological fractionation methods with a view to exploring the possibility of obtaining an immunologically active antigen component of the worm in a fairly purified form and further to establish the use of such an antigen in the immunologic tests for canine ancylostomiasis.

II. LITERATURE REVIEW

1. EFFECT OF ANCYLOSTOMA CANINUM IN THE DOGS

The major effect of A. caninum infection is anemia. This distinguishes A. caninum infection from the rest of the dog hookworm like Uncinaria stenocephala and Ancylostoma braziliense which do not cause anemia. It has been suggested that the mean daily blood loss due to one worm of A. caninum ranges from 0.01 to 0.2 ml (Miller, 1966). Higher losses of up to 0.8 ml per worm in 24 hours have been recorded (Wells, 1931). It is thought that the blood loss is due to haematophagy on the intestinal mucosae. It has not been confirmed whether haematophagy is obligatory for the survival of the worms or whether it occurs incidentally as the worms ingest intestinal mucosal cells (Miller, 1971). It has also been suggested that the worms secrete an anticlotting factor as they feed such that the area of attachment continues to bleed long after the worm has detached (Catcott, 1968). Emanating from this blood loss other signs ensue. These include weakness, oedema, diarrhoea or maleana which may be followed by dehydration (Ndiritu and Al Sadi, 1977). Banerjee et al. (1972), who worked with A. caninum in the mice reported that the liver of the infective mice tended to have lower carbohydrate content as compared to the controls. These authors noted that the liver of the experimentally infected mice were pyroninophillic and had an

increased amount of ribonuclease. Earlier on, Hara (1956) had observed that the livers of infected dogs had a reduced amount of glycogen and he also suggested that this could probably be due to the poor absorption of nutrients in the gut. Complications of A. caninum infection include acute dermatitis at the site of penetration by the infective larvae which is usually on the foot pads. The dermatitis is pruritic and results in erythema sometimes leading to eruptions (Buelke, 1971). Respiratory distress due to pulmonary haemmorrhage has been seen especially in puppies and can be fatal (Catcott, 1968).

2. ANIMAL RESISTANCE TO ANCYLOSTOMA CANINUM

Resistance to A. caninum is associated with two factors:

(1) Natural age resistance.

(2) Active acquired resistance from previous infection or vaccination with attenuated larvae.

Miller (1966) noticed that bitches manifest age resistance to worms at about 8 months and males at 11 month. He proposed that the difference was due to the fact that in the females, inhibited larvae tend to accumulate in the somatic tissues, a phenomenon that does not occur in the male dogs; and hence the female tends to get a higher degree of exposure.

The second type of resistance is acquired after the initial exposure to infective larvae (Catcott, 1968). In both cases the resistance is evidenced by reduced morbidity, lower severity of the clinical signs and lower mortality rate in the younger animals, following challenge by infective larvae. In such animals there is a reduced worm burden and the parasite fecundity is also reduced (Miller, 1971).

3. CONTROL OF ANCYLOSTOMA CANINUM

3.1. Chemotherapy of Ancylostoma caninum

The control of A. caninum has generally been confined to the use of antihelmentics in both curative and preventive medication. The main problems of this approach are drug resistance (Soulsby, 1968) and adverse drug reactions (Ndiritu and Enos, 1977). In the canine hookworm control the success with chemoprophylaxis has not been as good as that observed in canine heartworm (Soulsby, 1968) or human malaria (Maegraith, 1971). Due to these problems with chemotherapy and chemoprophylaxis, attempts to study the host-parasite interactions have been made with a prime aim of developing practicable immunoprophylaxis (Cohen and Sadun, 1976).

3.2. Hookworm Vaccines

3.2.1. Infective larvae vaccines

Initial experiments showing that dogs would develop resistance to hookworms after a primary exposure were conducted by Herrick (1928); Sarles (1929) and McCoy (1931). Later other workers (Foster 1935; Otto and Kerr 1939; Cort and Otto 1940 and Otto, 1941), obtained further evidence that a dose of hookworm larvae conferred a degree of protection subsequent to infection. The protection was gauged by decreased worm fecundity, arrested development of the challenge larvae and therefore fewer clinical problems in the protected animals. Vaccination with live larvae does not rid the animals of the worms but does seem to be capable of protecting the animal from being overwhelmed with the subsequent infection (Michel et al., 1973 and Herlich, 1980). This early method of vaccination exposed the animals to active live larvae whose infectivity could result in severe damage to the health of the animals before the development of immunity. It therefore became desirable to look for a method of attenuating infectivity and pathogenicity but without compromising the immunogenicity of the larvae (Miller, 1966).

3.2.2. Attenuated larvae vaccines

Ionising radiation experiments conducted by Dow et al. (1959), on Uncinaria stenocephala larvae showed that effective attenuation could be effected with 40 Kr of x-rays. This work confirmed the damaging effects of x-rays on larvae as observed much earlier by Tyzzer et al. (1916), and Semrad (1937) while working on Trichinella spiralis and Evans et al. (1941) working on various other nematodes. At about the time Dow et al. (1959) were working on Uncinaria stenocephala, many other workers also became involved in the study of irradiated larvae as a method of preparing vaccines. Among these were Villela et al.(1958), who carried out studies on the effects of cobalt-60 and x-rays on ascarid eggs, while Varga (1964) studied the radiation-related attenuation in the larvae of Ascaridia galli. The effect of irradiation on Schistosoma mansoni was studied by Villela et al. (1961); Smithers (1962), and Perlowagora - Szumlewicz (1966); while Hsu et al. (1962), and Hsu et al.(1963) did the irradiation studies on Schistosoma japonicum. Those researchers found that most irradiated vaccines produced some degree of immunoprophylaxis which was generally short-lived. The work of Jarrett et al. (1958, 1959) and Engelbrecht (1961) culminated in the development of the most successful irradiated vaccine against Dictyocaulus viviparus which is the only

commercial irradiated larvae vaccine in the market.

It was against such encouraging background that Miller embarked on his exhaustive study of the dog hookworm, Ancylostoma caninum in search for an attenuated larvae vaccine in 1963. Towards the end of 1960's, Miller had gained enough evidence regarding the use of the irradiated larvae as a vaccine (miller 1966, 1971, 1975, 1977). Field trials with his vaccine showed that vaccinated puppies had protection against challenge with infective larvae as shown by the reduction in challenge worm burden, reduction in the haematophagus activity of the challenge worms and the erythropoetic hyper-responsiveness of the vaccinated puppies, but even Miller recognised the fact that this vaccine did not effect total protection in the vaccinates (Miller, 1971). Other workers had earlier advanced a theory that enzymes secreted by the worms served as the effective antigens to which the host animals produced antibodies (Thorson 1956(a), 1956(b); Cinader, 1967). This theory encouraged and lead into the search for purified and hopefully effective and safe vaccines (Cox, 1978), from the physiological secretions of parasites, such as acetylcholine-esterase (Bremner et al., 1973; Murrell et al., 1974; Yeates and Ogilvie, 1975; Rothwell et al., 1976; Van den Bossche, 1976; Sai and Johri, 1977). The results reported by the above workers on the immune response to the parasite metabolic products revealed a very

low level protection to challenge by infective larvae but nontheless, a protection and hence the continuing trend in the search for extractable nematode antigens (Cox, 1978).

3.2.3. Nematode homogenate and extract vaccines

It is well known that the ideal vaccine for parasites would be an extract of a parasite consisting of the part that actually stimulates immune response. The development of this ideal vaccine is vital since it is also known that whole worm homogenate material can cause allergic reactions in the vaccinates (Monnig, 1950; Cox, 1978). The experiments of Jarrett et al. (1959), on Haemonchus contortus; Jennings et al. (1963), on Nippostrongylus braziliense, and those of Kemp et al. (1976); Bogitsh and Katz (1976); Scapin and Tendler (1977) on Schistosoma mansoni, were all directed towards a better understanding of the nematode antigens. Similar endeavours had been pursued for Trichinella spiralis by Despomier and Muller (1969, 1970) and later by Jenkins and Wakelin (1977) for Trichuris muris.

Antigen analysis of different components for the various stages and types of tapeworms has been carried out by several workers (Linsanti, 1976; Marcoullis and Grasbeck, 1976; Yarzbal et al. 1973 and Gathuma, 1977). In ancylostomiasis, the search for purified

antigens has been done by Rombert *et al.* (1967), followed by the serological studies of Ball and Bartlett (1969) in the human hookworm *Necator americanus*. Two years later Williams (1971) did a comprehensive analysis of *A. caninum* extract antigens using the Ouchterlony gel diffusion as well as electrophoresis. He found at least 13 definite and precipitating antigens in the saline extract. Since then studies on the antigenic nature of the *A. caninum* have been done by Mdivinshalli *et al.* (1974), who studied the effect of hookworm extract vaccines on the heart of the rabbits. Other ancylostoma antigenic studies done in the last ten years have been related to the development of serodiagnostic methods rather than vaccination *per se* as reviewed by Kagan and Norman (1976), particularly regarding the development of inhibition of haemagglutination as diagnostic methods for ancylostomiasis, which they found to be reasonably diagnostic.

4. FRACTIONATION OF CRUDE NEMATODE EXTRACT ANTIGENS

4.1. General Fractionation Methods

Initially extraction was the only method in common use (Biguet *et al.*, 1962). Other solvents of extracting soluble antigens included water (Kent, 1963), and potassium chloride (Kusel, 1972; Murrel *et al.*, 1974; Scapin and Tendler, 1977). These methods only helped to obtain a rather crude antigenic mixture (Williams, 1971). To further purify and

characterise the extract antigens, agar and cellulose gel fractionation methods have been used (Andrews, 1964; Chordi and Kagan, 1965; Williams,1971 and Novikou, 1975). With the newer methods like isoelectric-focusing and polyacrylamide gel electrophoresis being applied, the resolution of the nematode antigens has been more refined (Cox, 1978).

4.2. Sephadex Gel Chromatography

Sephadex gel chromatography applied in various patterns is one of the more common methods of antigen characterisation. The development of Sephadex by Pharmacia Fine Chemicals (Uppsala, Sweden), in 1950's made it possible to perform fractionation of many antigens. In most cases this method provides adequate protein and polysaccharide resolution enabling researchers to obtain more or less homogeneous groups of material for analysis (Porath and Flodin 1959; Nilsson, 1962; Lowrent and Kilandes, 1964; Galpin *et al.*, 1978, Fukuda and Hamada, 1978). In specific parasite studies the gel chromatography has been used to study tapeworms (Gathuma, 1977); protozoa like *Theileria parva* (Munyua, 1977); and *Plasmodium chabaudi* (David *et al.*, 1978). In such fractionation studies of parasites the complexity of the separated patterns makes it quite difficult to sort out the antigenic from

non-antigenic fractions (Pelley, 1977; Geerts et al., 1979). Since fraction volumes are emperically determined, homogeneous fractions are difficult to obtain (Von Hoeggeden, 1978), and often other methods have to be applied for further purification of the antigen. Such supplementary methods include polyacrylamide gel electrophoresis and isoelectrofocusing analysis (Hillyer and Cervoni, 1978). In addition to the general fractionation reviewed above, sephadex chromatography has been used to determine the molecular weights of various antigens (Andrews, 1964; Siegel and Monty, 1966; Locasio et al.,1969; Andrews, 1970 and Belew, 1978).

The recovery rate of the material applied in sephadex column is reasonably good. Working with the tapeworm material, Gathuma (1977), recovered over 75% of the material initially applied on sephadex G - 200 in an LKB 100 centimetre long column with a diameter of 2.6 centimetres (LKB Produkter A.B., Sweden, 1976). Gathuma (1977) and Munyua (1977) found that the middle-range fractions were the most antigenic when assayed on agar gel electrophoresis using homologous serum produced in cattle, for the Cysticercus bovis (Gathuma, 1977) and Theileria parva (Munyua, 1977). Pelley (1977) recovered 82% of the S. mansoni egg antigen applied in sephadex G- 200 column. He noted that in addition to the loss of antigen encountered during gel filtration, the fractions were not homogenous

and this caused problems while attempting to assay the antigen. This observation had been recorded by others working on Schistosomiasis (Sawada et al., 1970; Harris, 1975). Despite these problems, gel filtration remains one of the most practical systems for antigen separation. With the development of newer gels the heterogeneity is becoming less of a problem (Bratanova et al.,1978).

4.3. Isoelectricfocusing (IEF)

The application of isoelectricfocusing to immunology has provided a high resolution technique for the separation of antigen (Weir, 1973). The initial work of Williams and Waterman (1929) demonstrated the formation of a pH gradient between anode and cathode when ampholytes were subjected to an electric current. Protein separation using IEF was demonstrated by Kolin (1955). He showed that when a charged protein is applied to an ampholyte gradient in a constant electrical field the protein will migrate to a point where its net charge will be zero, this is the isoelectric point for that particular protein molecule. Following this observation Svensson (1962), and Vesterberg (1969) devised the practical technique for applying IEF technology to protein separation (Haglund, 1971; Jones et al.,1971; Freedman, 1972).

The greatest advantage of IEF separation is that it allows for more homogeneous fractions in a given

pH range (Montgomery et al.,1972). Since the pH range can be spread out in various lengths of columns or gel plates, it is possible to select fairly accurately homogeneously charged molecules (Askonas et al., 1970). This aspect of IEF has been exploited in antibody heterogeneity (Krause, 1970; Habes, 1970; Williamson, 1971). Apart from immunoglobulin separation, IEF has been used in characterisation of lymphocyte cells (Kreth and Williamson, 1971). In antigenic studies IEF has been extensively applied in virology (Talbot et al.,1973; Wiegen and Drzeniek, 1980), and bacteriology (Rodkey et al.,1970). While working with Echinococcus granulosus, Kumaratilake and Thompson (1979) demonstrated 63 resolvable bands from the saline homogenate of this sheep tapeworm. By using the banding patterns of the various tapeworm homogenates these authors demonstrated repeatedly the possibility of applying isoelectrofocusing as a taxonomic tool. So far no evidence of IEF studies in the A. caninum has been found in the literature.

4.4. Polyacrylamide Gradient Gel Electrophoresis

Polyacrylamide is a non-charged stable material which allows high resolution of antigens (Weir, 1973; Margolis, 1973; Verbruggen, 1975). It has been applied in the study of serum components (Hyslop, 1972; Macllwaine, 1973; Kholod, 1974; Gordon and Sachin, 1975; Gahne et al.,1977) as well as enzymes

(Gerhmann, 1974) in various species. This method has also been applied in antigenic studies of bacteria such as Clostridium perfringens (Enders and Duncan, 1977) and Neisseiria meningitides (Poolman et al., 1980). The development of the gradient gel has the added advantage of allowing for the estimation of molecular weights of the electrophoresed material (Shapiro et al.,1967; Weber and Osborne, 1969; Neville, 1971; and Lambin, 1978). Von Hoeggeden (1978) applied this method during his studies of Schistosoma antigens. However, such studies are still very few for parasitic agents.

5. IMMUNOLOGIC ACTIVITY OF THE PARASITE EXTRACT

5.1. Gel Immunodiffusion and Immunoelectrophoresis Tests

Gel diffusion technique for immunological analysis was first translated into reality by Bechold who in 1905 tested goat serum antigen in gelatin containing homologous antiserum (Weir, 1973). Nicolle et al.,(1920), adopted the methodology in the immunodiffusion studies of the diphtheria antitoxin in gelatin gels impregnated with Corynebacterium diphtheriae antigen. In 1927, Reiner and Kipp introduced the use of agar gel in which they demonstrated the precipitation of pig serum antigen by the anti-pig serum. Following these successful attempts, Ouidin (1946) and Ouchterlony (1948) reported the

technique of one dimensional and two dimensional immunodiffusion respectively. It was later noticed that Ficki's formula for free diffusion in one dimension was applicable to Ouidin's one dimensional immunodiffusion (Kwapinski, 1972). This meant that it was possible to quantitate the antigen antibody reaction. Equivalent development occurred for the Ouchterlony technique with the work of Korngold and Van Leeuwen (1957), who demonstrated the molecular weight relationship betweeen antigen and antibody.

The application of gel diffusion methods in parasitology have included analyses on trichinellosis, fascioliasis, echinococcosis, cysticercosis, schistosomiasis, amoebiasis, chagá's disease, malaria and theileriosis (Kagan and Norman, 1976; Gathuma, 1977; Munyua 1977; Scapin and Tendler 1977; Bruce and Wakelin 1977; Enayati and Pezeshi, 1977). In immunodiffusion one finds a multiplicity of parasite antigens and a high level of non-specific precipitation (Cohen and Sadun 1976 Denham et al. 1971). With the introduction of electrophoresis by Grabar and William in 1953, immunodiffusion studies were vastly improved (Weir, 1973; Verbruggen, 1975). Immunoelectrophoretic methods have been developed and subsequently used in antigen studies of many parasites including Schistosomes (Biguet et al. 1962), Echinococcus (Kagan and Norman 1963), Stephanurus (Tromba and Baisden, 1963), Fasciola (Van Tigelle and

Over 1976), Toxocara (Enayati and Pezeshki 1977).

Both human and animal hookworm infections have been studied using gel diffusion techniques. In humans the studies have involved screening of Necator americanus in hookworm endemic areas of West Africa and India (Schad et al.,1975). Similar epidemiologic studies had been carried out previously in Russia by Makhumudova (1969, 1970). In the dog, immunodiffusion tests have been performed on Ancylostoma caninum following the preparation of antisera to live irradiated larvae (Vieira and Rombert 1975), live non-irradiated larvae and adult worm homogenate saline extract (Williams, 1971). Williams observed five strong precipitin lines when using the antisera prepared against non-irradiated larvae whereas Vieira and Rombert (1975) found no discernible precipitation using the antisera prepared against irradiated larvae. In the studies of the adult worm extract Williams (1971) observed at least 13 precipitin components. He also demonstrated 5 common antigens between the human hookworm Necator americanus and the canine hookworm Ancylostoma caninum. Similarly cross-reacting antigens were shown to be present between filarie and the hookworms (Neppert, 1974). These observations were underlined by the more recent immunofluorescent and enzyme linked immunosorbent assay studies of Weiland and Schwarzhuber (1978) who showed that nematodes share several related

antigens.

To enhance the usefulness of the gel methods several formats of electrophoretic variations have been instituted quite successfully. Among the more commonly used modifications are the crossed immuno-electrophoresis and the rocket electrophoresis. These are basically used for quantitative immuno-electrophoretic determinations (Bio-rad laboratories, 1975). The methods have not as yet been widely applied in parasitic immunology (Von Hoeggeden, 1978).

