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Title: THE IMMUNOLOGICAL RESPONSE OF SHEEP TO IRRADIATED AND NON-IRRADIATED INFECTIOUS LARVAE OF HAEMONCHUS CONTORTUS (RUDOLPHI 1803) COBB, 1898

Abstract approved Redacted for Privacy
Dr. K. Stephen Pilcher

Ten helminth-free sheep were given two doses of 10,000, X-irradiated, H. contortus, third stage larvae 30 days apart. These animals and a group of ten non-vaccinated sheep were challenged with 50,000, normal, H. contortus, third stage larvae 60 days following the second vaccination. Antibody levels were measured in both groups by the Indirect Hemagglutination Test (IHA) with ground larval antigen. Significant levels of antibodies were not found in vaccinated animals following administration of either dose of vaccine. Following challenge, the vaccinated group contained antibodies of significant high levels as compared to the control group. The highest antibody titers were found in vaccinated animals which did not develop a protective immunity of a level high enough to resist challenge; and resistance in the individual vaccinated animals was

not related to the individual antibody levels.

Antigens extracted from H. contortus third stage larvae, ground with a Potter-Elvehjem grinder, were satisfactory for the IHA test. Tanned red blood cells did not have a limited adsorption capacity for ground larval antigen. Larvae ground in normal saline were found to yield the most sensitive ground antigens. Dextrose-Gelatin-Veronal-Buffer was found to be a satisfactory replacement in the IHA test for veronal buffer with serum. Saline extracts from metabolizing, early fourth stage, H. contortus larvae were satisfactory as antigens in the IHA test.

Antigen of extremely high sensitivity was extracted from third stage larvae in the process of exsheathment. When tested against selected positive H. contortus antisera, this exsheathment antigen was seven times more sensitive than the routine ground larval antigen.

Immunoelectrophoresis (IE), with rabbit antisheep globulin as antibody, was used successfully to determine qualitative changes in specific fractions of serum samples taken from vaccinated and non-vaccinated sheep. An unidentified fraction, seen as a precipitation arc in the slow beta or fast gamma region, appeared in all reactions of serum samples taken from sheep exposed to H. contortus larvae. Increases in $alpha_2$-II, $alpha_2$-III, $alpha_2$-IV, $beta_2$-I and $beta_2$-III globulin fractions, as evidenced by increases in densities of precipitation arcs representing these fractions in reacted serum

samples, were, in general, associated only with successfully immunized sheep. Of these fractions, $beta_2$-III globulin increases were found to be most closely associated with successfully immunized animals. Increases in $alpha_1$ globulins were seen to be limited to heavily infected, non-immune animals. No apparent changes in gamma globulins were observed in IE reactions of any serum sample. Increases and decreases in densities of the $beta_1$-I globulin fraction were directly correlated with the anemic status of the animal and the arc representing this fraction was absent or very light in reactions from animals with severe anemia.

When H. contortus antisera were subjected to electrophoresis and subsequently reacted against ground H. contortus larval antigen placed in the antigen trough, a precipitation arc, apparently indicating antibody against H. contortus, developed in the alpha globulin region. This fraction was easily washed out during processing and could not be stained with Amido Black 10B. It could not be identified.

Immuno-diffusion studies of serum samples from vaccinated and non-vaccinated sheep, when tested against ground H. contortus larval antigen, were inconclusive. Multiple precipitation arcs which developed between antigen and serum wells were, in general, associated only with serum samples taken from sheep successfully immunized against H. contortus.

The Immunological Response of Sheep to Irradiated and Non-irradiated Infectious Larvae of *Haemonchus contortus* (Rudolphi 1803) Cobb, 1898

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THE IMMUNOLOGICAL RESPONSE OF SHEEP TO IRRADIATED AND NON-IRRADIATED INFECTIOUS LARVAE OF HAEMONCHUS CONTORTUS (RUDOLPHI 1803) COBB, 1898

INTRODUCTION

In recent years, immune mechanisms between host and helminth parasite have received considerable study aimed at the control and eradication of serious parasitic diseases of man and animals. Most of the work in this area has been guided by concepts and techniques of immunology and serology indigenous to the field of microbiology.

Bacteria and viruses, after invasion of susceptible tissues, release relatively large quantities of antigen within the host during rapid multiplication. In most instances, the virulent microorganism releases this antigen directly into host tissues, thereby exerting considerable pressures on host defense mechanisms which result in rapid antibody responses.

Conversely, with the exception of Trichinella spiralis which stimulates an excellent immunological response, helminth parasites do not multiply within the host tissue and the rate of host acquisition and subsequent development of parasites is irregular.

The antigens concerned directly with the helminth immune-resistance-response are transitory and elaborated only during

certain invasive stages. Somatic antigens seem to play an insignificant role in most helminth immunities. The resultant antibody response to these antigens is directly related to the number of parasites invading the host, the development of the parasite within the host and the release of antigens by the parasite. Antigens, in the case of gastro-intestinal nematodes, must pass the mucosal barrier and be carried, in a relatively unaltered state, to antibody producing sites.

Mechanisms enabling the host to resist helminth infestations as a result of prior sensitization from a previous infestation, have prompted considerable research.

The development and successful use of irradiated vaccines to elicit an early antibody response to a number of helminth parasites, particularly ruminant lungworms, has stimulated interest in development of a similar vaccine against ruminant gastrointestinal nematodes. To date, research in this area has not been conclusive nor have vaccines been consistently successful in altering the course of such diseases. A "complete" serological and immunological study, involving the use of these vaccines, in animals, has not been reported.

The work described in this dissertation is one phase in the production of an irradiated vaccine for use against the ruminant gastrointestinal nematode, Haemonchus contortus. It is an

investigation of (1) the antibody response of sheep to the normal and irradiated parasite and (2) the changes of certain serum proteins in vaccinated and non-vaccinated sheep.

REVIEW OF LITERATURE

General Review of Nematode Immunity

Early work on helminth immunity dealt primarily with diagnostic procedures. The value of this work in disease detection cannot be overemphasized.

Silverman (93, p. 153) reports that Japanese workers in the early 1900's demonstrated experimentally-induced immunity to helminth reinfection but it was not until 1929 that Stoll (122) reported that two grazing lambs, after having lost their initial infection, were strongly resistant to reinfection with H. contortus. This demonstrated the stimulation of an immune-resistance-response. Soon after this initial report, other investigators reported that the phenomenon occurred during some infections with the following helminths: Trichinella spiralis (McCoy, 67); Strongyloides ratti (Sheldon, 90); Nippostrongylus muris (Schwartz et al., 88); Ascaris lumbricoides (Kerr, 51, 52 and Sprent and Chen, 114); Anclyostoma caninum (McCoy, 66 and Otto and Kerr, 72); Trichostrongylus calcaratus (Sarles, 83) and Dictyocaulus viviparus (Rubin and Lucker, 81).

Resistance by the immune host antagonizes the normal development of the helminth. These effects may be manifested in any of the following ways: complete refractiveness to reinfection; the

elimination of an existing infection; depression of growth, maturation and oogenesis and/or spermatogenesis (104).

Further investigations showed that with the acquisition of immunity, a humoral antibody response could be demonstrated by conventional serological techniques.

The presence of detectable antibodies does not always indicate a protective immunity in the host since little or no protective immunity can be demonstrated in animals which have received parenteral administrations of helminth materials (28, 101, 114, 117). In general, it is only when the living worm invades the animal that substantial immunity is produced (18, 28, 81, 82, 83, 85, 89, 114, 117, 118). A variable, but positive, induction of protective immunity may be demonstrated upon passive transfer of immune serum (6, 18, 61, 86). In 1955 Jarret (37) stated that the equivalent of four and one-half liters of donor anti-serum would be needed to protect a ten-week-old calf against D. viviparus. This immunity may also be transferred in utero and in colostrum milk as shown by Larsh (58) in the case of Hymenolepis fraterna infection in mice. But no protection can be demonstrated in suckling lambs on ewes immune to some nematodes even though detectable antibody is present (111). Nevertheless, some helminth antigens do function to stimulate protective immunity and Gonzales (26) has designated such antigens "functional antigens" and their homologous antibodies "functional

antibodies."