5.2. Cutaneous Activity of Parasite Antigens

Cutaneous reactions are basically classified as active cutaneous anaphylaxis (ACA) and passive cutaneous anaphylaxis (PCA). Generally the ACA reactions have mainly been used as screening methods, for example the famous mantoux test for tuberculosis (Rose and Friedman, 1976). This type of a test suffers a limitation in the sense that in actively sensitised animals only a very crude relationship can be drawn between the antibody level and the degree of anaphylactic activity (Kabat and Meyer, 1961). In the studies of the canine hookworm Vinayak et al., (1977), observed that the optimal protein concentration for the intradermal antigen was 30 micrograms of nitrogen per millilitre per dose. These authors obtained a sensitivity of 95% with 86.7% specificity. Similar level of sensitivity was obtained by Mukerji et al.

(1977) when they used an Ascaris lumbricoides (variety hominis) antigen to test infected individuals. In their case the amount of antigen required was 0.005 micrograms protein per dose of injection. This group reported 5% false positives which did not seem to be caused by cross-reaction with other parasites like Ancylostoma. The problem of non-specificity in intradermal tests has been highlighted in several nematode reports including those on canine filariasis by Schichinohe et al.,(1973) and Onchocerca volvulus (Bartlett, et al.,1978). Ishizaka (1973) attempted to lay out the minimum dimensions of a positive intradermal test which he projected as a bimodal reaction of wheal/erythema diameters at a ratio of 9:20 millimetres. He regarded the concentration of the antigen as not being critical. Unfortunately, active cutaneous anaphylactic tests lack specificity (Kabat and Meyer, 1961). It therefore became necessary to establish a better skin titration method for the antigen-antibody reaction. This was developed in the form of a passive cutaneous anaphylaxis (PCA) by Ovary in 1952. This work followed the observation that vasoactive substances were released during anaphylactic reactions (Schultz 1910; Dale 1913; Schild 1939; Kellaway and Trethewie, 1940). These substances were noticed to increase the permeability of the blood vessels as was shown by Ramsdell (1928) using intravenous trypan blue in rabbits and guinea pigs. It is now known that

mediators for the vasoreaction include histamine (Spector,1959; Ovary,1964), Serotonin and slow reacting substance of anaphylaxis (Orange et al., 1970; Norman, 1975). It is also known that these mediators are only released after the antigen has reacted with the cutaneously bound antibody. The antibody groups confirmed to be involved in this reaction include IgE, IgG and probably some non-classified heat stable homocytotropic immunoglobulin group in the rabbit (Lindqvist, 1968; Lindqvist and Osterland,1969; Ishizaka et al.,1969, Stromberg, 1979). In some parasites like Schistosoma japonicum, IgE studies regarding PCA in the rabbit have been well documented (Colwell et al.,1971; Kojima et al.,1976). Other parasites for which PCA has found considerable application include Trichinella (Gancarz, 1968) Echinococcus and Toxoplasma (Kagan and Norman, 1976). In ancylostomiasis there is very little information on serology. However, Ball and Bartlett (1969) attempted intradermal tests using Prausnitz-Kustner technique with Necator americanus antigens in human. In their studies they observed positive PCA one month post infection with the highest titres being observed at 3 months, becoming negative in about one year. Most PCA tests have been done on guinea pigs which seem to be the animal of choice. However, the rabbit system is satisfactory especially for the immediate reactions since the rabbit skin does not bind

heterocytotropic reaginic antibodies for long (Ovary 1964), and homocytotropic antibodies present no problems (Lindqvist, 1968).

5.3. Passive or Indirect Haemagglutination (IHA)

This test is commonly used due to its technical ease and relative accuracy as compared with such tests as ACA and PCA (Herbert, 1970 ; Plonka et al., 1972). Schichinohe et al., (1973) observed that IHA was twice as sensitive as the PCA using purified canine filarial antigen. Despite its sensitivity, IHA results have shown some inconsistencies. While studying canine ancylostomiasis and dirofilariasis, Makhumudova (1970); Schichinohe et al. (1973) respectively, found that IHA titres were highest in the early stages of infection. This was in contrast to the findings of Schad et al. (1975) who found very low IHA titre in young children suffering from ancylostomiasis as compared to the adults. These inconsistencies regarding the haemagglutination test had been noticed earlier in rodents by (Douns et al., 1955; Lunde and Jacobs, 1963). These works suggested the existence of haemagglutination inhibitors in rodents sera. Smith and Herbert (1976) working with Hyostrongylus rubidus observed no significant statistical difference in the total antibody production between pigs with varying levels of infection. However, these authors did not agree with Makhumudova (1970) in that they obtained high indirect

haemagglutination titres during the late infection phase of the disease. Enayati and Pezeshki (1977), compared the efficiency of indirect haemmagglutination test to that of counter immunoelectrophoresis (CIEP) in detecting antibodies to Toxocara canis antigen in guinea pigs. During the first five weeks the IHA antibody titres rose to 1:2560 in guinea pigs infected with either 500, 1000 or 1500 infective ova of the Toxocara canis. These authors pointed out that IHA was a much superior test to CIEP. This was also true in Sarcocystis of cattle (Sterzl, 1960; Lunde and Fayer, 1977). Carpenter (1965) showed that passive haemagglutination was capable of detecting 0.005 micrograms/ml of antibody nitrogen as compared to agar gel precipitation which has a lower limit of 5 micrograms/ml.

The value of passively haemagglutinating antibody was questioned by Herlich and Merkal (1963) while studying Trichostrongylus axei in calves. These authors failed to show a relationship between protection and indirect haemagglutinating antibody titres. Similar concern was voiced by Schichinohe et al. (1973) in canine filariasis and Sood et al. (1972) in human hookworm infections. Despite these shortfalls IHA test has been usefully and widely applied. This is because the IHA test if performed with purified antigen shows a low level of non-specific reactions (5%) as compared to immunodiffusion where

upto 10 - 15% non-specific reactions may occur (Kagan and Norman, 1976). Where IHA positive response indicates infection in hydatidosis, this is not the case with most other parasitic diseases like schistosomiasis in which IHA antibody persists long after the person is clear of parasites (Kagan and Norman, 1976).

5.4. Immunofluorescent Tests

The work of Kagan (1958); Sadun et al. (1960); Sadun et al. (1963) demonstrated the usefulness of the immunofluorescent technique in schistosomiasis studies especially in epidemiological surveys. A detailed study of this methodology in Trichinella spiralis was presented by Jackson (1959) and in 1960 the same author presented his work on Nippostrongylus muris. From this work,Jackson concluded that the effective antigens of the nematodes he studied were most likely excretions and secretions of the digestive and reproductive tracts of the worms. This observation supplemented the work of Thorson (1956(a)) who had suggested that hookworm enzymic secretions were acting as antigens. Using serum from hookworm infected dogs Klaver-Wesseling et al. (1978) found it difficult to show IgG binding on active infective A. caninum. However when the larvae were metabolically inhibited with sodium azide or low temperatures IgG antibody binding was seen on the outer coating of the larvae. When the inhibitory

factors were removed the fluoresecent substances were shed from the surface of the larvae. These authors suggested that the fluorescent antigen is continuously being shed and hence the difficulty in visualising it. This observation had been noted earlier by Soh and Kim (1973) who observed antigenic deposits on the entire wall of the intestines of dogs infected with A. caninum. These workers thought that the shed antigen was of cuticular origin.

Although the fluorescent techniques have allowed for antigen localisation in the worm, it should be noted that in some species non-immunological auto-fluorescence can be a handicap (Nairn,1964; Weir, 1973 ; Thompson, 1977).

III. MATERIALS AND METHODS

1. PREPARATION OF ANCYLOSTOMA CANINUM ANTIGEN

Free roaming dogs were screened for A. caninum eggs in the faeces. The ones found to have hookworm eggs in the faeces were sacrificed and the worms collected from the intestinal lumen. The worms were washed twenty times in phosphate buffered saline (PBS) pH 7.2, to get rid of the faecal material. After the final wash the worms were left in PBS overnight at $+4^{o}C$ and rewashed ten times more after which they were homogenised using a mortar and pestle previously kept at $-20^{o}C$. The homogenate was made up in PBS at the rate of 100 crushed worms for each millilitre of PBS. The suspension was kept at $-20^{o}C$ and thawed 12 hours later. This freeze-thawing and grinding was repeated three times. The homogenate was then centrifuged at 300 g for 30 minutes at $+6^{o}C$. The supernatant fluid was collected and analysed for protein content using Lowry's method (see Appendix 1), and stored at $-20^{o}C$ until required.

2. EXPERIMENTAL ANIMALS

2.1. Dogs

Two adult healthy bitches were treated for all intestinal parasites with nitroscanate (Ciba-geigy, Switzerland). When the faecal examination revealed no more parasite eggs, the bitches were mated on the

subsequent heat. The kennel floors where the bitches lived were washed weekly with 1% boric acid to reduce the possibility of infection. A total of 10 healthy looking puppies were selected from two litters and dewormed when they were one week old with thenium closylate and piperazine phosphate (Ancaris, Wellcome, U.K.). Deworming was repeated weekly for the first 12 weeks of life and thereafter every two weeks until the end of the experiments. At 12 weeks of age pre-immunisation sera was collected from all the puppies after which eight of the puppies were vaccinated as detailed below. The remaining two puppies were kept as controls.

Another four naturally and heavily infected non-dewormed dogs were bled, faecal egg count done, and thereafter vaccinated similarly to the puppies above. Faecal egg count was rechecked weekly to see if vaccination reduced faecal egg load.

In addition to the serum samples collected from the kennelled experimental dogs,serum was also collected from dogs caught within Nairobi City limits and kept at the Nairobi City Council dog pound. A faecal specimen for parasite egg count from each dog was also taken at the time of taking the blood sample. More sera and faecal specimens were collected from the dogs brought to the Veterinary Clinic of the University of Nairobi. Control serum from hookworm

free dogs was obtained from the University of Glasgow in Britain.

2.2. Rabbits

Twenty male New Zealand white rabbits were acquired at 10 weeks of age from the Veterinary Research Laboratories, Kabete. Pre-immunisation sera was prepared by collecting thirty millilitres of whole blood from each rabbits marginal ear vein. The blood was allowed to clot at 37°C for 2 hours and kept overnight at 4°C to allow the clot to shrink. The clot was removed and sera clarified by centrifugation at 1500 x g for 15 minutes. The serum was kept at -20°C until required. Five rabbits were thereafter vaccinated as explained below, the others were kept for cutaneous tests and as controls.

2.3. Guinea Pigs

Ten adult albino guinea pigs were acquired for passive cutaneous anaphylaxis tests.

3. PREPARATION OF ANCYLOSTOMA CANINUM ANTISERA IN DOGS AND RABBITS

The extract of A. caninum prepared earlier (Section 1), was diluted in phosphate buffered saline, pH 7.2, in order to contain 2 mg protein per millilitre. Equal volumes of this extract and Freund's complete adjuvant were properly emulsified. The dogs and the

rabbits were both vaccinated fortnightly for three months. Each animal received one ml of adjuvanted extract, half in the posterior thigh muscles and half subcutaneously. Prior to each vaccination the animals were bled and serum processed as above and kept at -20^{o}C until required for tests.

3.1. Lyophilisation of Canine Sera, Faecal and Mucosal Extracts

Two batches of canine sera from hookworm positive dogs were prepared for lyophilisation. One batch contained 50 ml of sera from a dog with hookworm infection and had been vaccinated. The other batch contained 210 ml of pooled sera from six infected dogs (35 ml from each dog). Lyophilisation was done in Edward's Pirani II freeze dryer (Edward's High Vacuum, Manor Royal Sussex, England) at -70^{o}C and a negative pressure of 630 mm of mercury for 36 hours. The lyophilised powder was redissolved in PBS pH 7.2 so as to make a 25x concentrate of the original material. For faecal extract, 2 kilogrammes of fresh faeces from 10 dogs with hookworm infection was suspended in 500 cc of PBS, pH 7.2 by stirring for 30 minutes. The homogenate was passed through a strainer and thereafter filtered through a No.1 Whatman filter paper (Whatman Limited, England). The filtrate was centrifuged at 3000 x g for 15 minutes and cleared by passing through a 0.45 micron

millipore filter. A 40 ml portion of the homogenate was freeze dried and reconstituted with 4 ml PBS to make upto 10 ml of 40 x concentrate.

Canine mucosal extract was prepared by grinding 500 gm of cleaned ileal mucosae obtained from 5 dogs which were heavily infected with hookworms. One hundred millilitres of PBS, pH 7.2 was added to the mucosal tissue homogenate and processed as described above for the faecal extract. The 100 ml of material was lyophilised down to an amorphous powder and later reconstituted to make 2 ml of 50 x concentrate.

3.2. Antibody Tests for the Lyophilised Serum, Faecal and Mucosal Extracts

The reconstituted lyophilised materials prepared above were tested for antibody activity using double diffusion, and rocket immunoelectrophoresis in 1% agar gel, using the antigens of the whole worm extract and the fractions prepared through the various methods namely Sephadex G - 200 filtration, polyacrylamide gradient electrophoresis and isoelectrofocusing.

4. FRACTIONATION OF ANCYLOSTOMA CANINUM EXTRACT

4.1. Gel Filtration Column Chromatography

Superfine Sephadex G - 200 gel of particle size 40 - 120 microns was soaked in PBS at the ratio of 1:40 and allowed to swell for 24 hours. The gel was carefully packed in a vertical K26/100 column (LKB,

Produkter A.B., Stockholm, Sweden). The column was rinsed with PBS pH 7.2 for 12 hours and calibrated with 2 mg each of bovine liver catalase of approximate molecular weight of 2.1×10^5 Daltons and hen egg ovalbumin of molecular weight 4.3×10^4 Daltons (Pharmacia Fine Chemicals, A.B. Uppsala, Sweden). After calibration, 4 ml of the hookworm crude extract (2 mg/ml which had been filtered previously) was added to column from the top. Fractionation was carried out at room temperature using PBS at a flow rate of 20 ml/hour. After a void volume of 154 ml was collected, fractions of 6.7 ml per tube were collected using an automatic fraction collector (LKB, Produkter A.B., Stockholm, Sweden). The optical densities of the fractions were recorded continuously at 280 nM using a Beckman spectrophotometer (Beckman Instruments, U.S.A.). The fractions were pooled into six portions according to their elution profile as shown in Appendix VI. These pooled portions were put into separate dialysing bags and concentrated against polyethylene glycol to approximately one tenth of the original volume per portion. They were tested for protein content and antigenicity with the prepared hookworm antisera. The portions were also tested for cutaneous and indirect haemagglutination activity.

4.2. Isoelectrofocusing (IEF) of the Crude Hookworm Extract

The hookworm extract used for isoelectrofocusing contained 5 mg/ml protein. Two methods of IEF were applied i.e. analytical and preparative.

4.2.1. Analytical isoelectric focusing on thin layer polyacrylamide gel (PAG)

An ampholine polyacrylamide plate of pH range 3.5 to 9.5 was laid on the template on the multiphor cooling plate (LKB, Produkter A.B., Stockholm, Sweden), and the electrode strips soaked in electrode solutions (appendix II) were applied. Crude hookworm extract and Sephadex G - 200 fraction III were soaked into the sample application absorbent paper strips. Each paper absorbing approximately 15 microlitres. The strips were placed on the ampholine plate 5 mm apart and 5 mm from the cathode. Power was run through at 210 V, 50 mA; by the end of one hour the power was 30 watts at which time the sample applicators were removed to avoid tailing. Electrofocusing was continued for one more hour. The pH across the gel was measured. This was done by punching out pieces of gel at various distances from cathode, soaking and crushing them individually in 1 ml saline pH 7.0 and the resulting pH measured. The plate was then immersed in fixing solution for one hour, placed in destainer for 5 minutes and then stained in 0.1% Coomasie blue at

60°C for 10 minutes (Appendix II). The gel was then destained and the bands evaluated by comparing the plate to the graph of pH and relative distance from the cathode.

4.2.2. Preparative electrofocusing in density gradients

LKB 8100 - 1 ampholine column with a capacity of 100 ml was connected to LKB gradient mixer 11 300 ultrograd, and the latter connected to the LKB 2121 varioperpex II peristaltic pump (LKB Produkter A.B., Stockholm, Sweden). Column cooling water was kept at 9°C. Electrofocusing electrode and gradient solution were made as shown in Appendix III. Five millilitres of the hookworm extract were introduced into the column, half with the dense gradient solution and the other half with light electrode solution. Electrofocusing was performed by applying a current of 13 mA for 16 hours at a temperature of 9°C. Elution was performed at the rate of 60 ml/hour and 130 1 ml fractions were collected. Absorbancy of the various fractions was measured at 280 nM and recorded automatically through an LKB 2 Channel recorder (LKB, Produkter A.B., Stockholm, Sweden). The pH of the various fractions was measured, after which the fractions were divided into 0.5 ml aliquots. One batch of the 0.5 ml aliquots was kept at -20°C until tested individually for antigenic activity.

The other batch of the 0.5 ml aliquots were pooled into seven composite portions according to the elution profile (Appendix VII) and tested for their antigenic activity thereafter.

4.3. Polyacrylamide Gradient Gel Electrophoresis (PAGE) of the Crude Extract

Eight hundred microlitres of the hookworm extract (of concentration 5 mg/ml) was mixed with 100 microlitres of saturated sucrose solution. The Pharmacia gel electrophoresis apparatus (GE - 4) was filled with borate buffer pH 8.35 and cooled down to 9.5^{o}C and a Pharmacia precast gradient gel 4/30 in a cassette was equilibrated for 15 minutes at 125 volts. The sample was run in a 70 volts for 20 minutes and thereafter electrophoresis continued for 15 hours at 125 volts. The gel was removed from the buffer and sliced vertically along the two sides (Figure 5). The slices were stained at 25^{o}C for 30 minutes in 0.7% amido black in 7% acetic acid. Destaining was done electrophoretically at 24 volts in 7% acetic acid for 45 minutes using a Pharmacia gel destainer GD - 4 II (Pharmacia Fine Chemicals, Uppsala, Sweden). By comparing the dye band migration distances to a standard pharmacia polyacrylamide gradient gel PAA 4/30 scale (Figure 6), the approximate molecular weight range at the various dye fronts was calculated. The stained slices were placed alongside the non-stained-

gel slab, in their original position and the unstained slab sliced horizontally at the corresponding dye fronts of the two stained slices. Twelve such slices were made (Figure 5) and each was suspended in 2 ml of PBS. Each slice represented a PAGE fraction and was used for antigenic analysis.

5. SEROLOGICAL ACTIVITY OF THE ANCYLOSTOMA CANINUM EXTRACTS

5.1. Double Diffusion Precipitation

The method of Ouchterlony (1948) was used to demonstrate antigen-antibody reactions. Microscope slides of the dimensions 25 mm x 76 mm were layered with 3 ml of 1% agar in sodium barbitol hydrochloride buffer pH 8.6 with 0.01% merthiolate as preservative. Appropriate well patterns were punched on the agar. The extract antigens were applied in the peripheral wells while the test rabbit, canine serum, faecal and mucosal extracts were put in the central well. Several dilutions (1:10, 1:50, 1:100) of both canine and rabbit sera were tested against undiluted antigen and various dilutions (1:10, 1:20, 1:40) of the antigen extract. The tests were done with sera obtained before and after absorption with canine liver powder. The liver powder was prepared from the slices of fresh liver washed in physiological saline and then homogenised in acetone. The material was deposited on filter paper and dehydrated with further acetone

washes, and dried at 37°C, after which the tissue, was ground in a mortar and sieved through a 1 mm wire mesh. Adsorption was done at the rate of 100 mg of liver powder per ml of serum, shaken for 2 hours and then centrifuged at 10,000 g for 30 minutes.

The slides were incubated in a moist chamber for 24 - 36 hours to allow for maximum diffusion. In slides with clear precipitation, photographs were taken using the scattered light technique. All the slides were pressed and washed for 12 hours in PBS containing 0.1% sodium azide as preservative. The slides were dried and stained in 1% Coomasie blue for 30 minutes. Destaining was done in a mixture of 10% acetic acid (v/v) and 25% (v/v) mixture for 15 - 30 minutes.

5.2. Immunoelectrophoresis

5.2.1. One dimensional immunoelectrophoresis

Electrophoresis was performed on either 25 by 76 mm or 75 by 100 mm glass slides depending on the number of samples to be tested. The plates contained 3 ml and 15 ml respectively of 1% agarose in sodium barbitol - hydrochloric buffer pH 8.4. After the agarose set, the wells and troughs were punched using LKB templates. The extract antigens were filled in the wells and electrophoresed in an LKB 2117 electrophoretic chamber LKB, Produkter A.B.,

Stockholm, Sweden), containing barbitone hydrochloric buffer pH 8.4, for approximately 60 minutes depending on how far the front of the indicator bromophenol blue was from the positive pole wick. Voltage was maintained at 32 volts/4 cm of the agar. Separate troughs were filled with canine or rabbit antiserum and diffusion allowed to continue in a humid chamber for 48 hours. The slides were processed similar to those of double diffusion (Section 5.1.).

5.2.2. Crossed immunoelectrophoresis

One per cent agarose was poured on microscopic slides (75 by 100 mm) as described above. The non-fractionated extract was electrophoresed in one direction just as in one dimensional electrophoresis. Only one sample was electrophoresed per plate. The agar was then slided vertically so as to separate the parts containing the electrophoresed antigens. Each part was transferred to a fresh slide and placed at the bottom after which the rest of the slide was filled with 10 ml of 1% agarose which had been mixed with 0.2 ml of rabbit antihookworm serum just before pouring. After the agar-serum mixture set on the slide, the antigen was electrophoresed in a direction perpendicular to the first direction so that the antigen migrated into the agarose-serum mixture zone. The plates were processed as above.

5.2.3. Rocket electrophoresis

One per cent agarose was poured on 25 by 76 mm glass slides. The agarose gel was then transferred to a larger slide (75 by 100 mm) after which wells were punched to contain the antigen samples. The rest of the slide was filled with a mixture of 10 ml 1% agarose mixed with 0.5 ml of either rabbit anti-hookworm serum, faecal or mucosae extract. The wells were then filled with the test antigens, which included crude extract, pooled Sephadex, polyacrylamide, and isoelectrofocusing fractions.

5.3. Cutaneous Tests

5.3.1. Passive cutaneous anaphylaxis (PCA)

Six 10 week old male New Zealand white rabbits and six 10 week old albino male guinea pigs were clipped on the back and flanks from the shoulder blades to the sacral region. Sites of injection were demarcated with an oil based ink marker and the animals rested for 36 hours so that any clipper damage would repair and therefore not affect the skin tests. Three rabbits and three guinea pigs were injected intradermally with the four sera specified below. The injections were done in a cranial-caudal direction using serial doubling dilutions ranging from undiluted to 1:32 for each serum with the first two sera on the left flank and the other two sera on the right flank. The remaining three rabbits and three guinea pigs

were done in reverse order to minimise the side effects on the skin reactions. The four sera used were:

Serum 1: Obtained from hookworm negative dog.

Serum 2: Obtained from hookworm vaccinated rabbit.

Serum 3: Obtained from hookworm infected-vaccinated dog.

Serum 4: Obtained from a control rabbit.

Each injection site received 0.1 ml of the appropriate serum, and in addition each animal received control injections of PBS and serum of non-vaccinated control rabbits. Four hours after the injection, three rabbits and three guinea pigs were each given intravenous injections of whole hookworm PBS extract in 2% Evan's blue dye at a dose rate of 1.5 ml for the rabbits and 0.5 ml for the guinea pigs. A reaction time of 60 minutes was allowed and thereafter the reaction read off as the diameter of the dye leakage. For the rabbits the reactions were read on the epidermal surface whereas the guinea pigs were sacrificed and the readings made on the dermal surface. The remaining animals were injected with hookworm antigen/dye mixture 24 hours after the intradermal serum injection and the reactions read off as above.

5.3.2. Active cutaneous anaphylaxis (ACA)

The hair was clipped from four dogs known to be infected with Ancylostoma caninum and also vaccinated with hookworm PBS extract; four dogs naturally infected with hookworm; four rabbits vaccinated with hookworm extract; two control dogs and two control rabbits. All these animals were rested as described previously (Section 5.3.1.). The animals were all given intravenous injections of2% Evan's blue dye at a dose rate of 20 mg/kg. One hour later the animals were divided into two equal groups. The first group was injected intradermally on both flanks with 0.1 ml of 7 dilutions of hookworm crude extract (10^{o} to 10^{6}); 6 portions of pooled Sephadex G - 200 fractions; 12 polyacrylamide gel electrophoresis fractions and 7 pooled preparative isoelectrofocusing portions. The animals also received injections of PBS and blank ampholine pooled in a sequence similar to the 7 portions of preparative IEF. An hour after the antigens were injected the diameter of the dye leakage was recorded.

The animals in the second group were injected on the right flanks with serial doubling dilutions of histamine solution made from a stock solution containing 0.2 mg/ml. Sixty minutes later the diameter of the dye leakage caused by the histamine was recorded, and the animals given an intramuscular injection of antihistamine at a dose rate of 15 mg/kg. Subsequently

these second group animals were injected on the unused left flanks with the antigen described above for the first group of animals. In addition a row of histamine intradermal injections was repeated. On hour later the dye leakage from the injection sites was recorded. In addition to the animals in the two groups above another hookworm infected dog and a vaccinated rabbit were both injected with the individual Sephadex G - 200 fractions after receiving intravenous Evan's blue dye.

5.5. Indirect Haemagglutination (IHA) Tests

5.5.1. Tanning of the sheep red blood cells

Four hundred millilitres of sheep blood collected in anticoagulant solution (Appendix IV) were washed 3 times in PBS pH 7.2 and subsequently adjusted to 2.5% (v/v) cell suspension in PBS. The cells were incubated with an equal volume of 1:20,000 (v/v) tannic acid solution for 10 minutes at 37^{o}C. The cells were centrifuged at 800 x g for 15 minutes and once again washed in PBS pH 7.2. The cells were resuspended in PBS pH 6.4 to a final concentration of 2.5% (v/v).

5.5.2. Determination of the optimal antigen concentration and testing of sera

All the antigens used in IHA test i.e. crude hookworm extract, Sephadex G - 200 fractions I to VI,

PAGE fraction II, analytical IEF fraction III, were prepared as 1:10, 1:20, 1:25, 1:50, 1:100 dilutions made in PBS pH 6.4. The tanned sheep red blood cells (SRBC) were sensitized by adding 2.0 ml of each antigen dilution to 2.0 ml of tanned SRBC and incubating the mixture at 37°C for 15 minutes. The cells were subsequently washed twice in PBS pH 7.2 containing 1% inactivated normal rabbit serum and finally made up into 2% suspension (v/v) in PBS pH 7.2 with 1% inactivated normal rabbit serum.

For each plate, the first vertical row of eight wells (labelled A to H) were filled with 25 microlitres of the test sera as follows:

Row A and C - Preimmunisation rabbit serum

Row B and D - Hyperimmune rabbit serum

Row E and F - Hookworm free canine serum

Row G and H - Serum of a hookworm infected dog.

The sera were diluted with 25 microlitres automatic dilutors down to the 12th well (i.e. to a final dilution of 1:4096). The tanned sensitized cells were then added as follows:

Plates 1 to 5 - Titrated with SRBC sensitized with the dilutions of crude extract.

Plates 6 to 35 - Titrated with SRBC sensitized with the dilutions of the six Sephadex G - 200 portions.

Plates 36 to 40 - Titrated with SRBC sensitized with the dilutions of PAGE fraction II.

Plates 41 to 45 - Titrated with SRBC sensitized with the analytical IEF fraction III.

Control plates for the four sera were set up and titrated with plain washed 2% SRBC and tanned washed 2% sheep red blood cells. Antigen control plates sere set up with PBS replacing the serum dilutions. All the plates were left at room temperature for 3 hours after which the red cell patterns were read out. The end-point was taken as the last well showing 100% haemagglutination of the SRBC's. The optimal dilution for each antigen was recorded and applied in testing the sera from dogs, rabbits as well as mucosal and faecal extracts.

5.6. Location of Antigens in the Adult Ancylostoma Caninum by Immunofluorescence

Rabbit and canine antihookworm globulin were precipitated separately with saturated ammonium sulphate solution (Rose and Friedman, 1976) and in each case the globulin was adjusted to 7.5 mg/ml

total protein. Ten millilitres of each globulin solution were labelled with fluorescein isothiocynate (FITC) using the method adopted from Clark and Shepard (1963). Briefly, ten millilitres of the globulin were mixed with 1 ml 0.5M carbonate buffer pH 9.5 and dialysed against 20 mg FITC dissolved in 200 ml of 0.5 M carbonate buffer in saline for 18 hours at 4°C. The conjugate was then chromatographed in Sephadex G - 25 column and the conjugate which migrated ahead of the dye was collected and tested for immunologic activity by immunodiffusion test. Using the formula after Goldman (Coons, 1958) it was possible to calculate the globulin protein - FITC concentration as follows:

$$\text{Concentration of FITC - Protein (mg/ml)} = \frac{\text{O.D.280} - (0.36 - \text{O.D.495})}{1.4}$$

The FITC - protein concentration in this case was found to be 6.52 mg/ml for the rabbit antihookworm globulin and 3.46 mg/ml for the canine globulin. The conjugate was stored as 1 ml aliquots at - 20°C until used in the various immunological tests.

Adult hookworms were embedded in gelatin and sectioned transversely to a thickness of 6 microns. The sections were fixed on glass slides using acetone. The slides were divided into four groups and treated as follows:

Group I Separate sections were layered with each globulin - FITC conjugate for 30 minutes and washed with PBS three times.

Group II The slides were treated as in group I but were counterstained with 0.01% Evan's blue dye for one minute to reduce non-specific fluorescence.

Group III The slides were washed with PBS and used as controls for non-specific tissue fluorescence.

Group IV The slides were layered with rabbit and canine antihookworm serum separately for 30 minutes and washed three times in PBS. This group was used as controls for non-specific fluorescence due to antisera.

All the slides were then mounted with glycerol: PBS mixture at a ratio of 9:1 (v/v) pH 9.0 and covered with glass cover slips.

Other adult hookworms were dissected longitudinally while others were crushed onto the slides. These were treated similarly to the transverse sections above. Fluorescence was read using Leitz BG 12 as primary filter and 510 nM filter (Leitz, W. Germany) as the secondary filter.

IV. RESULTS

1. FRACTIONATION OF THE CRUDE ANCYLOSTOMA CANINUM EXTRACT

1.1. Filtration Chromatography in Superfine Sephadex G - 200

According to the optical density profile (Figure 1) six fractions were obtained by grouping the tube contents as shown in Appendix VI. Fraction I contained the void volume. The actual optical density major peaks were in fractions II, IV and V. Fraction III peak was less prominent. The recovery of the material introduced in the column ranged from 70.3% to 85.0% with an average of 77.7%. The protein content of the various fractions was as shown in Table I. This can be compared with the values shown for the crude hookworm extract. From the elution profile it can be seen that the most bulky eluate was in fraction V with an approximate molecular weight of 4.0×10^4 Daltons, since it eluted just ahead of the marker ovalbumin whose molecular weight was 4.3×10^4 Daltons.

1.2. Preparative and Analytical Isoelectrofocusing (IEF) of the Crude Hookworm Extract

From the results of both analytical (Figure 2) and preparative (Figure 3) IEF, it is apparent that the hookworm homogenates presents a very heterogeneous

array of isoelectric points (PI). The pH profile of the analytical IEF (Figure 2) shows most of the proteins focused between pH 4.0 and 6.4, with a particular concentration at pH 5.6. Below pH 4.2 only little quantities of hookworm protein were discernible in the analytical plate. On the other end of the scale towards the cathode, a diffuse distribution of the protein could be traced down to about pH 8.0, part of which could be due to protein "tailing" from the sample applicators. The banding in the region of pH 6.4 to pH 8.0 was not clear-cut. In total, there were at least 26 discernible bands. The Sephadex G - 200 fraction III which was also focused on the same plate revealed a strong banding at pH 5.6 which implied a reasonable degree of purity for this fraction.

In the preparative IEF column, the hookworm proteins were detectable from pH 3.7 to pH 9.0, although most of the proteins were focused at pH 4.0 to pH 7.7. A very heavy protein concentration occurred at pH 5.3 which was similar to the findings of the analytical IEF. A precipitation seemed to have occurred at pH 6.3 to pH 6.8 which was thought to be a result of protein saturation rather than denaturation, since the material had comparable antigenicity to the protein before and after the precipitation region (Appendix VII),

1.3. Polyacrylamide Gradient Gel Electrophoresis (PAGE) of the Crude Hookworm Extract

Figure 5 shows the diagramatic representation of the protein band migration on the polyacrylamide gradient gel. Also shown on the diagram are the various levels of slicing on the gel to obtain Fractions for further immunological analysis. Heavy staining occurred in bands located at 29.0 to 31.0 mm, 38.5 mm, 44.7 mm, many small bands were also seen from 64.5 to 68.6 mm. The corresponding molecular weight estimates are shown on Table II as derived from the protein migration scale of Figure 6. It is to be noted on Figure 5 that the 12 slices made from the PAGE gel did not necessarily correspond to the stained bands. This was done on assumption that the quantity of protein in some of the non-stained bands was so low as to preclude visualisation. Analysis of the hookworm extract on PAGE showed that the molecular weight of the material was varied ranging approximately from 5.0×10^4 to 2.0×10^6 Daltons. The most abundant proteins occurred at position 31 mm to 33 mm corresponding to a molecular weight range of 3.4×10^5 to 4.0×10^5 Daltons.

2. IMMUNOLOGIC REACTIVITY OF THE ANCYLOSTOMA CANINUM EXTRACT

2.1. Double Diffusion Technique

The serum from the naturally infected and vaccinated dogs gave no precipitation with the various hookworm fractions. Such sera remained negative even after concentration by lyophilisation. No reactions were seen when faecal and mucosal extracts were tested with the hookworm antigen extracts. The serum of rabbits vaccinated with hookworm crude extract gave precipitation lines with the various hookworm antigens within 14 days of vaccination. Serial fortnightly vaccination enhanced the reactions with the strongest reactions being observed with the sera obtained 10 days post tertiary vaccination, after which more revaccination did not seem to improve the serum titres. As would be expected many precipitin lines were observed with the crude extract. The seven portions obtained by pooling isoelectrofocused material (preparative isoelectrofocusing) showed some degree of precipitation with portion V showing the strongest and most heterogeneous reactions. The polyacrylamide gel electrophoresis fractions also gave precipitating lines except fraction IV. The 6 pooled portions of Sephadex G - 200 showed weak immuno-precipitation with the exception of the strongly precipitating portions II and III. In fact

the reactions of these two Sephadex G - 200 portions was nearly as dense, although less heterogeneous, as the reactions caused by the crude extract. When the whole crude extract, Sephadex G - 200 portion III, IEF portion V and polyacrylamide fraction II were reacted around the same well of hyperimmune rabbit sera, it was observed that there were at least three commonly shared precipitin lines.

2.2. Immunoelectrophoresis

2.2.1. One dimensional immunoelectrophoresis

When the canine sera from naturally infected or vaccinated dogs were tested against electrophoresed antigens, only the serum from one heavily infected dog was found to show a weak reaction against an antigen contained in the crude hookworm homogenate. This reaction occurred along the region of antigen application well. Lyophilisation of this serum enhanced the reaction.

The rabbit sera on the other hand reacted against a wide range of the hookworm components, with the crude extract showing at least 10 precipitin lines, 4 of which had migrated towards the cathode, 10 to the anode and the others taking a middle position in the 1% agarose with a pH of 8.4 (Figure 7).

The rabbit sera reacted variably with the six

portions of the Sephadex G - 200 chromatography. Portion I was poorly reactive, and the reaction was confined to the limits of the antigen application well. Two short distinct lines were seen. Since the antigens were in the first eluate of the column, one can deduce that the antigens were relatively large. Portions II, III and IV contained the antigens found in portion I in addition to an abundant quantity of antigens migrating towards the anode. Portion V contained two distinct antigens that had low migration capacity, and finally portion VI did not show any precipitation with the rabbit antiserum.

Of the seven pooled portions of the preparative IEF, portions I, II and III contained positively charged antigens like those found in the Sephadex G - 200 chromatography portions III and IV. The reaction of IEF portions IV and V, especially portion IV resembled that of the non-fractionated extract in intensity. The pH range of portions IV and V were 6.09 to 7.55 and 4.88 to 6.04 respectively. Portions VI and VII were clearly negatively charged and they migrated towards the anode (Figure 7).

The twelve polyacrylamide gel fractions were rather disappointing in their reactions. Fraction 1A, 1B hardly showed any precipitation lines. Fraction 1C had 2 very distinct precipitation lines, both of which were cathodic. Fractions II, IV and V

were poor reactors while fraction III resembled the reaction described for fraction 1C. The precipitation of fraction VII extended from the cathodic side to more than half-way across the plate while the precipitation due to fraction VI, VIII, IX and X was confined to the centre of the plate.

2.2.2. Cross or two dimensional immunoelectrophoresis

This method showed that the bulk of hookworm antigens soluble in PBS were negatively charged since they migrated towards the anode. A total of 15 precipitin lines were observed and ten of them had migrated towards the anode (Figure 8).