The rise in humoral antibody during development of resistance within the host was considered by Soulsby (103) in 1957 to result from either of two processes. The first is direct stimulation by a large amount of antigen reaching the general circulation; he states that such a situation could arise during a massive invasion by infective larvae. The second involves the local hypersensitiveness of tissues where an increased reactivity of an area is reflected by an increased circulating antibody titer. This concept has been likened to the Arthus phenomenon (2) by Soulsby (103). In this phenomenon, local tissue hypersensitiveness is accompanied by a circulating precipitating antibody and the degree of local hypersensitiveness is related to the amount of applied antigen. The difference in these two processes is that antibody is increased rapidly in the former and slowly in the latter. The latter produces a state of altered reactivity of local tissues which is antagonistic to the worms residing there. The rise in antibody in response to large antigen volumes does not produce such a local sensitivity as explained by Soulsby.

The preceeding review leads to the "self-cure" phenomenon which consists of a sudden loss of parasitic infection in grazing sheep. In 1953 Stewart (40) reported that this phenomenon was associated with a hypersensitive type of immunological response.

The expulsion of helminths from the intestine was first reported by Norman Stoll in 1929 (122) while working with H. contortus in grazing sheep. He noticed that after pasturing infected sheep for a few weeks, the fecal egg counts rose to high levels and then fell and the worms were eliminated. Attempts at reinfecting these sheep with new populations of worms were futile, demonstrating the sheep to be refractory to re-infection. He designated this phenomenon "self-cure and protection." It has since been studied extensively. Sarles (83) observed this phenomenon in rabbits infected with Trichostrongylus calcaratus as did Michel (68) working with T. retortaeformis in the same host. In work on H. contortus, Gordon in 1948 (29) and Stewart in 1958 (119) emphasized that resistance to reinfection was variable and that, in fact, a new infestation was developing as the old infestation was eliminated. Mayhew in 1940 (65) also demonstrated this variability in resistance while working with Bunostomum phlebotomum and Oesophogostomum radiatum in calves. Stewart reported in 1955 (121) that inoculation of infective third stage larvae or third stage exsheathment fluid (described later) will also produce this response. It has been concluded by Soulsby and Stewart in 1960 (113) that the self-cure reaction in H. contortus infected sheep is provoked by antigens liberated from worms during their third ecdysis. Antibody relationships to this phenomenon will be discussed elsewhere, but it can be generally

stated that circulating antibody titers, as detected by all immunological tests used during experimentation, show a characteristic rise either during or soon after self-cure. Evidence that this phenomenon is a hypersensitive reaction has been given by Stewart in 1953 (120) as a rise in blood histamine was seen to occur two to four days after the per orum administration of infective larvae. He also observed that this rise could be suppressed by anti-histaminic drugs and, although the antibody response still occurred, self-cure was suppressed. As will be explained elsewhere, further research has indicated that the antibodies detected by various immunological tests, with the exception of precipitin revealed by agar diffusion, probably do not represent protective antibodies and therefore do not contribute to the immune status of the animal.

Another peculiar phenomenon, the "spring-rise," was first reported by Morgan et al. (69) in 1947 and was found to occur in H. contortus infected sheep in Great Britain. This phenomenon is characterized by a marked increase in worm egg out-put associated with an increase in worm burden. Subsequent immunological work by Soulsby in 1957 (103) has shown that the termination of this phenomenon has all the appearances of a flock-inclusive self-cure reaction. He reports a general decline in antibody response prior to termination followed by a marked increase in mean nematode antibody titer of the flock at termination. The mean egg count of the flock

declines rapidly and remains low even though the pasture contamination is high (Crofton, 16). This sudden reduction in egg counts accompanied by the elevation in antibody titer is characteristic of the self-cure mechanism (117, 120).

In summary, protective immunity is definitely shown to be related to self-cure, as demonstrated by the transient period of protection following it. The degree of immunity is dependent upon antigenic stimulation by the parasite.

Artificial Immunization

General Statements

Living parasites, in their developmental stages, or antigenic materials produced by them, are essential for stimulation of a satisfactory resistance. The use of living, fully virulent larvae as immunizing agents suffers from the obvious deficiency in that propagation of the disease, against which protection is sought, would most certainly occur.

Abnormal Routes

Attempts have been reported to immunize with the fully virulent nematode via abnormal routes (101, 123). These have consisted of intra-peritoneal and sub-cutaneous inoculations of living H. contortus

larvae. Success in establishing protection was demonstrated by both routes. Other investigators failed to confirm this work (94, 109).

Irradiated Vaccines

Another approach is to use the normal route of infection but use living parasites of reduced pathogenicity or reproductive capacity. Such an attenuation of virulent infective larvae can be brought about by suitable treatment with ionizing radiation.

X-irradiated Trichinella spiralis larvae were utilized successfully to induce immunity to reinfection in rats as early as 1942 by Levin and Evans (60). Gould et al. (30) conducted similar experiments in 1955 using both X-ray and cobalt^{60} attenuated larvae. Results in both experiments were similar.

An irradiated vaccine for ruminant nematodes was reported by Jarret et al. (38) at Glasgow, Scotland in 1958. This vaccine contained X-irradiated D. viviparus larvae and it was given to calves. The larvae seemed to be so attenuated that only mild damage was done when the larvae reached the lungs. Later reports by Jarret et al. (39, 40, 42, 43, 44, 45, 46) confirmed that a satisfactory immunity was imparted by the vaccine. It was also found that: a double vaccination with small numbers of irradiated larvae produced a more solid immunity than a single dose with four times the number of larvae (40); adult development is not necessary for immunity (40);

if irradiation is too great the larvae fail to penetrate the host and no immunity results (39); and some failures do occur due to an unknown reason (39). Subsequent reports by others (15, 21, 74) prove and substantiate the value of this vaccine in the field. Calfhood vaccination for the control of lungworms in cattle is now being used successfully in the British Isles, Denmark, Holland, France and Belgium.

Other investigators using irradiated larval vaccines successfully produced immunity against Uncinaria stenocephalus in dogs (20), Trichostrongylus colubriformis in sheep (46, 70), Haemonchus placeii in cattle (79), Dictyocaulus filariae in sheep, Cysticercus bovis in cattle (36, p. 8-9) and Oesophogastomum in cattle (75).

Jarret et al. (43) in 1959 reported that under experimental conditions a good immunity could be produced against H. contortus in sheep by a single dose of irradiated larvae. In these experiments, Roentgen (r) dosages of from 10,000 to 100,000 were used on infective larvae and vaccination dosages of 10,000 larvae were used. Each group of vaccinates and controls were challenged with 8,000 normal larvae four months post-vaccination. All animals were killed and necropsied one month post-challenge. Parasitological findings at necropsy indicated that larvae subjected to 40,000r (40Kr) and 60,000r (60Kr) stimulated a good immunity against reinfection. Further work by Jarret (47) showed that two vaccinal

doses of third-stage larvae exposed to 40Kr given 35 days apart would stimulate an immunity sufficient to withstand a challenge of 50,000 normal H. contortus larvae.

Mulligan and Stewart (70) administered two doses of 60Kr-irradiated larvae to lambs at intervals of five weeks followed by challenge with 10,000 normal larvae given five weeks later. Six of the ten vaccinated lambs resisted the challenge infection and the other four developed moderate infestations. The challenge infection developed in all ten control lambs but was slight to moderate in three of them. Of six lambs given two doses of normal larvae and subsequently challenged, resistance developed in two.

The differences between the results of these two authors suggests that immunity may not be easy to produce with irradiated H. contortus larvae. It was also found that all the irradiated larvae which developed into adult worms were females. Furthermore, irradiation of this order did not interfere with the third stage exsheathment thus suggesting males did not develop beyond this stage (70). This lack of male development has also been noticed by Reik and Keith in work with Oesophagostomum radiatum (75) and Ciordia and Bizzel with T. axei (13).

Chemically Abbreviated Infection

This method of immunization depends upon the removal, with an anthelminthic, of previously established infections of worms. Campbell (8) proposed this phrase and described this approach in his study of immunity. Christie, Brambell and Charleston (12) described the elimination of a two week-old H. contortus infection from a sheep using phenothiazine. Upon challenge with a "trickle" infection of normal larvae, the vaccinated sheep were less susceptible than the controls. Later two immunizing doses of 1×10^4 normal larvae were given daily for ten consecutive days to 16 lambs. One and four days after the last immunizing dose, a therapeutic dose of Thibenzole[1] was given to terminate the pre-patent infection. These sheep were later challenged with 5.3×10^4 normal H. contortus larvae. All vaccinated animals resisted the challenge but six sheep were affected by the challenge dose. Five of the control animals died of severe anemia, ten others were clinically affected and only one sheep was without anemia.

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[1] Brand of thiabendazole, produced and marketed by Merck Chemical Division, Merck & Co., Inc., Rahway, New Jersey.

In Vitro Nematode Exsheathment

Numerous methods of in vitro exsheathment of nematode larvae have been used. This process which normally is initiated in the abomasal compartment of the stomach of the host, is the physiological shedding of the second stage outer sheath from the infective third stage larvae. This ecdysis is of considerable immunological importance.

Sommerville (98) and Rogers and Sommerville (77), in 1957, found in H. contortus that the process occurred in two stages. The first stage was a stimulus which caused the larvae to secrete "exsheathing fluid" and the second was the attacking of parts of the sheath by this fluid so that the larvae, aided by their own movements, were able to escape from the sheath. Soulsby, Sommerville and Stewart (112) found this exsheathing fluid contained an antigenic, dialyzable factor which activated larvae in vitro. They used this factor as an antigen in gel-diffusion studies. Later studies (78) on the properties of this exsheathing fluid found that it could be stored at 1^{o} C and remained active for two weeks. It was inactivated by 0.003M magnesium chloride and had the highest activity at pH 7.5.

Taylor and Whitlock (125) stimulated rapid exsheathment of H. contortus larvae with various dilute saline solutions well saturated with carbon dioxide. This exsheathment was carried out

in a water bath at 37° C.

Silverman and Podger (95) studied the carbon dioxide gas phase in balanced salt solutions and noted that after exsheathment was initiated, all larvae capable of exsheathment would undergo it and a cumulative effect was demonstrated. The best results were obtained under carbon dioxide gas with a saline solution containing one percent ammonium hydroxide at pH 6.5.

Antigen Preparation

Early work with helminth antigens centered around polysaccharides and lipids. In the late 1920's Fairley (22, 23) and Kellaway (50) prepared alcoholic extracts of helminths and found that they reacted with corresponding antisera and that they could act as functional antigens. Wharten (129) considered lipids to be only haptens and noted that the specificity of the lipids that he prepared was low. In 1943 Gonzales (25, 27) reported that helminth polysaccharides have many characteristics of bacterial polysaccharides. In 1947 Culbertson *et al.* (19) and Warren (128) studied aqueous extracts of helminths.

Jennings (48), in 1949, prepared serologically active lipid fractions of eggs, larvae and adults of *H. contortus*. He macerated the material in a ground-glass bacterial mill and a mortar and pestle. From this macerated material he prepared aqueous

suspensions and boiled antigen preparations which were lyophilized before extraction. Lipid materials were stored under nitrogen and kept cold since rancidification caused deterioration of antigenic activity. Most extractions were done with absolute alcohol. This material was filtered, distilled and brought to isotonicity for use in the complement fixation (C. F.) test. Prepared polysaccharide and protein fractions were found not to fix complement (Stewart, 116).

Excellent results were obtained with both the lipid antigen and boiled antigen and greater amounts of serologically active lipid were found in the eggs and third-stage larvae. This lipid content did not diminish in "old" larvae.

In 1950 Stewart (116) extracted egg, larvae and adult worm material from H. contortus and Trichostrongylus spp. at 100° C for ten minutes. Normal saline suspensions had the greater degree of specificity and excellent reactions were found with H. contortus antiserum in the C. F. test. The larvae were ground in one gram lots (48) and suspended in distilled water for extraction. The extracted material was centrifuged and used. The most potent antigens were in egg and larvae extracts. Ether extraction of adult H. contortus completely destroyed the activity of antigen and the prepared boiled antigen was positive to the Biuret but negative to the Molisch test confirming the lipid-protein nature of the antigen.

In 1956 Soulsby (99) investigated H. contortus antigens

prepared at low temperatures. This method was prompted by Gonzales' work with A. lumbricoides in 1943 (25). Soulsby partially dried the worms on filter paper and placed them in dry ice at -79° C as soon as possible after collection. He then alternately froze and thawed this material five times to liberate the cellular protein constituents. After the final freezing the material was ground in a saline diluent from the frozen state to a fine 20 percent emulsion. This material was extracted for two hours and centrifuged for 30 minutes at 6,000 RPM. The supernatant was stored in five ml aliquots in dry ice. Excellent results were obtained when this antigen was used in both the C. F. and indirect hemagglutination (IHA) tests. He routinely used unheated antigens prepared as described by Stewart (116).

Bird and Rogers (5) prepared H. contortus sheath extracts at a high pH. Paper electrophoresis of this material yielded poor results. A band migrating to the anode appeared but was easily washed out. Work by these authors suggested that glycoproteins, consisting mainly of fructose and acidic amino-acids, were present in the cuticle. This material was similar to collagen.

Rogers and Sommerville (78) prepared homogenates of H. contortus at a low pH. This material was centrifuged and successfully used. An antigen containing metabolic products was also prepared from hypochlorite treated larvae in distilled water

containing antibiotics. These larvae were held for 72 hours and the liquid used. Exsheathment fluid antigen was prepared with ovine rumen fluid and when stored at 1° C, activity was retained for at least two weeks. Exsheathment antigens and larvae homogenates were active against both T. axei and H. contortus sheaths. Gel-diffusion studies with H. contortus anti-serum demonstrated good antigenicity with homogenates and exsheathment antigens but no activity in metabolic antigens.

Silverman (92), in 1963, compared the solubility of disrupted H. contortus contents in water, normal saline and phosphate buffered saline. Maximum soluble nitrogen content of the extraction solution was found after 16 hours with water and saline showing advantages over buffered saline. Extractions longer than 24 hours produced non-specific results. He found no advantage in further purifying these antigens for routine study.

He also compared three different disruption methods: 1) ultrasonic disintegration, 2) the Hughes bacterial press and 3) the tissue grinder. Ultrasonic disintegration was found to be the best disruption method and the Hughes press was least effective as it left variable numbers of intact larvae. He considered manual and mechanical tissue grinders were satisfactory but suffered from the disadvantages of heat at grinding surfaces and a variable disintegration efficiency. Silverman also found that lyophilized antigens

showed little loss of activity when stored either at room temperature or refrigerator temperatures for six to nine months (96).

Baisden and Tromba (3) extracted swine kidney worm antigens after ultrasonic disruption. Disruption was carried out at 9KC/sec with five treatments of five minutes each, per sample. These samples were centrifuged at 17, 300 for 30 minutes and supernatant antigen-carrying suspensions were preserved with 0.003 percent sodium ethylmercurothiocyanate and stored at 4^{o} C for use in gel-diffusion studies.

Immunological Techniques

General

Immunological techniques provide additional tools for assessing the affects of age, diet and stress on the response of the host to parasites. They prove useful in taxonomic, phylogenetic and epidemiological studies and assist in understanding interrelationships of different parasites in the same host (92).

It is desirable, when assessing immunity in parasitized animals by serological techniques, to use tests which demonstrate functional antibody and to use functional antigens for this detection (99). Most in vitro immunological studies in host-parasite relationships have centered around this principle.

The Complement Fixation Test (CF)

The detection of circulating antibodies to nematode infestation of sheep by means of the CF test was first recorded by Stewart (115) in 1948. Sera were collected from sheep experimentally infested with either H. contortus or Trichostrongylus spp., or from naturally infested sheep in the field. Since it was not known whether circulating antibodies were present in infested sheep, antisera were also prepared in rabbits by intra-venous injections of H. contortus worm material. These sera were used for technique development.