2.2.3. Rocket electrophoresis

This method was fairly sensitive in detecting the various components of the hookworm extract. When the non-fractionated extracts were tested against the rabbit antiserum, at least three precipitin lines were observed to migrate towards the cathode, with four at the centre of the plate and another six migrated towards the anode. The components migrating towards the cathode were generally weakly staining as opposed to the ones at the centre of the plate and the ones migrating towards the anode were strongly staining, which may imply the scantiness of the cathode oriented material. Similarly strong staining material was observed in fraction II and IV of the electrophoresed PAGE extracts, (Figure 9a) leading

one to conclude that these precipitates were possibly due to a mixture of compounds of fairly small molecular weight, ranging from 2.7×10^5 to 4.0×10^5 Daltons (Table II). When a slow electrophoresis of the PAGE fractions, II and IV, was performed on the same plate with crude extract, an antigen common to all the fractions and the crude extract was observed. This antigen was most abundant in PAGE fraction II. The Sephadex G - 200 chromatography portions I, II and IV gave faint precipitation while portions V and VI had none. Only portion III had appreciable degree of precipitation. When the individual preparative isoelectrofocusing tubes were tested for immunoprecipitation they showed a strong precipitation from tubes 25 to 105 (pH range 3.3 to 8.5), with the strongest reactions occurring between tubes 67 and 87, whose pH range was 4.7 to 6.3 respectively. Compared to the rest of the rocket profile tubes 38 to 55 were the least heterogeneous. These tubes had basically only two antigens, a fast and a slow migrating antigen, while in the rest of the tubes more than four antigen fronts were observed (Figure 10).

On testing the seven pooled portions of the IEF it was clear that each portion contained a mixture of antigens. This was to be expected since even the individual tube eluates were not homogeneous. The faecal and mucosal extracts did not show any

precipitation lines.

2.3. Cutaneous Tests

2.3.1. Passive cutaneous anaphylaxis (PCA)

In the rabbit PCA it was observed that when hookworm infected canine sera were applied, the PCA, read out as average wheal diameters was slightly more prominent at 4 hours as compared to the 24 hour reading (Table IIIa, IIIb). It is to be noted that the canine sera either at 4 or 24 hours were less reactive in the rabbit skin as compared to the rabbit sera. In this system it appeared that dilution of either canine or rabbit sera down to 1:32 did not cause an extinction of the PCA reaction, and in fact the dilution down to 1:4 did not make any practical difference (Figure 11). In the guinea pig, however, the canine serum reaginic antibodies failed to fix in the skin, either at 4 or 24 hours, largely contrasting the rabbit antisera (Figure 12). In the guinea pig system it was observed that the rabbit antihookworm serum behaved similar to the rabbit PCA system with the 4 hour reading being marginally better than the 24 hour reading in both species. It is to be noted that for the guinea pig, the PCA reading was easier done from the dermal side (Figure 13).

2.3.2. Active cutaneous anaphylaxis (ACA)

Dogs and rabbits which had previously been sensitized to hookworms either by infection or vaccination seemed to be more sensitive in ACA tests as compared to the rabbits as shown on Figure 14 and Table IV. In the dogs it appears that whether the reaction was allowed to continue for 15, 30 or 60 minutes the end point still held at log 10^5 dilution of the original 2 mg/ml material which is equivalent to 0.02 micrograms/ml crude hookworm extract. Figure 15 shows the behaviour of the extract antigens on both the rabbit and canine skin. With the Sephadex G - 200 portions it appears that the sensitising eluate was confined to the molecular weight range of 4.3×10^4 to 2.1×10^5 Daltons, as determined by the ovalbumin and catalase markers. It was also noted here that the rabbit skin was less sensitive than the dog skin. When the PAGE fractions were applied it was shown that the ACA activity was very spread out starting at fraction 1A to fraction VI (Table V) with corresponding approximate molecular weight range of 1.3×10^5 to 2.0×10^6 Daltons. The IEF portions were much more difficult to evaluate due to the base ampholine reaction of an average 8 mm, but over and above the ampholine reaction the seven IEF fractions were all very active with the most active material occurring in portion I, II and III whose pI range was 7.62 to

11.65. It was also shown that the above ACA reactions were not wholly due to histamine release since anti-histamine (Mepyramine maleate, May and Baker, U.K.) was unable to block the reaction substantially in either the dog or the rabbit (Table VI and Table VII).

2.4. Indirect Haemagglutination (IHA)

When the serum of non-vaccinated rabbits was tested with the crude extract, Sephadex G - 200 portions I to VI and polyacrylamide fraction II it was found to be negative. After vaccination the geometric mean IHA titre rose to between 1:107.6 and 1:4096 in four weeks when tested against the crude hookworm extract, Sephadex G - 200 portions I, II and III and PAGE fraction II. The Sephadex G - 200 portions IV to VI gave very poor titre (Table VIII and Figure 16). Isoelectrofocusing portion V caused severe hemolysis during IHA test and therefore no results were available.

The serum of non-vaccinated control dogs showed a higher baseline titre (1:8) as compared to the control rabbits baseline titre of less than 1:2. After vaccination with the crude extract and testing the dog sera with the antigens used to test the rabbit sera above, the serum titres rose to 1:32 with crude hookworm extract and Sephadex G - 200 portion II and 1:16 with PAGE fraction II (Table VIII).

It was observed that in the infected/vaccinated dogs an increase in the number of eggs per gram of faeces seemed to correspond with a steady rise in the serum IHA titres in corresponding dogs (Figure 17). Sera taken at post-mortem from the 37 dogs initially having eggs in their faeces and hereafter considered as chronically infected, gave high titres with Sephadex G - 200 portions I, II and III (1:80.6 to 1:107.6) and low titres (1:34) with the other antigens specified above. At post-mortem it was noted that 50% of these dogs had no faecal eggs; that only 30% had worms in the intestines and that 90% of the corresponding sera were positive in the IHA test.

When the non-concentrated faecal and mucosal extract were tested for IHA activity it was observed that the mucosal extract had no IHA titres with any of the antigen preparations. The faecal extract gave 1:16, 1:64, 1:8 and 1:4 IHA titres with Sephadex G - 200 fractions III, V, VI and crude extract respectively.

2.5. Location of the Antigen-Antibody Complexes in the Hookworm by Immunofluorescence

The buccal cavity of the hookworm showed strong autofluorescence which was not enhanced by the fluorescein isothiocyanate staining. Specific immunofluorescence was observed to occur in the upper oesophageal region, gut lining, secretory glands and on the cutical. In

all cases, fluorescent reaction was stronger when the serum of infected dogs was used as the source of IgG as compared to the immunised rabbit sera.

V. DISCUSSION

Antigenic analysis of the phosphate buffered saline extract of Ancylostoma caninum showed a marked heterogeneity. Soulsby (1968) reached a similar conclusion regarding most nematodes. When the hookworm extract was subjected to Sephadex G - 200 chromatography it was observed that no truly homogeneous antigenic component could be isolated. In the Sephadex G - 200 column it was observed that more than 37% of the material eluted as small molecular weight substances, approximately 4.0×10^4 Daltons or less. This material was contained in portions V and VI (Table I). However, this material was found to be active in immunodiffusion, electrophoresis and cutaneous tests. The immunologically active material eluted in portions I and IV whose molecular weight range was 4.3×10^4 to 2.1×10^5 Daltons. This finding could be compared to that of Gathuma (1977) who observed that in the bovine tapeworm Cysticercus bovis, the molecular weight of the antigenically active components from the Sephadex G - 200 column fractionation was approximately 1.5×10^5 to 2.0×10^5. However, Gathuma (1977) who worked on the bovine tapeworm, Baisden and Tromba (1963) working on the antigens of the kidney worm; and Pelley et al. (1977) who worked on the antigens of Schistosoma mansoni, all observed that it was not

possible to obtain a single homogeneous entity with Sephadex extraction. This finding was confirmed in this study on the canine hookworm, A. caninum. It was observed here that among the 10 entities identified during the immunoelectrophoresis of the crude extract none of these entities eluted singularly. Although Sephadex chromatography did not yield highly purified antigenic components of A. caninum the partially purified antigens were found useful in diagnostic tests especially the indirect haemagglutination (IHA) test. When immune rabbit sera were tested for IHA test, the crude extract material gave titres with a geometric mean of 107.6, while the Sephadex G - 200 portions I, II and III gave geometric mean IHA titres of 1:3444.3, 1:4096 and 1:2496.5 respectively (Table VIII). Chronically infected dog sera also had higher IHA titres with Sephadex G - 200 portions I, II and III as compared to the crude hookworm extract. This is a significant finding since many hookworm chronically infected dogs did not show any hookworm eggs in their faeces, despite their having been initially infected with hookworms, hence the diagnostic value of the IHA test using the semi-purified antigens obtained through Sephadex G - 200 column chromatography.

It was shown that of the 37 dogs initially having eggs in their faeces 50% of them were negative for faecal egg count 3 months later, and that only 30%

of these dogs had worms recovered at post-mortem concurrently. Moreover when IHA test was applied to sera obtained at post-mortem, about 90% of the dogs were found positive. It is not clear how the dogs lost the worms, however the loss could be attributed to either self-cure or that the dogs had been treated just before they were acquired. The possibility that some of the dogs acquired the faecal eggs by ingesting faeces of other infected dogs cannot be ruled out. Thus since dogs that were initially having eggs later proved to have no worms in their guts it was not possible to establish a true positive diagnosis for the dogs either by egg count or presence of worms. Therefore it was not possible to work out the specificity/ sensitivity of the IHA test in the absence of the relevant data.

In addition to the fact that some chronically infected dogs did not show faecal eggs, it was observed that sera from infected dogs were very poor in immunoprecipitation tests. This was also also observed in the faecal extract test where no immunoprecipitation was observed but IHA titres with Sephadex G - 200 fraction V of up to 1:64 were seen. The mucosal extract was shown to have no immunologic activity, in either the immunoprecipitation or haemagglutination tests. This was unexpected since

it was thought that the injured gut lining would have released the antibodies into the faeces or onto the mucosa. Only one serum sample from a heavily infected dog was found to be immunoprecipitating giving a very poor reaction. Since the electrophoretic gel had a pH of 8.4 and the antigen migrated slightly towards the cathode it was concluded that the infected dogs could possibly produce precipitating antibodies to an antigen with a low positive charge whose pI value was slightly lower than 8.4.

On testing the Sephadex G - 200 eluate on the skin of vaccinated rabbits and infected dogs it was observed that the compounds active in cutaneous tests were present in portions II through to portion V with a molecular weight range of 4.3×10^4 to 2.1×10^5 Daltons. It was observed however that the wheals observed on the dog skin were bigger than those on the rabbit skin (Figure 15). The least amount of hookworm extract protein required to elicit active cutaneous anaphylaxis (ACA) was found to be approximately 0.2 micrograms per site. This compares well with the findings of Carpenter (1965), Herbert (1970) and Mukerji *et al*. (1977), who found that the ACA reaction required approximately 0.005 to 0.05 micrograms of protein per site. Fractionation of the crude extract in Sephadex did not really enhance ACA activity as was the case in IHA tests.

These active cutaneous anaphylaxis tests were shown not to be wholly dependent on histamine as the mediator since mepyramine maleate was unable to completely block the ACA reaction caused by the hookworm extracts (Table VI and VII). This observation agrees with others recorded earlier by Orange et al. (1970) and Norman (1975), who subsequently proposed that other vasoactive substances would be involved. Such compounds namely, serotonin and the slow reacting substance of anaphylaxis (SRS-A) have since been shown to mediate ACA (Orange et al. 1970). In the dog ACA, histamine accounted for only 15% of the reaction induced by crude hookworm extracts as shown by the degree of reaction blocked by the antihistaminic compound, mepyramine maleate. In contrast there was more than 70% blockage of histamine activity when pure antihistamine was injected into control dogs followed by injection of the histamine.

On performing the passive cutaneous anaphylaxis (PCA) test it was shown that the infected canine serum elicited large PCA reaction on the rabbit skin (Figure II). It was noted that the infected dog sera which elicited PCA in the rabbit was not able to produce immunoprecipitation. This could imply that one was dealing with the reaginic (IgE) antibodies. This would not be surprising since it has been suggested that the PCA reaction is mediated mainly by the reaginic I_{gE} antibodies (Colwell et al. 1971, and Kojima et al

1976). It was observed that this canine serum which did not produce immunoprecipitation but elicited PCA had an IHA titre 1:64 which would have been interpreted as positive for canine hookworm. Herbert (1970) suggested that the PCA assay closely approximates the IHA assay and that the methods are particularly useful especially in cases where immunoprecipitation is non-discernible, and since failure of detecting antibodies to nematodes in agar gel diffusion is not rare it appears that the standardization of IHA and PCA (Wide et al. 1967; Augustin 1973) is of prime importance. In addition to other diffusion independent assay methods like indirect solid-phase microradial immunoassay should be given paramount consideration (Rhodes et al. 1981). The findings of this study further confirm this notion in specific reference to A. caninum. Vaccinated rabbit serum elicited satisfactory PCA and there was little difference when the serum was allowed to fix in the skin for 4 or 24 hours. Unlike the canine serum the immune rabbit serum caused immunoprecipitation in agar gel diffusion and had a high IHA titre (1:2048).

When the guinea pig was used in the PCA test, the results of the immune rabbit sera were similar to those obtained in the rabbit (Figure 12 and 13). A major difference between the rabbit and guinea pig PCA was however observed with the serum of infected canines. It appears that the canine reaginic antibody

fixed very poorly on the guinea pig skin regardless of whether it was allowed to fix for 4 or 24 hours.

Isoelectrofocusing of the crude extract showed that the pI values of the hookworm extract ranged from 3.7 to 9.0 with most of the extract eluting at pH 4.0 to 7.7, and most concentrated at pH 5.3 in the preparative IEF column. In the analytical IEF heavy banding was noticed to occur at pH 5.6. In both systems, it was shown that the material with pI value range of 4.0 to 8.0 was strongly reactive in the immunoelectrophoretic tests. This was especially evident when the IEF preparative eluates (Appendix VII) were tested individually using the fused rocket electrophoresis (Figure 10). Once again, here as in the Sephadex G - 200 chromatography, it was realised that the antigen groups did not elute as single pure entities at any one level. This was the experience of Kumaratilake and Thompson (1979) who in their work on Echinococcus granulosus observed 63 resolvable antigen bands but were unable to separate a single antigen moiety. In this work at least 26 discernible bands were observed. It is possible that some of the bands observed here in the gel having a wide pH spectrum were due to denatured material. This could imply that after the fractionation one may end up with a more complex antigen spectrum than the one occurring naturally. It is likely therefore that when the IEF fractions were applied to the immunological

reactions, some of them could have been cleaved to hapten type molecules making them incapable of reacting immunologically. In order to have a better resolution, an ampholine plate with a narrower pH range would need to be used. This is so because resolution is inversely proportional to the square root of the pH gradient as expressed below (LKB Produkter A.B., Sweden, 1976):

$$\Delta pI = k\sqrt{\frac{D(dpH/dx)}{E(-du/dpH)}}$$

where

Δ pI = Minimum difference in pI between resolvable proteins.

D = Diffusion coefficient of proteins (constant).

du/dpH = Electrophoretic mobility of proteins (constant).

dpH/dx = pH gradient

E = Electrical field strength

In this study the least heterogeneous material occurred at pH range 7.18 to 8.43 where only two migrating fronts were seen in the fused rocket electrophoretic test (Figure 10). This material was contained in portion III and IV of the pooled preparative IEF material and was found to be active in the immunoelectrophoretic systems (Figure 7). When these

pooled IEF portions were applied in the IHA test it was noticed that they caused severe hemolysis especially with those in the extreme pH ranges and it was therefore difficult to test their value in this system. In the cutaneous tests, pooled IEF portions had some ACA reaction but being highest in portions I, II and III. Compared to the Sephadex portions, the IEF eluates showed a higher level of ACA wheal-diameters in both the vaccinated rabbits and the infected dogs. Part of the reason may be due to the considerable irritation due to ampholine pH (Table V). From this experience, it would seem that the IEF extracts would be limited to immunoprecipitin tests due to their good reactivity in this system and their unfavourable effect on the cutaneous and indirect haemagglutination tests.

Polyacrylamide gel electrophoresis (PAGE) fractions revealed a wide spectra of molecules in the hookworm PBS extract. In this system the reactive hookworm extract material was contained in the molecular weight range of 2.2×10^5 to 4.0×10^5 Daltons (Table II). The fractions in this range were intensely reactive in rocket electrophoresis system (Figure 9). Due to the innertness of the polyacrylamide gel it is hypothesized here that the PAGE fractions were more likely intact molecules whose mobility was entirely due to molecular weights as opposed to the possibly cleaved IEF molecules.

In the electrophoretic tests it was observed that the PAGE fractions II and IV (Figure 9) had strong immunoprecipitin reactions, with fraction III showing a weak reaction. In the IHA tests PAGE fraction II gave the highest titre with the vaccinated rabbit sera although it only gave low titres with the infected canine sera as compared to the antigens of Sephadex G - 200 portions I, II and III and the crude hookworm extraction (Table VIII). This demonstrates once again that the purified antigen gave better results in the IHA test. This is perhaps due to the fact that antigen purification removes haemagglutination inhibitors that have been reported to be present in some worm extracts (Douns et al. 1955; Lunde and Jacobs, 1963). In the skin tests, it was shown that PAGE extracts were in fact more reactive than the Sephadex G - 200 extracts both in the vaccinated rabbit and the infected-vaccinated dogs. The reactive antigens were contained in fractions 1A to V (Table V) with molecular weight range of 1.7×10^5 to 2.0×10^6 Daltons. This material was considerably larger than the skin reactive material of the Sephadex G - 200 where molecular weight range was 4.3×10^4 to 2.1×10^5 Daltons. For diagnostic purposes it would appear that the most useful material was in PAGE fraction II with an average molecular weight of 3.7×10^5 Daltons.

An attempt was made to localise the specifically reactive antigens in the worm using the direct

fluorescent antibody technique. It was shown that the labelled IgG was bound to the cuticular tissue and the alimentary tract as well as excretory orifice and the gut lining. This may imply that in addition to the structural components of the hookworm the antigens which induced immune reactions against the hookworm were part secretory and excretory. Since the dog is the natural host of this hookworm and comes into contact with the live worms it would benefit most from the excretory/secretory antigens, produced in the course of the worms life. This is true here since the fluorescent antibody reaction was stronger with the infected canine serum unlike the vaccinated rabbit serum which produced a weak reaction. This complements the observation of Jackson (1959) on Trichinella spiralis and N. muris (1960) in which he suggested that the effective antigens were excretory. If this is the case it would partially explain the poor performance of homogenate vaccines (Cox, 1978), and the success of the live larvae vaccines like the one against Dictyocaulus viviparus (Jarret et al. 1958, 1959).