Serum samples were heat inactivated at 56° C for 30 minutes and diluted 1/10, 1/20, 1/40, and 1/80 in 0.5 ml. amounts. The appropriate test serum, hemolytic and positive and negative serum controls were added. The antigen routinely used was heated H. contortus antigen, as later described by the same author (116), and two or four units contained in 0.5 ml were utilized with two and one-half minimal hemolytic doses of complement. The tubes were incubated at 37° C for 60 minutes with agitation every ten minutes. Hemolysin-sensitized-erythrocytes were added and incubated for 60 minutes more with shaking at ten minute intervals. Results were recorded as the degree of fixation of complement with the last complete fixation as the titer end-point.

Jarret et al. (42) reports a modified CF test similar to

Stewart's but one which was based upon a 50 percent hemolysis end-point.

Successful use of this test in demonstrating antibodies to helminths have been reported by other authors, working with normal D. viviparus (44), irradiated D. viviparus (15, 40, 45, 74), Trichostrongylus spp. (118), H. contortus (99, 117, 119) and helminth infections in general (92).

The Indirect Hemagglutination Test (IHA)

In 1951 Boyden (6) introduced the IHA test. The basic principle governing this test concerns the adsorption of antigenic proteins onto erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein serum.

Soulsby (99, 107) and Soulsby and Stewart (113) report the application of this test to the field of helminth immunity, principally in H. contortus studies. The primary objective in investigating this test was to demonstrate functional circulating antibody in higher titer than the CF test could detect and to eliminate periods of negative serological reaction as shown by the CF test (116). Soulsby's investigations showed that this test was satisfactory for demonstrating antibodies to helminth infestation. The basic technique of Boyden was followed with some modifications. Three times washed, sheep red blood cells were treated with a solution of

commercial tannic acid at a dilution of 1:20,000 in veronal buffer (VB) at pH 7.2. Tannic acid solutions were made up fresh daily. The sensitized cells were suspended in this buffer at a three percent concentration and an equal volume of a one in five dilution of prepared antigen was adsorbed onto the surface of the sensitized erythrocytes. As a routine, unheated H. contortus larval antigen, prepared as previously described, was used. After a suitable period of adsorption, the antigen coated cells were recovered by centrifugation, washed once in buffer containing 1:250 guinea pig serum and suspended in the same solution to give a three percent concentration. This buffer plus serum allowed a stable suspension of cells to be produced since it was found that coated cells in buffer alone had a tendency to auto-agglutinate. Boyden originally used rabbit serum for this purpose but Soulsby reports that most normal rabbit serum contains antibodies against helminth antigens. This serum-buffer solution was also used as a diluent for test sera. Soulsby's studies (99) indicated that iso-agglutinins do occur in sheep sera and they must be removed. As a routine, sera samples were adsorbed against packed sheep red cells for 10 minutes to remove these unwanted isophile antibodies. Dilutions of test sera in 0.5 ml quantities were made in concavities of perspex, WHO, influenza agglutination plates. Two drops of a sensitized erythrocyte suspension (approximately 0.125 ml.) from a Pasteur pipette were

added to each serum dilution and mixed with a glass rod. These plates were left at room temperature for two hours after which the degree of hemagglutination was recorded.

Kagan and Bargai (49) reported the successful use of this test in the detection of antibodies against T. spiralis in experimentally infected rabbits.

Herlich and Merkel (32) used the test in studying pure infections of T. axei in calves.

Vasington et al. (127), while applying this technique to immunological studies of infectious bronchitis infection in chickens, reports the use of Wasserman test tubes, 15 x 100 mm., for dilution of test sera. They also found that sensitized cells adsorbed with antigen at 37° C for 15 minutes gave better results than when adsorption was carried out at room temperature or 4° C for 30 minutes.

Silverman (92) stresses that careful selection of a suitable sheep cell donor is imperative and that cells should be stored at least 24 hours before use. He found that solutions of helminth antigens, which are most satisfactory for coating tanned cells contain about 1×10^4 larval equivalents per ml (LEQ/ml). For somatic antigens, this represents 7.5 micro-grams of nitrogen per ml (μgN/ml) and for exsheathing fluid and metabolic antigens, 1.25 μgN/ml. These antigen solutions were found to be effective after being used

to coat tanned cells several times.

Immuno-diffusion (Gel-diffusion) Tests

Most immuno-diffusion studies in helminth immunity have followed the general technique of Ouchterlony (73). This technique is based upon the principle that specifically reacting antigen and antibody form visible precipitates when mixed in suitable proportions. The two reactants are allowed to diffuse toward each other through a semi-solid aqueous medium and precipitation occurs at the point at which they meet and react. A variety of diffusion media have been used: agar, gelatin, cellulose acetate, pectin and paper (92). The simplest and most widely used is agar-gel.

Soulsby (100) first reported the use of this technique for helminth studies in 1957 with Ascaris spp. A later report (113) in 1960 described its use in H. contortus immunological studies. Granulated New Zealand agar was washed for two weeks in water then dried in acetone and stored as a powder. The agar was used at a 0.7 percent concentration in isotonic saline and merthiolate was added at a concentration of 1:10,000 for preservation. The melted agar was poured onto glass plates to a depth of 4 mm and after solidification small wells were cut into the agar with a metal, no. five cork borer. The agar core was removed by suction and the floor of the well was sealed with a drop of melted agar. As a routine the

distance between wells was 6 mm but for more detailed studies this distance was increased to 10 mm. Wells were replenished daily for three days. Plates were incubated in a humid atmosphere at 30° C for at least 14 days but many reactions appeared as early as four days. Various antigens and non-heat-inactivated sera were used.

Excellent precipitation bands have been observed by various authors (78, 110, 111). Silverman (92) stresses the importance of preparation of good antisera for optimal results. One modification reported by him (upon suggestion of Dr. Soulsby) was the increase of the ionic strength of the agar-gel. A 10 to 20-fold increase in salt concentration from 0.85 percent enhanced both the speed and density of precipitate formation. The most satisfactory ionic conditions found for ovine serum were 12-14 percent sodium chloride in one percent agar.

A partial assessment of the above listed techniques has been made (Section II, General Immunity). Further analysis of the literature yields other significant points.

Soulsby found, while following seasonal antibody levels in a flock of sheep undergoing the spring-rise phenomenon, that the same general trends were indicated by the CF and IHA tests but there was no constant relationship between the two (99, 113). Although these tests used the same antigens it was possible to completely absorb out the hemagglutinin and still obtain significant, although somewhat

reduced, titers in the CF test. This indicated each test was detecting a different antibody type (113). The reactions of the IHA test were difficult to assess and as was the case with the CF test the titers of the IHA test reaction do not give any indication of the ability of the animal to resist H. contortus infection (113). This is also pointed out by Poynter (74) in D. viviparus infections. He found that immunity may develop before a rise in titer and may persist strongly as the CF titer is falling.

Silverman (92) has run a critical analysis of the CF test, bentonite flocculation test, latex agglutination test and IHA test and indicated a strong preference for the IHA test as the primary quantitative serological test on the basis of specificity, sensitivity and ease of operation.

The normal serological reaction in sheep resistant to H. contortus as judged by immuno-diffusion consists of four distinct bands with reactions against exsheathing fluid being the strongest (113). In general there is a marked response at the time of self-cure in all tests, particularly in immuno-diffusions, but there is also a marked variation in response of individual animals during the infection (106).

Serum Electrophoresis and Immunoelectrophoresis (IE)

A limited number of serum electrophoretic studies of helminth

infected animals have been reported. In 1957 Shumard, Bolin and Eveleth (91) observed a depression in total serum protein and an increase in albumin-globulin ratio in young animals infected with T. colubriformis, H. contortus and N. spathiger. Leland and Drudge (59) reported the same findings but also an increase in the alpha-2 globulin in calves experimentally infected with T. axei. This was also reported in H. contortus parasitized animals by Kuttler and Marble (57) in 1960. They found a marked increase in all globulin fractions including alpha-1, alpha-2, beta- and gamma-globulins. Taffs (124) in 1962 reports increases in gamma globulin concentrations and decreases in albumin concentrations in ascarid antiserum.