In conclusion it was shown in this study that although the hookworm Ancylostoma caninum is antigenically very heterogeneous, it was possible to obtain and evaluate partially purified antigens using such methods as Sephadex G - 200 filtration chromatography, isoelectrofocusing and polyacrylamide gel electrophoresis,

and that the partially purified antigens were found useful in the immunologic tests evaluated. The tests were immunoprecipitation, cutaneous anaphylaxis, and indirect haemagglutination.

VI. TABLES

Table I: Total protein content (mg) of the various hookworm Sephadex G - 200 portions as compared to the crude extract.

Crude Extract	Sephadex G - 200 Portions					
	I	II	III	IV	V	VI
8.0	0.4	0.3	1.2	1.0	3.0	0.7

Table II: Polyacrylamide gradient gel electrophoresis fractions with approximate molecular weight limits for each fraction.

PAGE Fraction	Width of band (mm)	Distance from point of application to limit of fraction (mm)	Approximate mol. wt. range (Daltons)
1A	10	0 - 10	2.0×10^6 - 1.0×10^6
1B	10	11 - 20	1.0×10^6 - 2.0×10^5
1C	10	21 - 30	7.0×10^5 - 4.0×10^5
II	3	31 - 33	4.0×10^5 - 3.4×10^5
III	4	34 - 37	3.4×10^5 - 2.7×10^5
IV	4	38 - 41	2.7×10^5 - 2.2×10^5
V	5	42 - 46	2.2×10^5 - 1.7×10^5
VI	5	47 - 51	1.7×10^5 - 1.3×10^5
VII	8	52 - 59	1.3×10^5 - 1.5×10^4
VIII	9	60 - 68	8.5×10^4 - 5.4×10^4
IX	5	69 - 73	5.4×10^4 - 5.0×10^4
X	5	74 - 78	5.0×10^4

Table III (a): Passive cutaneous anaphylaxis (expressed as average wheal diameters (mm)) in rabbits and guinea pigs 4 and 24 hours after injection with the canine sera.

		Negative canine serum dilutions						Positive canine serum dilutions					
		UD	1:2	1:4	1:8	1:16	1:32	UD	1:2	1:4	1:8	1:16	1:32
Average wheal diameter (mm) in rabbits	4 hours Fixing Time	5.3	5	4.6	5.6	4.3	3.6	23.3	23	21	21	20	18.3
	24 hours Fixing Time	0	0	0	0	0	0	19.6	19	18.6	15.6	14.6	15.6
Average wheal diameter (mm) in guinea pigs	4 hours Fixing Time	2.6	2.3	2	2	3	2	6	6.3	5.3	5.6	4.6	3.6
	24 hours Fixing Time	2	0	0	0	0	0	4.6	4	4.3	3	2	6

Note: Each average figure is a mean of 3 readings.
In all animals PBS controls were 0 mm at 4 and 24 hours.

UD = Undiluted

Table III (b): Passive cutaneous anaphylaxis (expressed as average wheal diameters (mm)) in rabbits and guinea pigs 4 and 24 hours after injection with the rabbit sera.

		Negative rabbit serum dilutions						Positive rabbit serum dilutions					
		UD	1:2	1:4	1:8	1:16	1:32	UD	1:2	1:4	1:8	1:16	1:32
Average wheal diameter (mm) in rabbits	4 hours Fixing Time	3.3	3	3.6	3.6	1	0.6	25	25	24.6	22.3	20.6	20.3
	24 hours Fixing Time	0	0	0	0	0	0	24	23	23.3	20	19	18.6
Average wheal diameter (mm) in guinea pigs	4 hours Fixing Time	2.3	2	6	0	0	3	25	24	24	22	19.6	18
	24 hours Fixing Time	0	0	0	0	0	0	25.3	20	20	18	18	17

Note: Each average figure is a mean of 3 readings.
In all animals PBS controls were 0 mm at 4 and 24 hours.
UD = Undiluted

Table IV: Active cutaneous anaphylaxis (mm of wheal diameter) elicited with various crude extract dilutions in infected and vaccinated dogs.

Time (minutes) lapsed since injection of antigen	Antigen Dilutions							Control sites injected with phosphate buffered saline
	UD	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
15	15.5	13.5	10.7	8.7	5	5	4	3.1
30	18.2	14	11	8.5	8.3	7	5.2	2.5
60	24.3	18.3	12.8	11.3	8.5	6.6	4.5	2.6

UD = Undiluted

Table V: Active cutaneous anaphylaxis expressed as average wheal diameter (mm) 60 minutes after intradermal injection of various hookworm extracts in the skin of infected-vaccinated dogs and vaccinated rabbits.

Hookworm antigen used to inject skin		ANIMALS USED			
		Dogs		Rabbits	
		Antigen	Ampholine control	Antigen	Ampholine control
ISOELECTRO-FOCUSED FRACTIONS	I	20	10	17	8
	II	22	10	18	8
	III	20	9	14	7
	IV	18	10	13	8
	V	16	12	12	9
	VI	17	8	10	7
	VII	17	9	10	7
SEPHADEX G - 200 POOLED PORTIONS	I	14		12	
	II	13		10	
	III	12		11	
	IV	15		13	
	V	16		13	
	VI	13		10	
POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS	IA	16		20	
	IB	17		18	
	IC	16		15	
	II	18		16	
	III	17		15	
	IV	16		14	
	V	16		14	
	VI	10		12	
	VII	5		6	
	VIII	4		5	
	IX	4		4	
	X	4		4	

No discernible swellings with control Sephadex G - 200 or polyacrylamide gel.

N.B. Each reading is a mean of 4 animals.

Table VI: Effect of Antihistamine (Mepyramine maleate) on the wheal diameters (mm) in dogs and rabbits sensitized to hookworm antigen by natural infection and vaccination.

Test Animals		Crude Hookworm Extract Dilutions						
		UD	1:2	1:4	1:8	1:16	1:32	1:64
Dogs	Without Antihistamine	22.4	19.3	17.1	13.6	13.3	11.0	9.9
	With Antihistamine	19.0	16.5	13.5	12.3	10.8	8.8	8.3
Rabbits	Without Antihistamine	19.1	16.5	14.5	11.3	12.0	10.1	7.7
	With Antihistamine	17.0	15.0	13.0	10.5	6.8	5.0	5.3

N.B. Each reading is a mean from four animals.

UD = Undiluted

Table VII: Effect of antihistamine on the wheal diameters (mm) caused by histamine injections in dogs and rabbits.

	Histamine (0.2 mg/ml) dilutions	Wheal Diameters (mm)	
		Before administration of antihistamine	After administration of antihistamine
Dogs	UD	25	7
	1:2	23	8
	1:4	23	6
	1:8	20	6
Rabbits	UD	20	6
	1:2	19	7
	1:4	20	5
	1:8	18	5

N.B. Each reading is a mean from four animals.

UD = Undiluted

Table VIII: Indirect haemagglutination titres of vaccinated rabbits, vaccinated dogs and chronically infected dogs as tested with the hookworm Sephadex G - 200 Portions, Polyacrylamide Fraction II and Crude Hookworm Extract.

Antigens used		Vaccinated rabbits (5)	Vaccinated dogs (8)	Chronically infected dogs (37)
SEPHADEX G - 200 PORTIONS	I	1:3444.3	1:26.3	1:80.6
	II	1:4096.0	1:32.0	1:107.6
	III	1:2496.5	1:16.0	1:107.6
	IV	1:32.0	1:29.0	1:11.3
	V	1:5.7	1:12.1	1:16.0
	VI	1:2.8	1:4.0	1:11.3
POLYACRYLAMIDE GEL FRACTION	II	1:6501.2	1:16.0	1:32.0
CRUDE HOOKWORM EXTRACTION		1:107.6	1:32.0	1:34.3

Number in parenthesis indicates number of animals used.

VII. FIGURES

Figure 1: Sephadex G - 200 chromatographic profile of the adult Ancylostoma caninum phosphate buffered saline extract; elution levels of marker proteins, catalase and ovalbumin are indicated by the arrows

(a) Catalase

(b) Ovalbumin

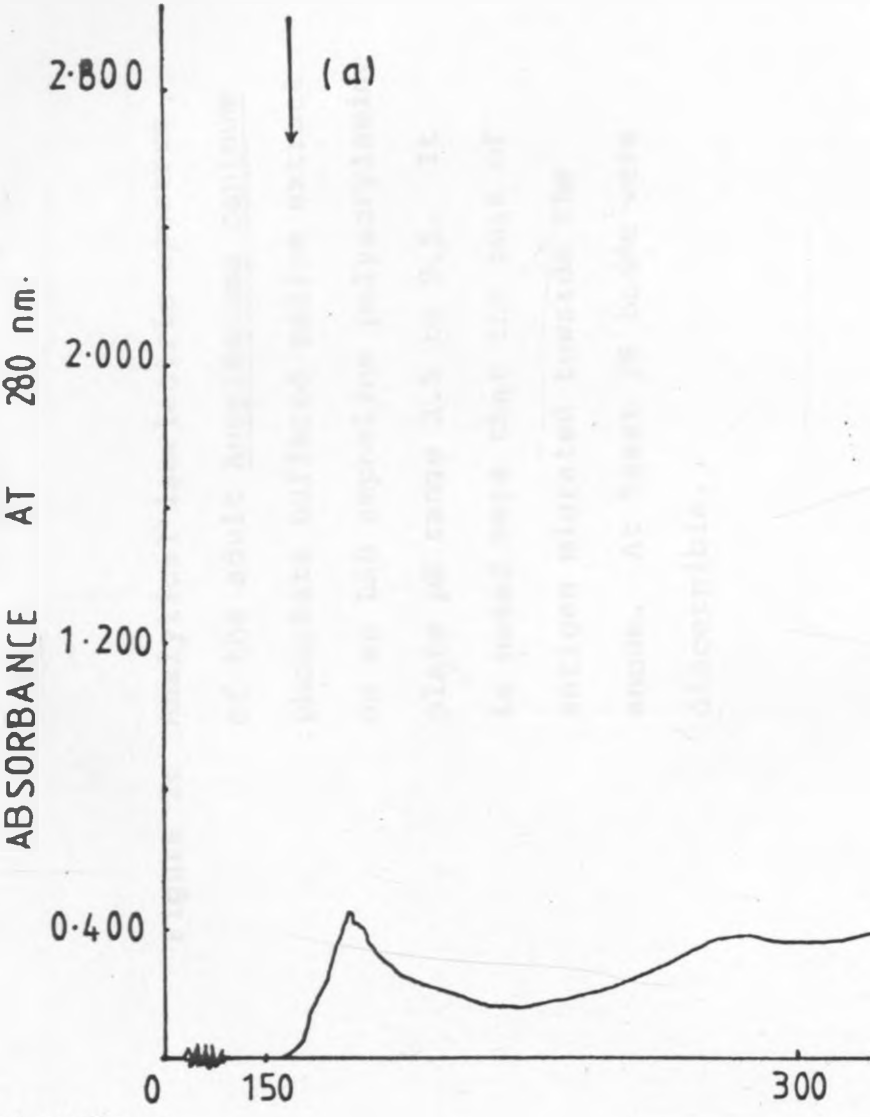


FIGURE 1

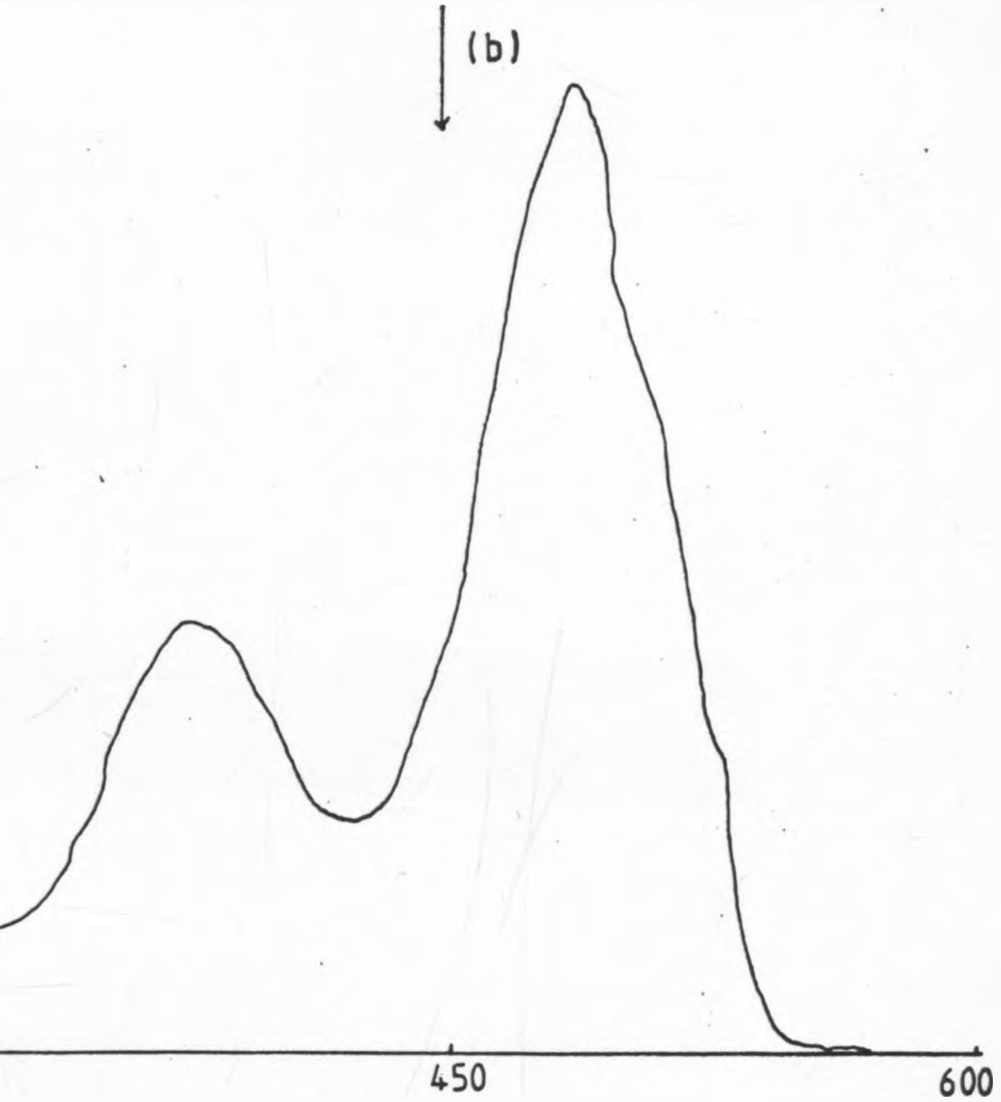

(b)
450
600
VOLUME (Ml)

Figure 2: Analytical isoelectric spectrum of the adult Ancylostoma caninum phosphate buffered saline extract on an LKB ampholine polyacrylamide plate pH range 3.5 to 9.5. It is noted here that the bulk of antigen migrated towards the anode. At least 26 bands were discernible.

FIGURE 2

Figure 3: Preparative isoelectric profile of Ancylostoma caninum extract (_____): also shown are the ampholine pH gradient (----------) and the ampholine base absorbance (.....). Most of the material eluted between pH 4.0 to 7.7 with a particularly heavy banding at pH 5.6

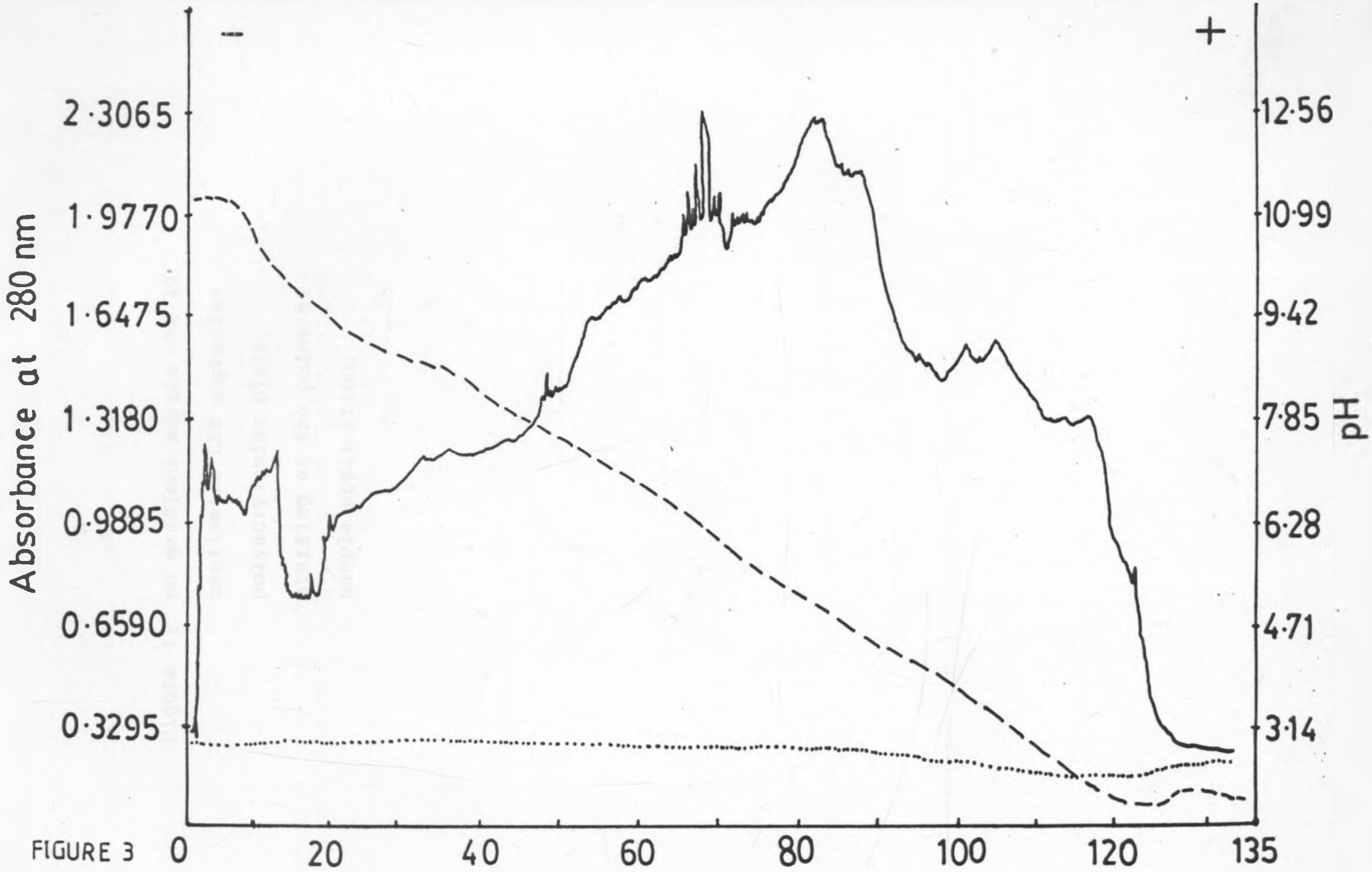

−
+
2·3065
1·9770
1·6475
1·3180
0·9885
0·6590
0·3295
Absorbance at 280 nm
12·56
10·99
9·42
7·85
6·28
4·71
3·14
pH
0
20
40
60
80
100
120
135
ELUTION VOLUME (ML)

FIGURE 3

Figure 4: pH gradient across the 10 centimetre LKB ampholine polyacrylamide plate, starting at the point of sample application.