Herlich and Merkel (32) related the IHA test results to serum electrophoresis studies in pure T. axei infections in calves. They found general increases of antibodies and gamma-globulin but no protective functions associated with these elevations. They also found that alpha and beta globulins fluctuated slightly, with beta globulins increasing greatly in some animals, and a slight increase in alpha globulins occurred after challenge. Bird (4) experimentally produced antiserum against the plant nematode Meloidogyne javonica by inoculating the parasite into rabbits. He showed by gel-filtration and ultracentrifugal analysis that the antibody was located primarily in the gamma globulin fraction but that an alpha-2 globulin was also involved.

Mahrt et al. (62) demonstrated the same protein changes in animals infected with Ostertagia ostertagia but found these changes to be transient.

The introduction of the IE technique by Scheidegger in 1955 (87) provided a useful additional tool for the evaluation of specific functional serum fraction changes in immunological studies.

Silverstein et al. (97) demonstrated immunoelectrophoretic patterns of adult sheep and fetal lamb sera and discussed the four globulin arcs (beta-2A, beta-2M, fast gamma and slow gamma) which they considered of immunological significance. Only in the case of the beta-2M was the arc identified with a protein having a sedimentation constant of 19s whereas both slow and fast gamma arcs were 7s proteins. Overall they demonstrated 21 arcs of precipitation: one prealbumin, one albumin, three alpha-1, six alpha-2, nine beta-1, four beta-2 globulins and gamma globulins. The beta-2 and gamma globulin arcs were similar to those of other mammalian sera. Upon stimulation of fetal lambs with a variety of antigen mixtures, globulin precipitation arcs representing antibody were found to be primarily beta-2M and fast gamma-globulins.

AAlund, Osebold and Murphy (1) have reported studies with ovine gamma globulins, which they characterized as 7s gamma-2, 7s gamma-1 and 7s gamma-1M. These fractions were shown to be counterparts of those proteins in human serum. Circumstantial

evidence also was presented that ovine serum contains a protein analogous to gamma-1A of human serum.

Chordi and Kagan (10) were able to demonstrate 23 antigenic components in normal sheep serum: one prealbumin, one albumin, two alpha-o, four alpha-1, six alpha-2, three beta-1, five beta-2 and one gamma globulin. These fractions were then related to human fractions. A later report (11) of experimentation with sheep hydatid fluid, using the fluid as antigen, showed that IE bands could not be demonstrated in immune sera with hemagglutination titers of less than 1:3,000.

Hirshfeld (33, 34, 35) was able to resolve and identify 30 precipitation arcs in human sera using the LKB[2] apparatus. This method is described in detail in Section III.

Immunological Cross Reactions

Cross reactions between various antigens and antisera utilized in the serological tests described above have been studied. Jennings (48) prepared saline suspensions of serologically-active lipid fractions of various helminths and animal tissues and tested these antigens against sheep anti-H. contortus serum with the CF test.

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[2] LKB-Productor AB, P. O. Box 12220, Stockholm 12, Sweden

Positive reactions were obtained against: H. contortus adults, larvae, and eggs; Nematodirus spp., adults; Trichostrongylus spp., larvae; Oesphogostomum spp., adults; Monezia expanza, adults; A. lumbricoides, adults; Chabertia ovina, adults; D. filaria, adults, and Strongyloides spp., larvae. Negative reactions occurred against antigens of: Fasciola hepatica; Paramphistomum cervi; beef heart; beef liver; sheep brain; sheep intestinal mucosa; and hen's egg yolk. He suggested that it is possible that there are essential lipids involved in the basic metabolic structure of the helminths concerned.

Reactions of boiled antigens and alcoholic extracts of other helminths were tested against H. contortus natural antisera with the CF test by Stewart (116). He found the same positive cross reactions as Jennings but found antigens of C. ovina and F. hepatica to be anti-complementary and that from Paramphistomum was weakly positive.

E. J. L. Soulsby (44) in 1956, working with the IHA test found marked cross reactivity with antigens of H. contortus, Cooperia certicei, Ostertagia spp, Trichostrongylus spp. C. ovina, Cooperia onchophora and Bunostomum spp.

Rogers and Sommerville (78) in 1960 found homogenates of H. contortus to be highly active against sheaths of T. axei but the reverse reaction was not true.

Silverman (92) while using antigens enhanced by Freund's adjuvent, which consists of mycobacteria emulsified in mineral oil,

to produce experimental antisera against nematodes, found that the resultant antisera produced in this manner gave certain non-specific precipitations in gel-diffusion plates. He traced these reactions to the mycobacteria which contained antigen(s) in common with some of the nematodes.

Although these cross reactions did occur, Soulsby (111), in a discussion of nematode immunity, explains that functional immunity appears to be specific, at least at the generic level, but it is not known whether this phenomenon extends to the species level within the various genera. He further states that since functional antigens are thought to be specific, they essentially are not somatic antigens which, as has been shown, produce only a low level of protective immunity and show a wide range of cross reactions. If the protective property existed in the somatic antigens, an immunity toward one parasite would extend toward another parasite which shared common antigens. In general, this does not occur.

MATERIALS AND METHODS

Division of Labor

The following parts of this research project were my responsibility and all work involving these parts was done by me.

1. Larvae harvest and storage.
2. Antigen preparation.
3. Serological and immunological tests.
4. Animal bleeding and harvest of serum samples.

The experiment as outlined in Table 1 was designed and carried out by Dr. S. E. Knapp (54). Data included in this dissertation concerning microhematocrits, body weights, fecal egg counts and intestinal worm counts at necropsy were supplied by him.

Experimental Design and Immunization (54, 55)

Preparation of Immunizing Larvae

Ten day old third-stage H. contortus larvae were exposed to 40,000 roentgens of X-irradiation. These larvae were from the same culture source i.e. donor animal, day of collection and larval recovery. The larvae, covered with distilled water, were irradiated in a plastic petri dish. The irradiation was carried out at room temperature using a General Electric Maximar unit. Irradiation

Table 1. Schematic of experiment no. I, 1962-1963 to test the immune response of X-irradiated Haemonchus contortus larvae.*

Experimental Group	Number of Lambs	Procedures 11-1-62 (Day 1)	12-1-62 (Day 30)	2-5-63 (Day 94)	3-28-63 (Day 148)
I	10	10, 000 X-irradiated larvae	10, 000 X-irradiated larvae	50, 000 normal larvae @ 10, 000 per day.	Kill and examine
II	3	----	10, 000 normal larvae (culture viability control)	Kill and examine	----
III	5	30, 000 normal larvae	10, 000 normal larvae	Kill and examine	----
		Control on Presence of Adult Haemonchus contortus at date of challenge			
IV	3	10, 000 normal larvae (culture viability control)	Kill and examine	----	----
V	10	----	----	50, 000 normal larvae @ 10, 000 larvae per day	Kill and examine

*Compiled from: Knapp. S. E. 1964. Immune responses by domestic ruminants to irradiated gastro-intestinal nematodes. Termination report to United States Atomic Energy Commission. contract AT(45-1) 13-76.

conditions were: 250 KVP (Kilovolt-peak), 11.4 ma, 3 mm of aluminum inherent filtration and a target to sample distance of 38.5 centimeters. This resulted in a first half-value layer of approximately 0.32 mm of copper. Under these conditions, the exposure rate was 200 roentgens per minute measured in air with a Victoreen 250R chamber.

Procedure (Table 1)

Vaccine Group I contained ten lambs and each lamb was given per orum as a drench, 10,000 X-irradiated larvae on day one (week 0) and on day 30 (week 4). Ninety-four days (13 1/2 weeks) after administering the first dose of irradiated larvae, each of these animals received 10,000 normal larvae per day for five consecutive days. These animals were killed and examined on day 148 of the experiment (week 21).

Control Groups II and IV each contained three lambs and were given 10,000 normal larvae as culture viability controls; group two lambs were given larvae on day 30 and group four lambs were given larvae on day one. These animals were killed and examined 64 and 30 days post-inoculation respectively.

Lambs in Control Group III were given 30,000 normal larvae on day one and 10,000 normal larvae on day 30. This group was a control on the presence of adult H. contortus at the date of challenge.