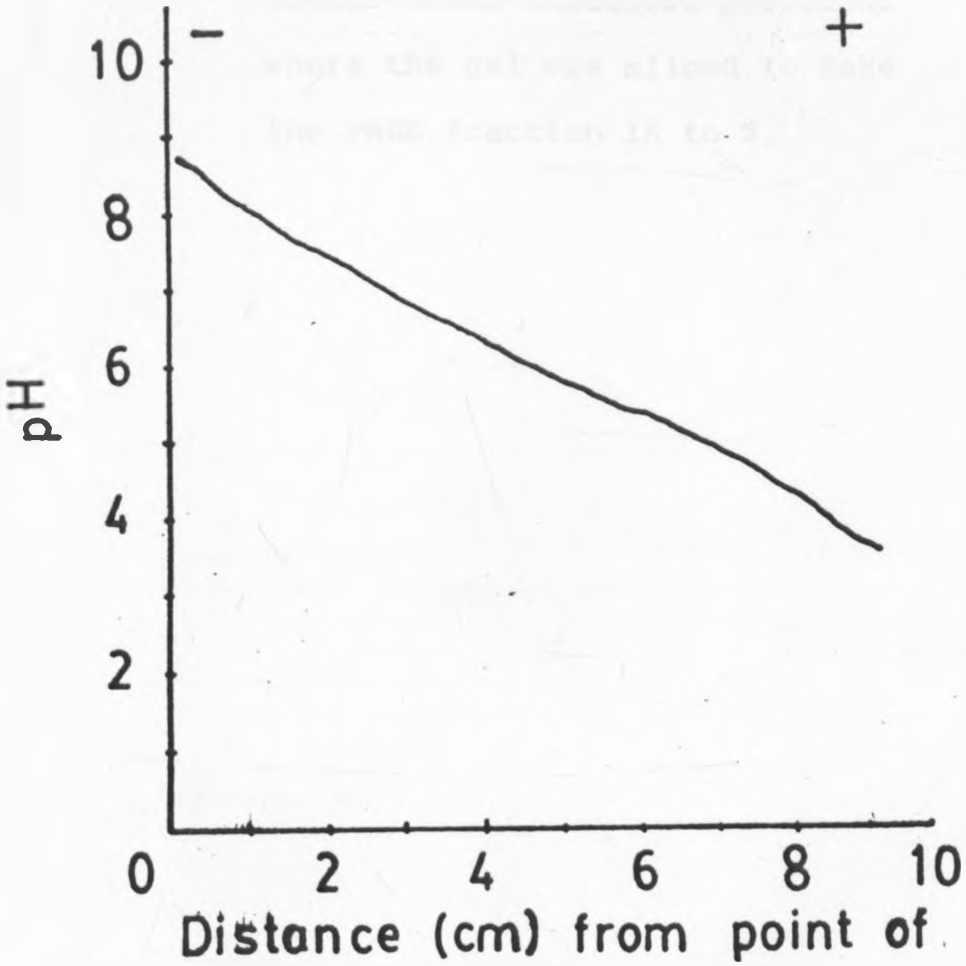
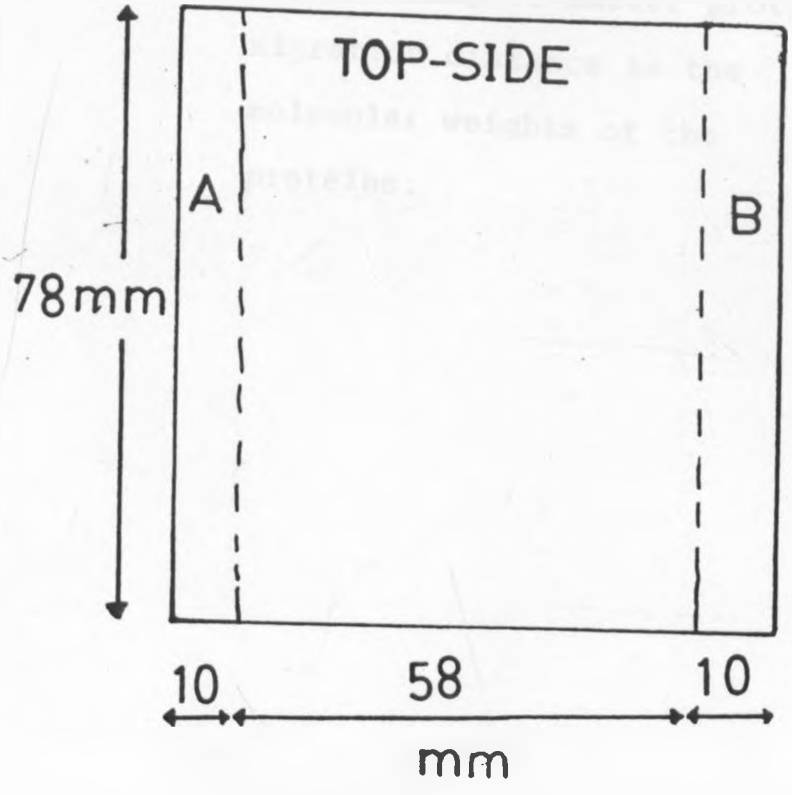

−
+
10
8
6
4
2
pH
0
2
4
6
8
10
Distance (cm) from point of application of samples

FIGURE 4

Figure 5: Diagramatic representation of
(a) the polyacrylamide gel and
(b) the observed hookworm bands;
dotted lines indicated positions
where the gel was sliced to make
the PAGE fraction 1A to X.

(a)



Right side

(b)

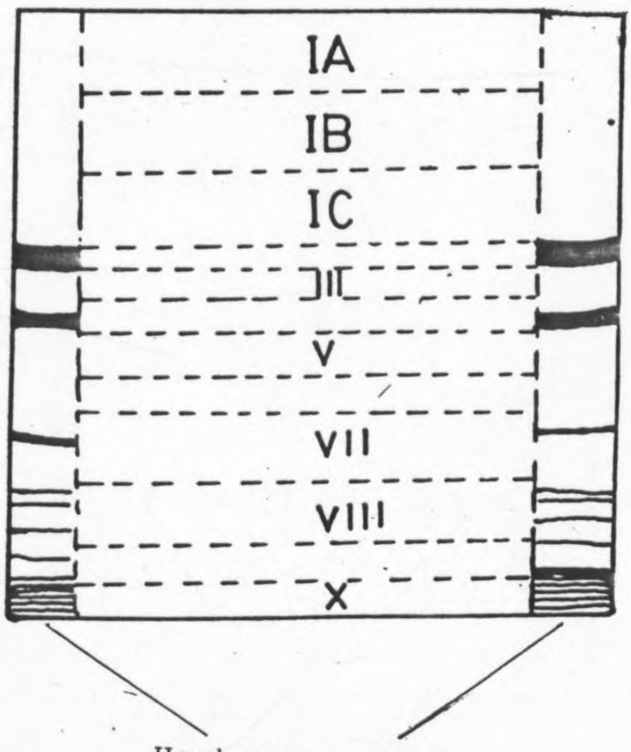


FIGURE 5

Hookworm protein bands after staining with Commassie blue.

Figure 6: Relationship of marker proteins migration distance to the molecular weights of the proteins.

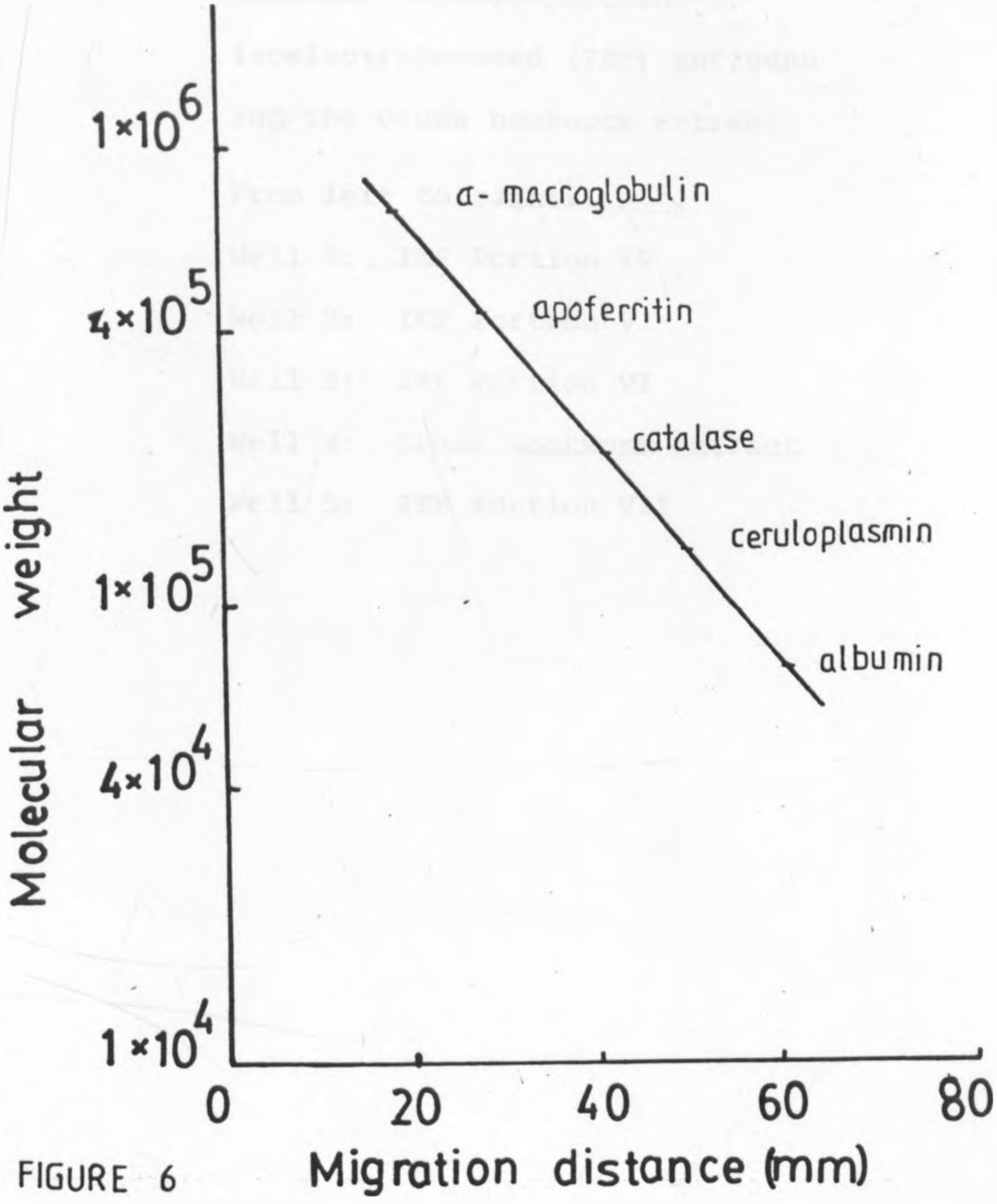

1×10^6
4×10^5
1×10^5
4×10^4
1×10^4
Molecular weight
α- macroglobulin
apoferritin
catalase
ceruloplasmin
albumin
0
20
40
60
80
Migration distance (mm)

FIGURE 6

Figure 7: Immunoelectrophoretic activity of the rabbit antihookworm serum with the various portions of isoelectrofocused (IEF) antigens and the crude hookworm extract:

From left to right;

Well 1: IEF Portion IV

Well 2: IEF Portion V

Well 3: IEF Portion VI

Well 4: Crude Hookworm extract

Well 5: IEF Portion VII

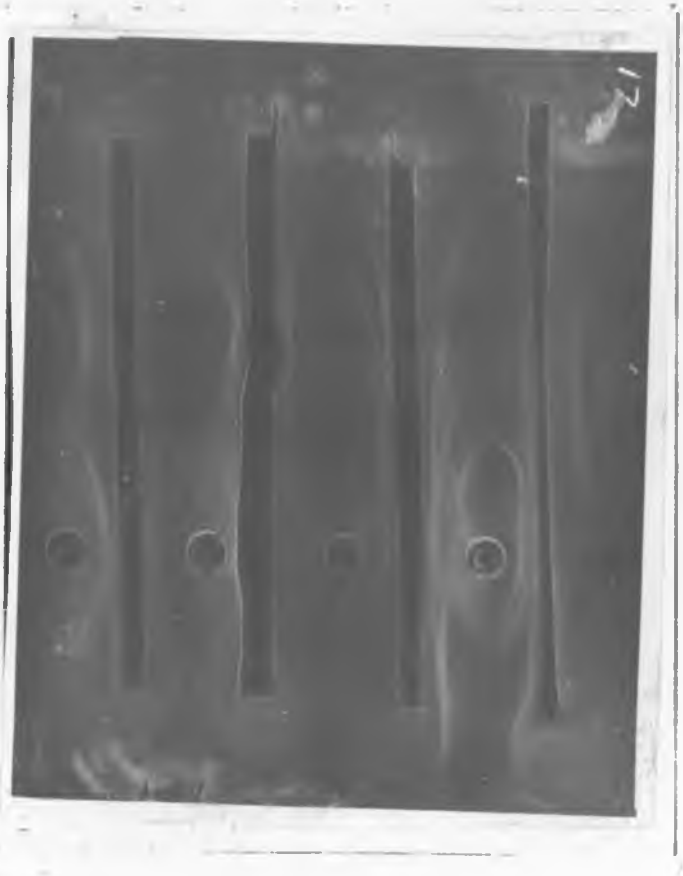


FIGURE 7

Figure 8: Two dimensional electrophoresis of the hookworm crude extract showing the migration of the various proteins. Most of the material migrated towards the anode.

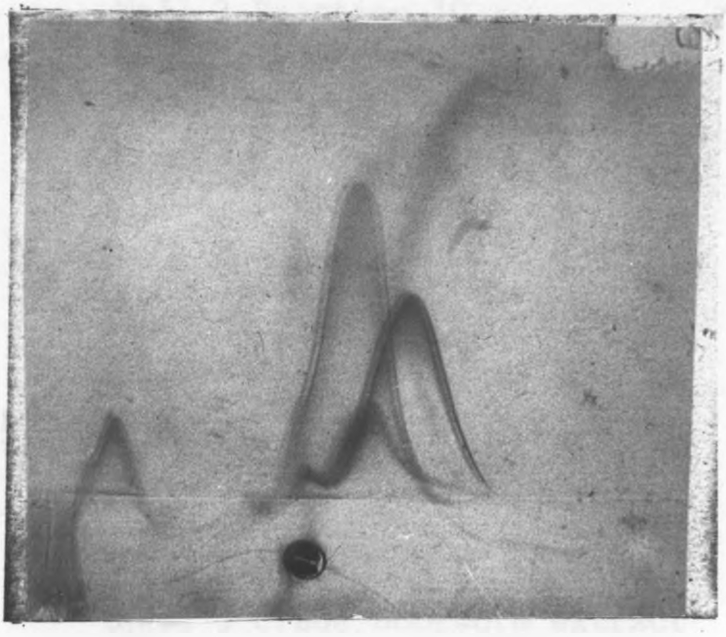


FIGURE 8

Figure 9: Rocket electrophoresis of:

(a) Polyacrylamide fractions

(Left to Right)

Well 1 Fraction 1A

Well 2 Fraction 1B

Well 3 Fraction 1C

Well 4 Fraction II

Well 5 Fraction III

Well 6 Fraction IV

(b) Polyacrylamide fractions and crude hookworm extract

(Left to Right)

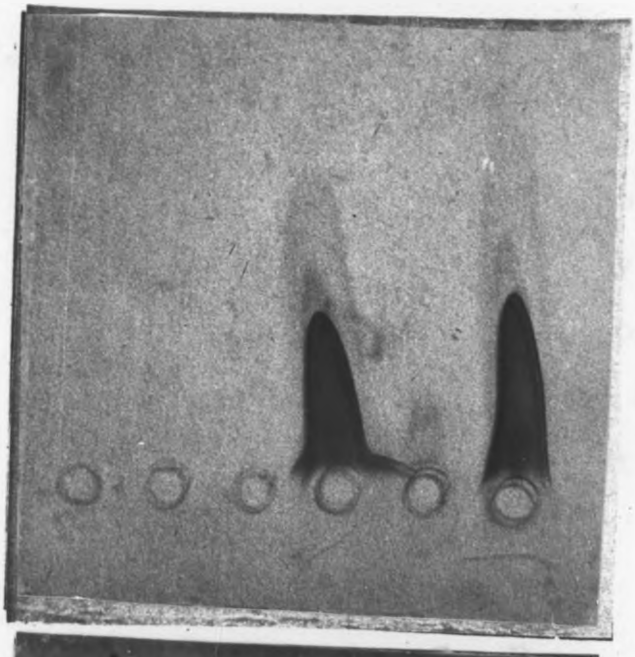
Well 1 PAGE Fraction V

Well 2 PAGE Fraction VI

Well 3 Crude hookworm extract

Well 4 Crude hookworm extract

(a)



(b)

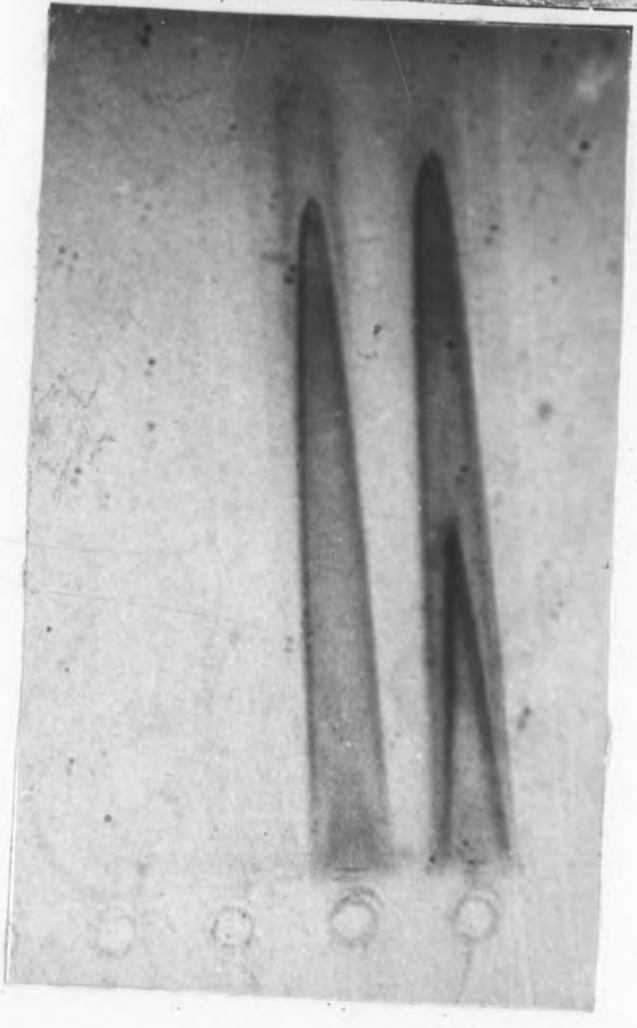


FIGURE 9

Figure 10: Fused rocket electrophoresis of the individual preparative isoelectrofocused fractions showing the heterogeneity of the hookworm extract. Note the occurrence of common bands continuing from one fraction to the next.