Surviving animals were killed and examined on day 94 (week 13).

Group V contained ten lambs and was used as a challenge control group. Each animal in this group was given 50,000 normal larvae at 10,000 per day for five consecutive days starting at day 94 (week 13). The surviving animals were killed and examined on day 148 (week 21).

All lambs used in this experiment were raised by Dr. S. E. Knapp at the Department of Veterinary Medicine, Oregon State University. These lambs were separated from their dams at birth and raised under isolated conditions, indoors, in stalls with concrete floors which were covered with wood chips. The lambs were seven months of age at the time of initiation of the experiment and no nematode larvae were observed in the feces of these animals prior to this date.

The lambs were grouped by weights with each group having a similar weight average.

Experimental and Control Sera

Five ml of whole blood were obtained with a needle and hypodermic syringe from the jugular vein of each animal at the beginning of each week during the course of the experiment starting at day one (week 0). No blood was collected during week one. These samples were immediately expelled into heavily siliconed 15 ml glass

centrifuge tubes, slanted and allowed to clot. The clots were loosened from the side of the tube with a wooden applicator stick and incubated at 37° C for two hours after which they were refrigerated at 4° for two hours. Each sample was then centrifuged at 1500 RPM for 15 minutes and the serum drawn off by pipette. The serum samples were placed in two dram screw-cap vials and held frozen at -70° C until used. A large blood sample was obtained from each animal at the completion of the experiment and the serum was harvested by centrifugation in 250 ml centrifuge bottles and frozen. This serum was used for test standardization and experimentation during development of the immunological techniques. Serum samples from week five were lost.

In addition to the serum samples from the animals in this experiment, other H. contortus negative samples were obtained from isolated helminth-free sheep from the same source. A serum sample with low antibody titer was obtained in quantity from an H. contortus donor lamb which had undergone self-cure and had subsequently been challenged with 50,000 normal larvae. The serum from this lamb was extracted from blood taken ten days post-challenge.

Larvae Culture

Donor Animals

All animals used as H. contortus donor lambs were five to eight months of age and had been raised helminth-free in isolation as described above. As an added precaution and to insure against contamination of the pure culture of H. contortus used in this experiment, each donor animal was given a therapeutic dose of Phenothiazine or Thibenzole as an antihelminthic eight days prior to inoculation. Despite this precaution it was found that the culture was contaminated with Nematodirus fillicollis. At various times during passage, this contamination ranged from two to eight percent of the resultant larval culture as determined by actual count.

H. contortus Culture

The culture of H. contortus used in this experiment was originally obtained from Dr. Irwin B. Wood, Lederle Division, American Cyanamid Company, (now of Princeton, N. J.) in September, 1959. Dr. Wood obtained his culture in 1958 from Dr. J. H. Drudge, Kentucky Agriculture Experiment Station, Lexington, Kentucky. OSU cultures were passed to susceptible lambs every four months. Prior to inoculation of each donor lamb, a fecal sample was checked for possible nematode eggs. Only

helminth-free lambs were used. As a routine, 10,000 viable normal third-stage H. contortus larvae, 10 to 30 days of age, were given to the donor lamb. These larvae were diluted in ten ml of distilled water and given either by dose syringe or divided and given in two, no. ten, gelatin capsules. The donor lambs were placed in an isolation unit measuring five feet by five feet. This pen had an expanded-metal steel floor and a pull-out hardware cloth screen placed one foot below the flooring. The holes in the metal flooring were large enough to allow the fecal pellets to drop through.

On the 19th day post-inoculation a fecal sample was obtained from the animal and an H. contortus egg count was made using the McMasters' technique with a saturated solution of sodium nitrate as the flotation medium. The counts were made and recorded daily for the duration of donation. When the egg count rose above 2000 per gram of feces (EPG), all fecal pellets were collected for culture each day. Collection continued until the egg count decreased to below 1000 and another lamb was inoculated to continue the culture.

Harvest and Storage

All fecal pellets harvested on a given day were transported in new paper sacks since it was found that polyethylene bags inhibited hatching and development of the nematode eggs. The feces collected were either held overnight at 15° C or prepared for culture

immediately.

The pellets were screened over a 1/4 inch galvanized metal screen to allow debris and disintegrated pellets to pass through. The retained pellets were pooled and placed in plastic, one pint, polyethylene cottage cheese cartons to a depth of about two inches. It was found later in the experiment that if the incubating pellets were sprayed with about five ml of water each day a higher yield of viable larvae could be obtained. This daily application of water also helped to control mold formation by maintaining humidity within the cup at a high percentage. This method was adopted and followed daily as a routine procedure. A solution consisting of Mycostatin[3] prepared in distilled water at a concentration of 200 units per ml was used experimentally in the incubating cultures to control mold growth. This technique proved moderately successful but was not used extensively due to the possible adulteration of the larval culture and unknown detrimental effects that it might have.

The culture cups were then capped with a plastic cap which contained 12 to 20, one mm holes to allow an exchange of air during incubation. The cups were marked with date of collection and culture identification and placed in a 26° C incubator with forced air

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[3]Brand of Nystatin produced by E. R. Squibb & Sons, Division of Olin Matheson, 745 Fifth Avenue, New York, New York. 10022

circulation. A pan of water was placed at the bottom of the incubator to insure high humidity.

The cultures were allowed to incubate for a minimum of nine days. At this time the eggs had hatched and the larvae had developed into third stage infective larvae. The cultures were then removed and each cup was placed in a large modified Baerman apparatus. The apparatus consisted of: a large 8 inch diameter glass funnel with a four inch piece of rubber tubing attached at the bottom. A 28 x 57 mm screw-cap capsule vial was inserted into the tubing. This apparatus was filled one-half full with lukewarm tap water and four thicknesses of cheese cloth were placed over the funnel. The contents of each culture cup were placed in individual funnels and the ends of the cheese cloth folded over the feces. The cups and lids were rinsed thoroughly and this water was added to the funnels since it was found that most of the larvae were adhering to the cup walls. The collection by sedimentation was usually complete and all larvae had gravitated to the glass vial by four hours time at room temperature. The vials were then removed, capped and refrigerated for use.

The larvae were then washed onto a one cm column of sterile sand which was contained in a conical fifteen ml glass centrifuge tube with the bottom nipped out. The sand was held in this tube by a small pledget of glass wool at the tube bottom. Distilled water was

allowed to drip onto the sand column at a rate of 8-10 drops per minute for 18 hours. At this time most of the viable larvae had gravitated or moved through the sand column and into a collecting vessel under the tubes. For large scale sand filtration the bottoms were removed from number ten tin-cans and heavy cheese cloth secured over the lower end. This was then placed on a screen over a pan of water with the bottom of the can just touching the water. The layer of sterile sand was placed on the cheese cloth and the contents of 10-15 vials were poured onto the sand. The sand and larvae were sprayed with water at intervals to keep them moist. After 18 hours, most of the viable larvae had migrated into the pan. This treatment effected the removal of most non-viable larvae and extraneous particulate fecal debris. The larvae were then concentrated, pooled, counted, checked for viability, washed, checked for other helminth contamination and stored. Five-hundred-thousand larvae were suspended in ten ml of distilled water in a 28 x 57 mm screw-capped capsule vial and these vials were stored at 4^{o} C in an upright position until used.

Antibiotic sterilizing solutions, consisting of (1) penicillin, 500 units per ml (2) dihydro-streptomycin sulfate, 0.5 milligrams per ml and (3) Mycostatin, 200 units per ml, were used without success in attempts to eliminate microflora from the cultures.

Serological and Immunological Tests

The Passive Hemagglutination Test

Experiment I

Weekly serum samples from all experimental animals were subjected to the passive hemagglutination test. In general, the technique followed the procedure of Boyden (6) as modified by Soulsby (99, 107). Pertinent details of the test are described.

Red Blood Cell Source and Preparation

Sheep red blood cells were used in all experiments. The animal used as cell donor was a cross-bred ewe raised in isolation and was one year of age at the time of first bleeding. Fecal samples were obtained periodically from this animal and at no time were nematode eggs of any species found in these samples; the animal, therefore, was considered H. contortus-free.

Blood was taken aseptically directly into an equal volume of sterile modified Alsevers (79) solution, pH 6.1. After mixing, the cells were refrigerated at least 18 hours before use. Cells remained stable for six weeks in this solution but at no time were cells used which were older than four weeks. Red blood cells were prepared fresh each day. They were washed three times in veronal buffer (VB)

at pH 7.2, centrifuged at 1700 RPM for 15 minutes and then suspended at a concentration of 3% in VB.

Buffers and Diluents

1. Saline consisted of 0.85% heat-sterilized sodium chloride solution. It was used routinely as a diluent for "crude" antigen and larval suspensions.

2. Veronal buffer (76) was made according to the following formula:

sodium chloride	85.00	gm
5,5 diethyl barbituric acid	5.75	gm
sodium 5,5 diethyl barbiturate	3.75	gm
magnesium sulfate (heptahydrate)	2.028	mg
calcium chloride (dihydrate)	39.2	mg
distilled water	2000	ml

This diluent was made up as a sterile 5 x stock and fresh buffer was prepared from it each day by dilution of four parts sterile, glass-distilled water plus one part stock buffer. This solution was routinely used as a diluent for blood cell suspensions and tannic acid solutions. As a diluent for serum and a washing agent for antigen-coated tanned cells, a 1 in 250 dilution of guinea pig serum in VB was used. This was necessary since the antigen caused some degree of agglutination of tanned red cells and dilute guinea pig serum

exerted a "protective" effect and allowed a stable suspension of cells.

3. Commercial reagent grade tannic acid[4] was used at a dilution of 1 in 20,000 in VB at pH 7.2. This solution was made up fresh each day.

Antigen and Antigen-coating of Cells

A given volume of 3% cells was mixed with an equal volume of 1:20,000 tannic acid solution in VB and incubated, with intermittent shaking, at 37° C for 15 minutes. The treated cells were recovered by centrifugation at 1,700 RPM and resuspended in VB at a 3% concentration.

Immediately before use, the larvae were washed six times, with agitation, in sterile saline and recovered by centrifugation at 500 RPM for six minutes. The larvae were again counted and checked for viability before each experiment. Antigen was prepared from ground third stage larvae at a concentration of 5×10^4 larvae per ml of sterile saline. The larvae were ground with a Potter-Elvejhem glass grinder with a plastic-tipped, stainless-steel pestle. The grinder was kept submersed in an ice bath throughout the grinding operation. The larvae were ground for 20 minutes at

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[4] J. T. Baker and Co., Phillipsburg, New Jersey.

3300 RPM. The ground suspension was then refrigerated at 4° C for 18 hours, during which time it was remixed every four hours so that all sedimented material was washed for maximum antigen extraction. This suspension was centrifuged in a refrigerated centrifuge set at 6000 RPM for 30 minutes. The button was discarded and the supernatant was used for antigen.

This larval antigen containing 5×10^4 LEQ/ml was diluted one in five in sterile saline and then adsorbed onto an equal volume of tanned cells for a final antigen concentration of 1×10^4 LEQ/ml. The suspension was incubated at 37° C for 20 minutes and cells recovered by centrifugation. The cells were washed once with serumized VB and suspended at 3% in the same diluent.

Serum Preparation and Dilution

All test sera samples were heat-inactivated at 56° C for 30 minutes. These samples were diluted one to ten with a 20% suspension of washed red blood cells in VB and incubated for 15 minutes at 37° C with mixing at five minute intervals, then the serum was recovered by centrifugation. Further doubling dilutions of serum in 0.5 ml quantities through 1:1280 were made in 13 x 100 mm glass test tubes. Controls were included in each test. Two drops of sensitized erythrocytes (0.125 mls) from a plastic, drop-calibrated pipette were added to each dilution and mixed. The tubes were left

at room temperature for two hours, after which the degree of hemagglutination was recorded. Reactions were graded 1^+, 2^+, 3^+, or 4^+ with 4^+ reactions denoting complete agglutination. In final tabulations, 3^+ and 4^+ reactions were considered positive and 1^+ and 2^+ reactions negative.

The Geometric Mean Titer (GMT)

The IHA titers of each experimental group were compared statistically by the Geometric Mean Titer (GMT) according to the method of Markham (63). This is the method of choice when analyzing antibody titers because it is less distorted by atypical high or low titers and it gives a specific value rather than a range such as that represented by the mean. This method of determining the GMT is based upon the sum of the titers of the serum samples expressed as the powers of two. The powers of two in a dilution series are illustrated below:

0	2	4	8	16	32	64
2^0	2^1	2^2	2^3	2^4	2^5	2^6

By multiplying the number of samples at each titer by the power of 2 represented by the titer, the totals are determined. These totals are added and divided by the number of samples and converted to GMT.

To conform to this method, the doubling dilutions used in this experiment i.e., 20, 40, 80, et cetera, were uniformly reduced by a factor of ten, the mean number of powers of two determined and converted to GMT. This figure was returned to the original series by multiplication by ten.

In the development of the IHA technique, experiments were needed to elicit a sensitive test.

Experiment 1a

Saline suspensions of 10^5 larvae/ml were ground for 10, 20 and 30 minute intervals to determine minimum grinding time needed for maximum worm disruption. Samples of the ground suspensions were then counted.

Experiment 1b

Ground larval suspensions were prepared at (1) 1×10^4 LEQ/ml, (2) 2.5×10^4 LEQ/ml, (3) 5×10^4 LEQ/ml and (4) 1×10^5 LEQ/ml and these were adsorbed onto tanned cells. This experiment was done to determine the optimum concentration of ground larval extract that could be used to coat tanned cells for IHA use.

Experiment 1c

Larvae at concentrations of 5×10^4 were ground for 20 minutes

in diluents of (1) distilled water, (2) isotonic saline, (3) VB and (4) Mayers buffer (76) to determine the best method of extracting a stable antigen. These suspensions were extracted and tested against selected H. contortus positive and negative serum samples. Mayers buffer was made as a 5 x solution according to the following formula:

sodium chloride	83.8	gm
sodium bicarbonate	2.52	gm
sodium 5, 5-diethyl barbituric acid	3.09	gm
5, 5-diethyl barbituric acid	4.6	gm
magnesium sulfate (heptahydrate)	1.0	gm
calcium chloride (dihydrate)	0.2	gm
distilled water	2000	ml

This buffer was diluted one in five with distilled water immediately before use.

Experiment 1d

Larvae ground in saline at 5×10^4 larvae/ml were extracted at 4° C for periods of (1) zero hours, (2) four hours, (3) twelve hours, (4) eighteen hours and (5) twenty-four hours to determine the minimum extraction period needed to yield a satisfactory antigen. Antigen coated cells were prepared and tested against selected serum samples as described.

Experiment 1e

Dextrose-gelatin-veronal buffer (DGVB) (76) was tested as a replacement for serumized VB for both antigen-coated cell washing and suspension and serum diluent. DGVB was made according to the following formula:

5,5-diethyl barbituric acid	0.589	gm
gelatin	0.60	gm
sodium 5,5-diethyl barbituric acid	0.38	gm
calcium chloride (dihydrate)	0.02	gm
magnesium sulfate (heptahydrate)	0.12	gm
sodium chloride	8.50	gm
dextrose	10	gm
distilled water	1000	ml

Antigen-coated cells, prepared as described in Experiment 1, were washed and suspended in this buffer and tested against selected positive and negative serum samples.

A series of experiments were done to increase the sensitivity of the IHA test as described in Experiment 1. The following modifications were made and IHA tests were run using selected positive and negative serum samples.

Experiment 1f

Exsheathment fluid-coated cells were tested for antigenic potency. The method of exsheathment was patterned after the method of Taylor and Whitlock (125). This consisted of placing saline suspensions of washed third stage larvae in a 125 ml Erlenmeyer flask and agitating the flask in a 37° C water bath for four hours. During the first 30 minutes of incubation, carbon dioxide gas was bubbled into the larval suspension. The gas bubbling apparatus was removed after 30 minutes and the flask sealed with aluminum foil and allowed to agitate for the remaining three and one-half hours at which time exsheathment was considered complete. The exsheathment fluid-containing portion of the suspension was harvested by pipette from samples centrifuged at 6000 RPM in a refrigerated centrifuge. The supernatant fluid from this material was either (1) frozen at -70° C in sealed ampules, (2) lyophilized or (3) stored at 4° C until used.