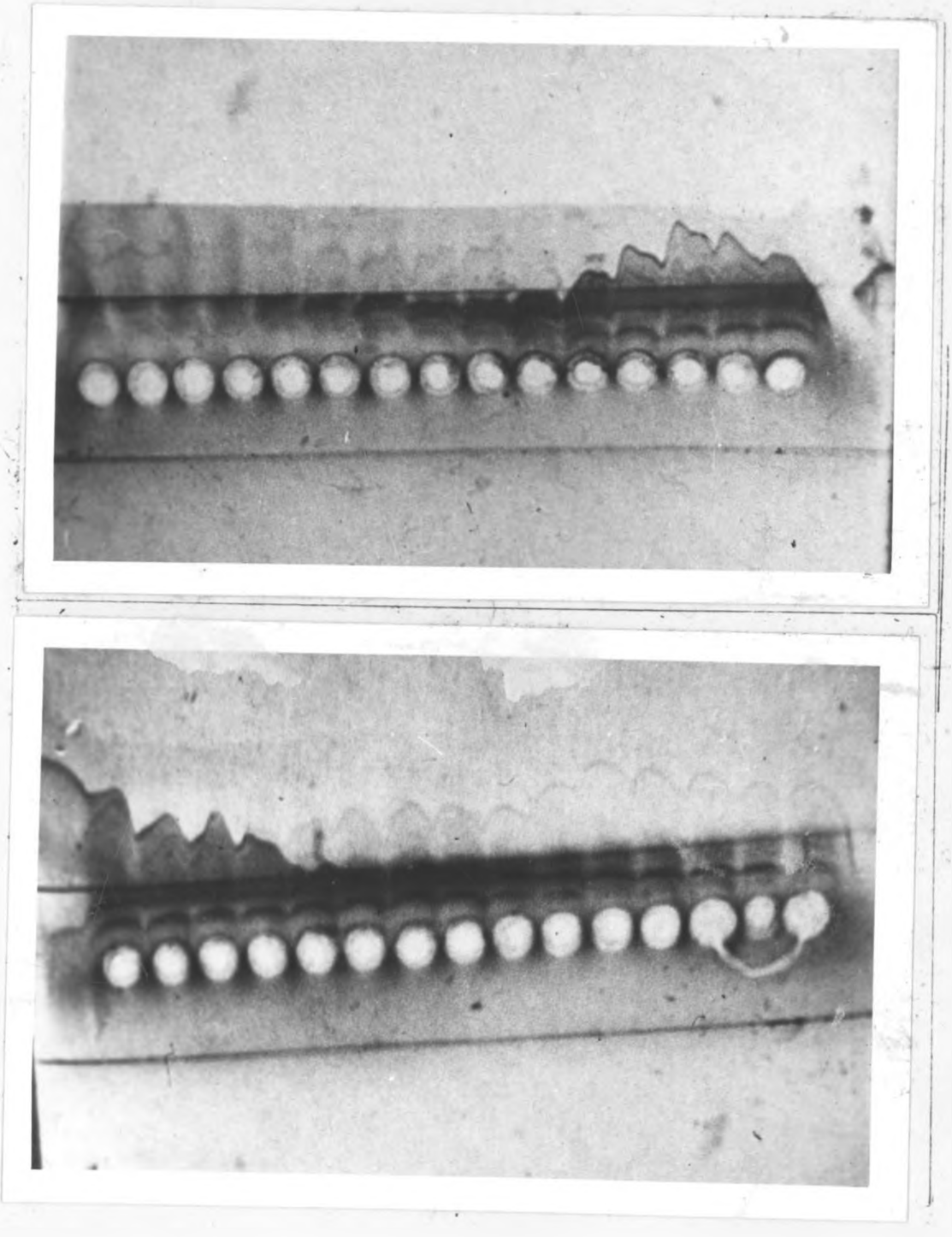
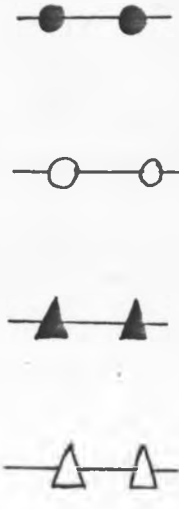


FIGURE 10

Figure 11: Wheal diameters expressed as dye leakage (mm) indicating passive cutaneous anaphylaxis on rabbit skin determined at 4 and 24 hours after injection of various dilutions of hookworm vaccinated rabbit and hookworm infected dog sera.



Vaccinated rabbit serum (4 hours)

Vaccinated rabbit serum (24 hours)

Infected canine serum (4 hours)

Infected canine serum (24 hours)

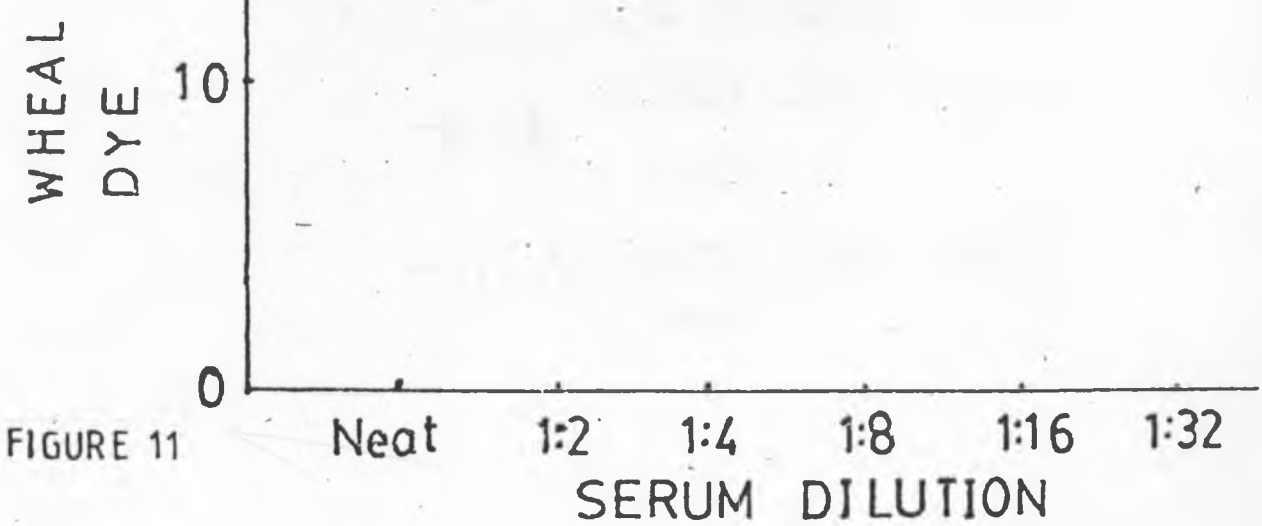


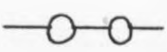
FIGURE 11

DIAMETERS EXPRESSED AS LEAKAGE (mm)

20 30

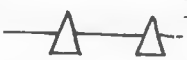
Figure 12: Wheal diameters expressed as dye leakage (mm) indicating passive cutaneous anaphylaxis on guinea pig skin, 4 and 24 hours after injecting various dilutions of sera obtained from vaccinated rabbit and hookworm infected dog.

Vaccinated rabbit serum (4 hours)



Vaccinated rabbit serum (24 hours)

Infected canine serum (4 hours)



Infected canine serum (24 hours)

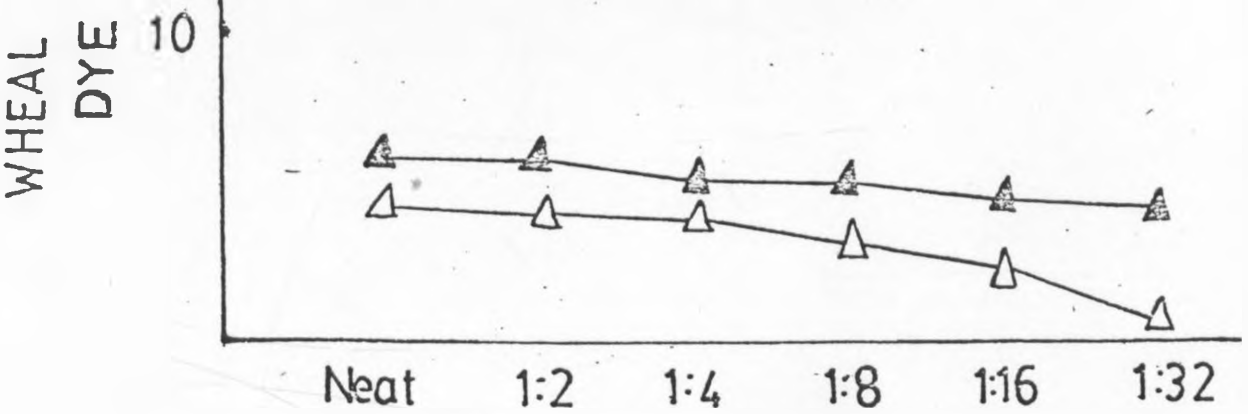


FIGURE 12

DIAMETERS EXPRESSED AS LEAKAGE (mm)

20 30

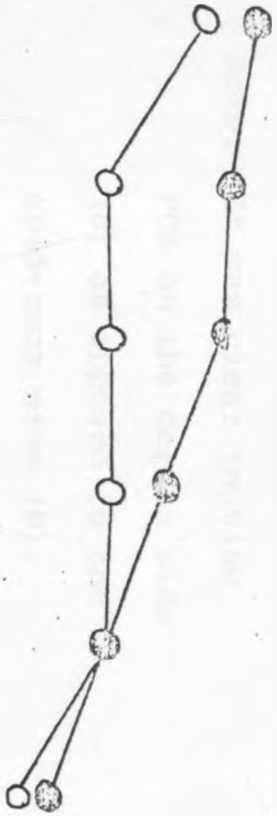
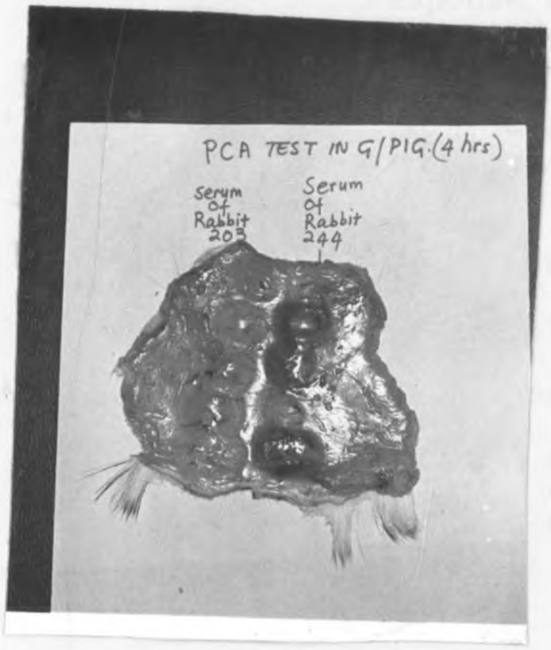
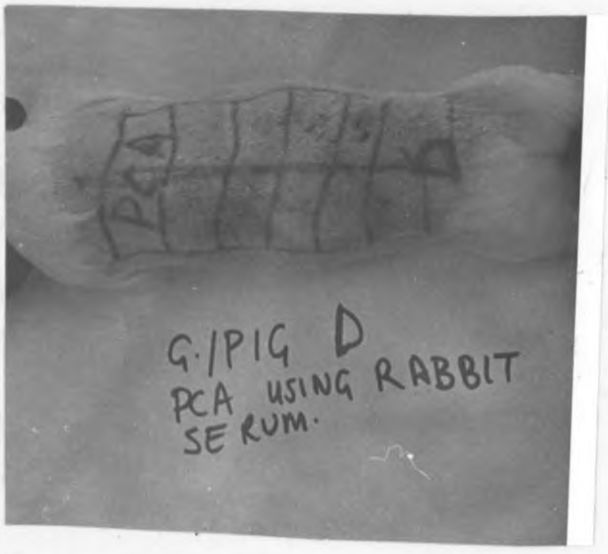


Figure 13: Passive cutaneous anaphylaxis in the guinea pig using the serum of two rabbits immunised with hookworm crude extract. The serum was allowed to fix for 4 hours in the guinea pig skin.

Note: It was clear to view PCA on the dermis side (a) as opposed to the epidermis side (b).


PCA TEST IN G/PIG.(4 hrs)
Serum of Rabbit 203
Serum of Rabbit 244

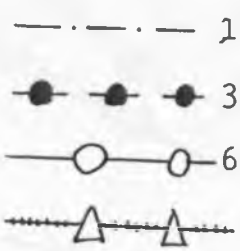
(a)


G.PIG D
PCA USING RABBIT SERUM.

(b)

FIGURE 13

Figure 14: Active cutaneous anaphylaxis response of hookworm infected dogs and hookworm vaccinated dogs, injected with various dilutions of crude hookworm antigen and reactions read at various times.
(Note: each point represents the mean of 3 naturally infected and 3 vaccinated dogs).



and, active cutaneous anaphylaxis on the rabbit skin, 60 minutes after injection with various dilutions of crude hookworm extract.

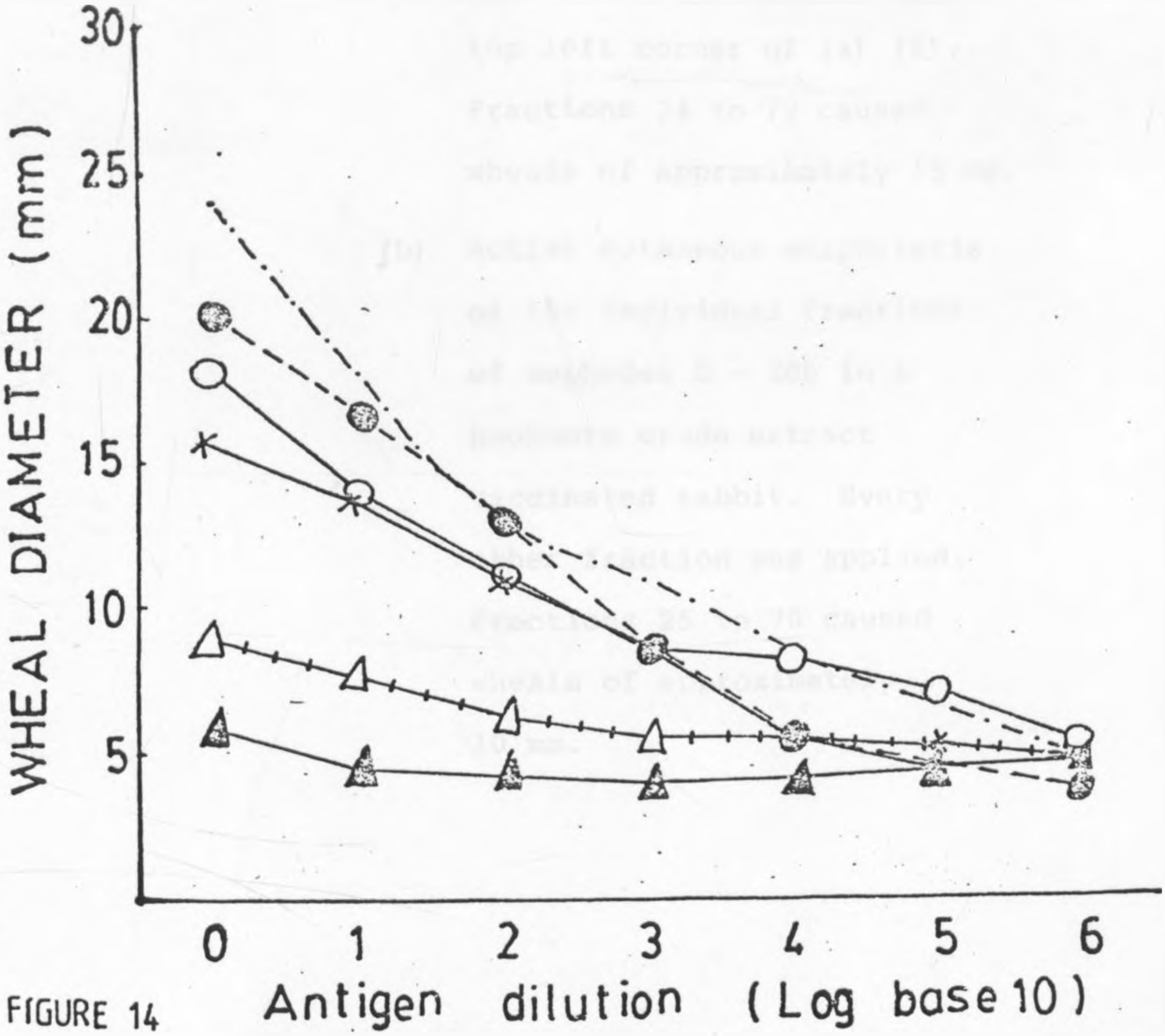
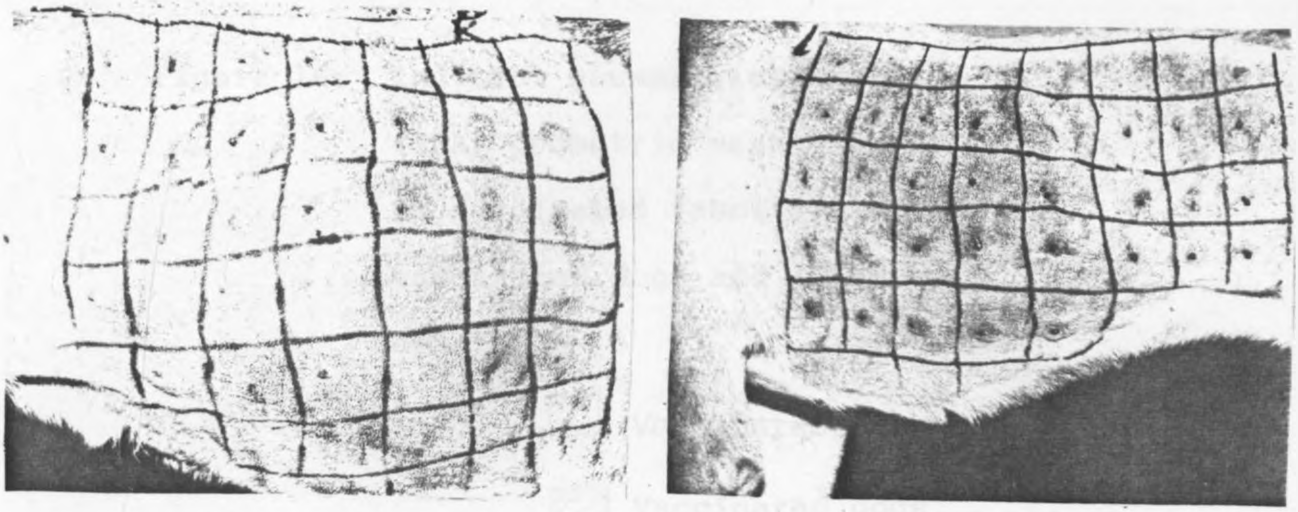

WHEAL DIAMETER (mm)
30
25
20
15
10
5
0
1
2
3
4
5
6
Antigen dilution (Log base 10)

FIGURE 14

Figure 15: (a) Active cutaneous anaphylaxis of the individual fractions of sephadex G - 200 in a hookworm infected dog. Every other fraction was applied starting with fraction one at top left corner of (a) (i). Fractions 24 to 72 caused wheals of approximately 15 mm.

(b) Active cutaneous anaphylaxis of the individual fractions of sephadex G - 200 in a hookworm crude extract vaccinated rabbit. Every other fraction was applied. Fractions 25 to 70 caused wheals of approximately 10 mm.



(i) (ii)

(a)

(b)

FIGURE 15

Figure 16: Indirect haemmagglutination (IHA) geometric mean titre in vaccinated rabbits, vaccinated dogs and infected dogs.

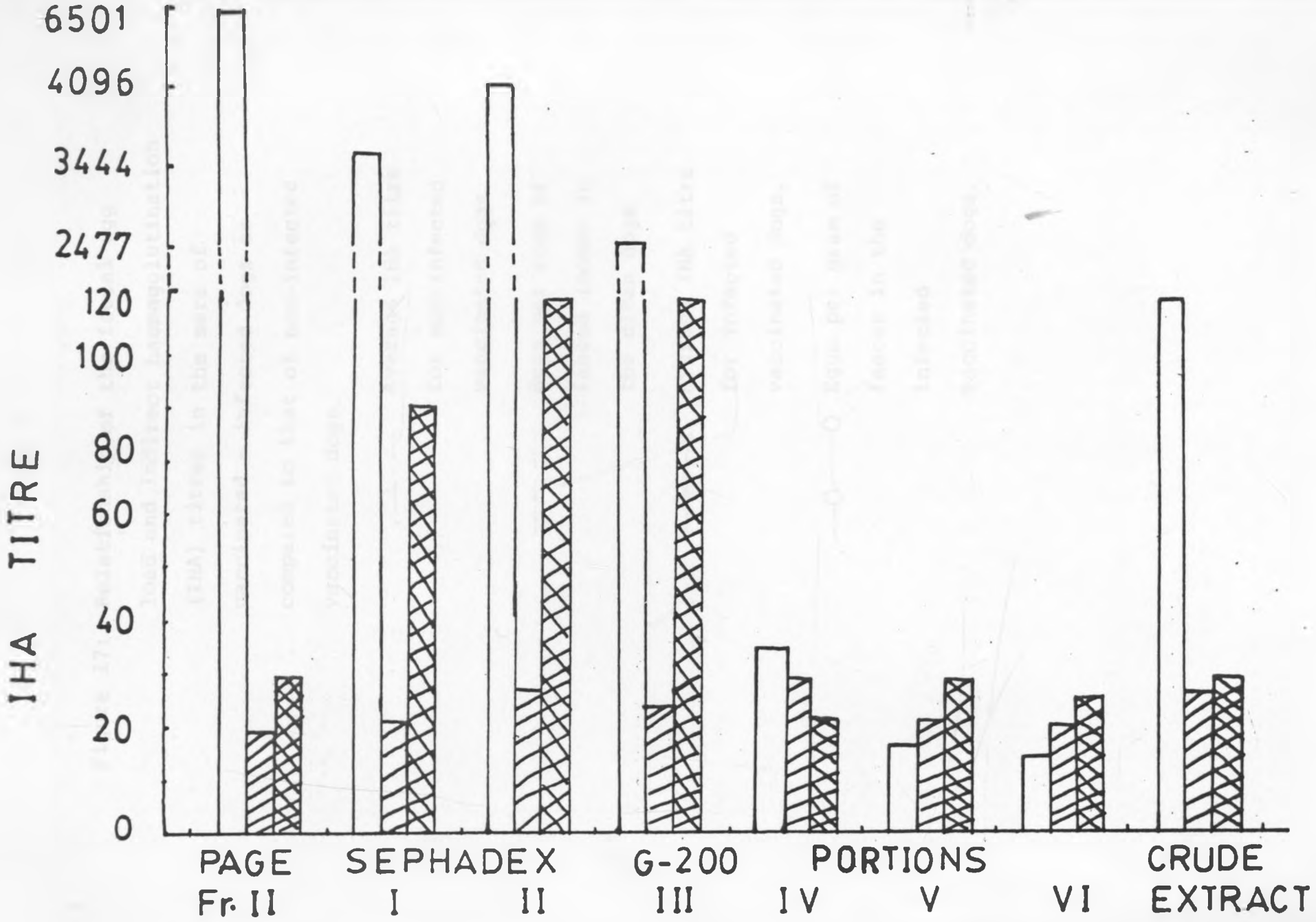
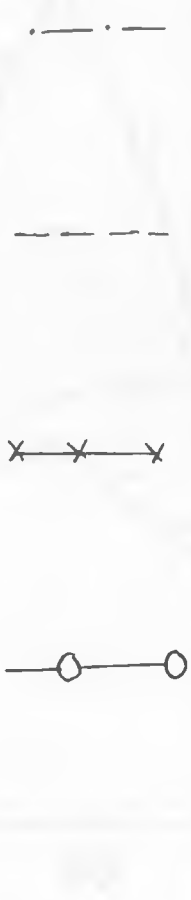


FIGURE 16 Antigens used in the IHA test.

Figure 17: Relationship of the faecal egg load and indirect haemagglutination (IHA) titres in the sera of vaccinated - infected dogs as compared to that of non-infected vaccinated dogs.



.—·— Average IHA titre for non-infected vaccinated dogs.

— — — — Eggs per gram of faeces (none) in the clean dogs.

×—×—× Average IHA titre for infected vaccinated dogs.

—○—○ Eggs per gram of faeces in the infected vaccinated dogs.

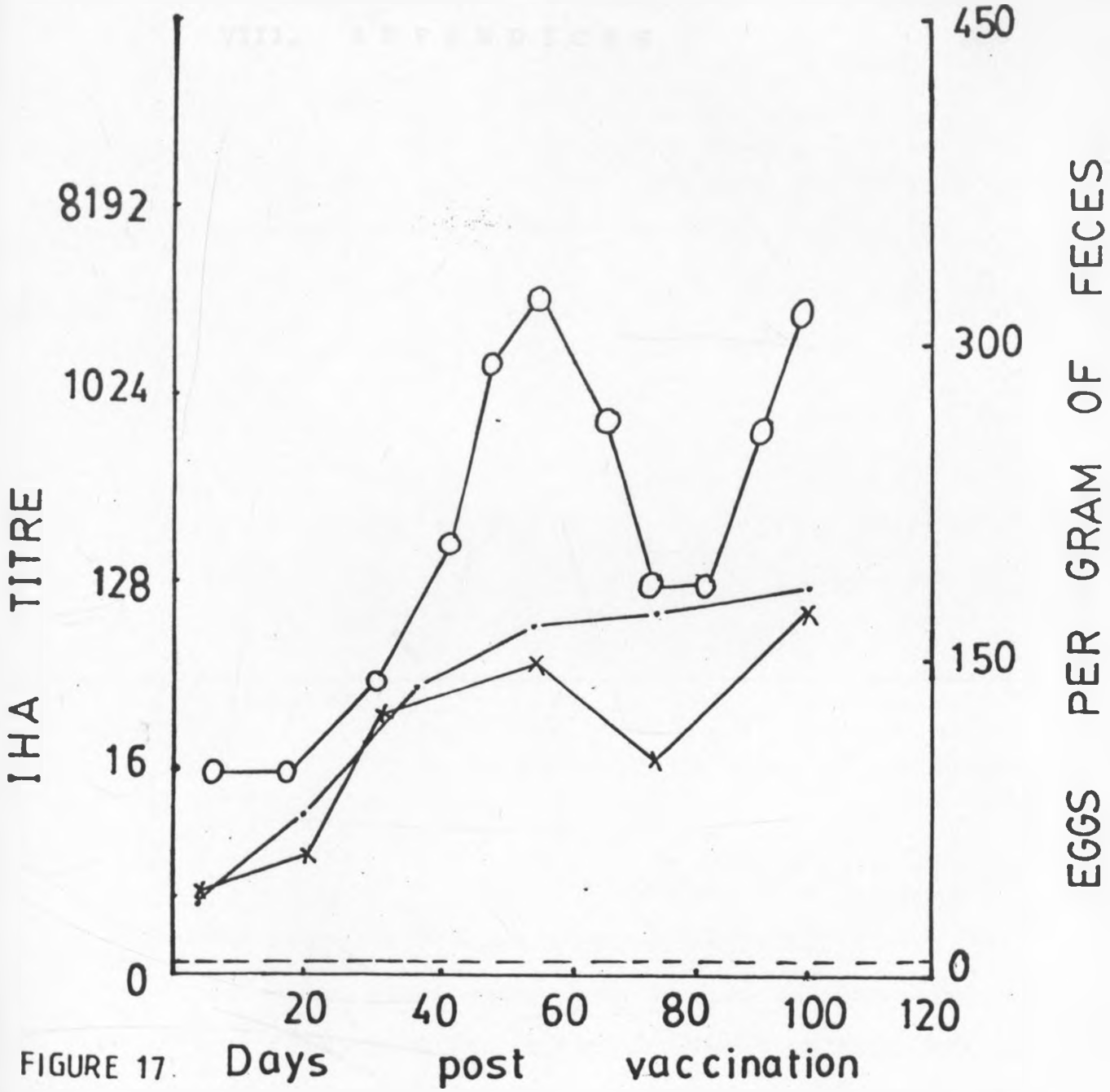

IHA TITRE
8192
1024
128
16
0
EGGS PER GRAM OF FECES
450
300
150
0
20
40
60
80
100
120
Days post vaccination

FIGURE 17.

VIII. A P P E N D I C E S

Appendix I

Lowry's (Folinciocalteu) method of determining protein content of adult hookworm extract.

Bovine serum albumin (BSA) standard solutions were prepared in phosphate buffered saline (PBS) at concentration of 10, 20, 30, 30, 50 micrograms/ml. Lowry's reagents A and B were prepared as follows:

Reagent A:

1 ml of 1% copper sulphate was added to 20 ml of 11% sodium carbonate in 0.55 N sodium hydroxide. To the above mixture was added 2 ml of 2% sodium-potassium tartarate.

Reagent B:

Was prepared by adding Folin in water at the ratio of 1: 1.2 w/v.

Into four millilitres of each BSA standard solution and triplicate samples of the hookworm extract was added 0.8 ml of reagent A, left at room temperature for 10 minutes and 0.8 ml of reagent B added to each sample mixed and left for one more hour at room temperature. Optimal densities were read at 750 nM using deionised distilled water as the blank.

Appendix II

Materials for analytical isoelectrofocusing

Ampholine polyacrylamide plate pH 3.5 to 9.5. Polyacrylamide gel concentration 5%, degree of cross-linkage 3%, ampholine concentration 2.4% w/v. Gel dimensions (mm) 245 x 110 x 1.

Electrode solutions on the strips:

Negative electrode 1M (NaOH) sodium hydroxide

Positive electrode 1M (H_3PO_4) phosphoric acid

Fixing solutions:

Trichloro acetic acid	10%
Sulphosalicylic acid	3%
Methanol	26%
Distilled water	61%

Destaining solution:

Ethanol	19%
Acetic acid	6%
Distilled water	75%

Staining solution:

Coomassie blue	0.1%
Destaining solution	99.99%

Appendix III

Preparation of solutions for preparative electro-focusing.

Dense electrode solution:	
Distilled deionised water	15g
Sucrose	10 ml
1M sodium hydroxide	6 ml
pH of solution was	11.7
Light electrode solution:	
1M phosphoric acid	1.5 ml
Distilled deionised water	8.5 ml
pH of solution was	1.2
Dense gradient solution:	
Glycerol (99%)	27 ml
Ampholines	3 ml
Sample	2.5 ml
Distilled deionised water	17.5 ml
Light gradient solution:	
Glycerol (99%)	6 ml
Ampholines	3 ml
Sample	2.5 ml
Distilled deionised water	38.5 ml
Ampholine mixture	
Isoelectric point	PI 3.5 to 10.0 : 4 ml
	PI 2.5 to 4.0 : 1 ml
	PI 9.0 to 11.0 : 1 ml

Appendix IV

Anticoagulant solution used to collect sheep blood for indirect haemagglutination test.

Citric acid (anhydrous)	4.4 g
Sodium citrate (dihydrate)	13.2 g
Dextrose (monohydrate)	14.7 g
Distilled water	1000 ml

Appendix V

Immersion media for immunofluorescent samples

Propylene glycol	6 ml
Ethylene glycol	4 ml
Glycerol (99%)	1 ml
Water (distilled)	2 ml

Appendix VI

Constituent tubes of the six Sephadex G - 200 fractions.

Fraction	Tubes
I	1 - 23
II	25 - 32
III	36 - 48
IV	51 - 63
V	69 - 79
VI	82 -100

N.B. Each tube contained 6.7 mls of the eluate.

Appendix VII: pH and absorbance (at 280 nm) of the individual IEF hookworm fractions and the pooled IEF portions (P)

PI			PII			PIII			PIV			PV			PVI			PVII		
Tube No.	pH	O.D.	Tube No.	pH	O.D.	Tube No.	pH	O.D.	Tube No.	pH	O.D.	Tube No.	pH	O.D.	Tube No.	pH	O.D.	Tube	pH	O.D.
1	11.65	0.243	21	9.14	1.107	31	8.76	1.288	51	7.55	1.282	71	6.04	1.751	86	4.78	2.079	111	3.09	0.904
2	11.25	0.670	22	9.08	1.307	32	8.71	1.258	52	7.48	1.397	72	5.94	1.842	87	4.69	2.006	112	3.02	0.867
3	11.22	0.870	23	9.06	1.251	33	8.66	1.255	53	7.36	1.383	73	5.86	1.816	88	4.60	2.021	113	2.94	0.917
4	11.28	0.730	24	9.05	1.224	34	8.61	1.203	54	7.24	1.616	74	5.78	1.827	89	4.52	1.889	114	2.86	0.838
5	11.30	1.170	25	9.01	1.265	35	8.55	1.226	55	7.18	1.648	75	5.68	1.840	90	4.44	1.606	115	2.75	0.817
6	11.30	1.100	26	8.95	1.349	36	8.50	1.250	56	7.12	1.776	76	5.60	1.920	91	4.37	1.573	116	2.60	0.833
7	11.30	1.760	27	8.89	1.160	37	8.43	1.255	57	7.10	1.749	77	5.52	1.925	92	4.32	1.364	117	2.34	0.812
8	11.30	1.788	28	8.86	1.186	38	8.37	1.264	58	7.05	1.704	78	5.44	1.981	93	4.24	1.235	118	2.14	0.741
9	11.30	1.600	29	8.83	1.211	39	8.32	1.265	59	7.00	1.653	79	5.36	2.049	94	4.19	1.169	119	1.89	0.609
10	11.06	1.537	30	8.80	1.205	40	8.26	1.269	60	6.92	1.660	80	5.31	2.226	95	4.15	1.164	120	1.77	0.440
11	10.38	1.520				41	8.20	1.258	61	6.87	1.650	81	5.24	2.224	96	4.10	1.179	121	1.66	0.436
12	10.09	1.735				42	8.16	1.289	62	6.83	1.620	82	5.15	2.282	97	4.05	1.091	122	1.63	0.433
13	10.06	1.580				43	8.12	1.277	63	6.74	1.685	83	5.05	2.306	98	3.98	1.000	123	1.66	0.307
14	9.93	1.430				44	8.07	1.291	64	6.67	1.693	84	4.96	2.303	99	3.93	1.022	124	1.73	0.181
15	9.92	1.130				45	7.97	1.326	65	6.57	1.837	85	4.88	2.113	100	3.88	1.052	125	1.83	0.085
16	9.64	1.094				46	7.90	1.388	66	6.47	1.885				101	3.82	1.107	126	2.05	0.044
17	9.40	0.903				47	7.84	1.394	67	6.37	1.891				102	3.73	1.045	127	2.33	0.030
18	9.51	1.062				48	7.76	1.476	68	6.24	2.199				103	3.67	1.057	128	2.70	0.020
19	9.37	1.064				49	7.69	1.681	69	6.14	1.935				104	3.62	1.152	129	2.87	0.009
20	9.25	1.143				50	7.62	1.370	70	6.09	1.835				105	3.54	1.019	130	2.97	0.006
															106	3.46	1.045	131	3.08	0.005
															107	3.38	1.083	132	3.17	0.008
															108	3.32	0.984	133	3.25	0.005
															109	3.25	1.088	134	3.30	0.004
															110	3.16	0.949	136	6.03	0.002

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