Prior to test, exsheathment fluid samples were diluted to contain (1) 1×10^4 LEQ/ml, (2) 2.5×10^4 LEQ/ml, (3) 5×10^4 LEQ/ml and (4) 1.36×10^5 LEQ/ml and adsorbed to tanned cells. These cells were prepared as antigens as described in Experiment 1.

Experiment 1g

One exsheathment fluid antigen and two metabolic antigens were prepared and tested.

Metabolic antigen A was prepared from 5×10^5 early fourth stage larvae which had been incubated in 6 ml of sterile saline for 30 days at 4° C. Antigen B was prepared from 5×10^5 larvae which had been incubated in 6 ml of sterile saline for 24 hours at 4° C. The antigens were prepared as centrifuged supernatants of these suspensions and adsorbed to tanned cells in the usual manner.

The exsheathment fluid antigen (antigen D) was prepared by the following technique which was devised to capture possible labile antigens excreted by the exsheathing larvae.

Tanned cells were prepared in the usual manner and suspended at 15%. A 20 ml saline suspension of washed larvae at 5×10^4 larvae/ml was placed in a 50 ml centrifuge tube which was suspended, partially submerged, in a 37° C water bath equipped with an electrical stirrer. A motor-driven small glass microstirrer, in the form of a propeller at the tip of a glass rod, was used to keep the larvae suspension in constant agitation. This stirrer was rotated at about 500 RPM. Carbon dioxide gas was bubbled into the suspension through a Pasteur pipette. After 30 minutes the gas apparatus was removed and 20 ml of 15% tannic acid treated

treated sheep red blood cells were poured into the agitated larval suspension and the reaction allowed to proceed for three and one-half hours.

The suspension containing early fourth stage larvae, spent sheaths, non-exsheathed larvae and red blood cells was then poured onto a saline-washed #613 Whatman filter-paper pad in a Buchner funnel. This funnel was fitted into a 125 ml filtering flask and a very low vacuum was applied at the side arm. The cells were collected in the flask after about five minutes. Fifty ml of sterile saline was then washed through the filter to complete cell harvest. The collected cells were then washed in the usual manner and tested as a three percent suspension. This antigen contained 1×10^4 LEQ/ml.

Control antigen (C) was prepared as described in Experiment 1.

Immunoelectrophoresis (IE)

Experiment 2

This experiment involved separation, on the same slide, of pre-exposure and experimental serum from the individual animals. Commercial rabbit anti-sheep globulin[5] was reacted against the

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[5] Nutritional Biochemicals Corporation, 21010 Miles Avenue, Cleveland, Ohio.

separated fractions to determine specific serum fraction changes in the animals during the experiment. Studies were concentrated on serum samples taken at the dates of highest IHA titer and experiment termination. These slides were analyzed macroscopically and microscopically. The individual fractions were identified as completely as possible by position and interband relationships. Terminologies for fraction identification by other authors (1, 10, 97) for normal sheep serum, with corresponding fractions in human serum as presented by Chordi and Kagen (10), are given in Table 2. Fraction designations as given by the latter authors are used to analyze the reactions obtained in this study. The same lot of anti-sheep serum was used for all IE experiments.

All IE studies were carried out using an LKB 6800A Standard Immunoelectrophoresis Set. The immunoelectrophoresis was carried out in two main stages. The first stage comprised an electrophoretic separation of antigen or serum and the second comprised an immunological reaction occurring when the electrophoretically separated samples and added antibodies, through diffusion, met each other and formed visible precipitation arcs. IE was performed by means of an electrophoresis apparatus with two polystyrene buffer chambers in which were inserted the electrically charged electrodes. Into frames divided in two sections were placed six, one by three inch, glass slides. The frames were

Table 2. Terminology and identification of serum fractions.*

Precipitinarc in sheep sera A[1]	B[2]	C[3]	Corresponding arc in human serum[4]
ρ	ND	ND	ρ_1
Albumin	ND	ND	Albumin
α_0 - I	ND	ND	ρ_2, Lipoprotein
α_0 - II	ND	ND	Seromucoid
α_1 - I	ND	ND	α_1 - A, Glycoprotein
α_1 - II	ND	ND	α_1 - B
α_1 -III	ND	ND	α_1 - C
α_1 -IV	ND	ND	α_1 - D, Macroglobulin
α_2 - I	ND	ND	Hapto globulin
α_2 - II	ND	ND	α_2 - A
α_2 - III	ND	ND	Ceruloplasmin
α_2 - IV	ND	ND	α_2 - B
α_2 - V	ND	ND	α_2, Macroglobulin
α_2 - VI	ND	ND	α_2, Lipoprotein
β_1 - I	ND	ND	Siderophilin
β_1 - II	ND	ND	Hemopexin
β_1 - III	ND	ND	β_1 - A
β_2 - I	β-2A	ND	β_2 - A
β_2 - II	ND	ND	β_2 - B
β_2 - III	β-2M(19S)	ND	β_2 - Macroglobulin
β_2 - IV	ND	ND	β_2 - C
β_2 - V	ND	ND	β_2 - X
γ	ND	ND	γ
ND	7s fast γ	7s gamma 1	ND
ND	7s slow γ	7s gamma 2	ND

ND = No designation

*Compiled from:

1, 4, Chordi, A. and I. G. Kagan. Identification and characterization of antigenic components of sheep hydatid fluid by immunoelectrophoresis. Journal of Parasitology 51: 63-71. 1965.

2, Silvertein, A. M., et al. Fetal response to antigenic stimulus. III. γ-globulin production in normal and stimulated fetal lambs. Journal of Immunology 91(3): 384-395. 1963.

3, Aalund, O., J. W. Osebold, and F. A. Murphy. Isolations and characterizations of ovine gamma globulins. Archives of Biochemistry and Biophysics 109: 142-149. 1965.

fastened to a horizontally-set leveling table and a layer of agar-gel was poured over the slides. Into each frame section was poured ten ml of one percent buffered agar solution at a temperature of between 75° C and 85° C. A uniform layer about one mm thick formed on the slides. After five minutes the agar solution gelled and the frames were transferred to a holder, which was inserted into a high-humidity chamber where it remained for 30 minutes. A pattern of long, narrow troughs and wells was then punched in the gel layer with a punch which could be moved along the frame. The construction of the punch made it possible to choose between two different dies which would allow variation in both diffusion distance and width and position of the trough in relation to the wells.

After punching, the gel was sucked out of the wells with a suction needle. Into the wells were put one to two microliters of the antigen sample by means of a micropipette. The frames were then placed in the electrophoresis apparatus. Electrical contact between the gel layer and the electrode in the vessel was achieved by means of rayon wicks saturated with buffer solution. One end of the wick was laid upon the gel layer and the other end was allowed to hang down into the electrolyte. A D. C. voltage was applied between the electrodes and the electrophoretic separation was allowed to continue for one hour, during which time some of the antigens or serum components were separated. After the completion of the run the gel

was removed from the troughs with a gel knife. Anti-sheep globulin was added to the troughs and the frames with the glass slides were placed in an incubation chamber at room temperature for about 20 hours. In the course of this process the electrophoretically separated antigens and antibodies (in the anti-sheep globulin) diffused towards each other. When the antigens met their specific antibodies, opalescent arcs of precipitate were formed. The gel layer was then dried onto the slide and stained after excess unprecipitated protein was washed out with a one percent sodium chloride solution.

Experimental runs, in general, utilized this system with the following reagents: (1) buffer: Michealis type, pH 8.6, ionic strength 0.1 (0.05-M sodium diethyl-barbiturate, 0.01-M diethyl-barbituric acid and 0.05-M sodium acetate in glass-triple distilled water); (2) buffered agar-gel: agar-buffer-water in proportions of 1:25:75 and 1:10,000 merthiolate; (3) impregnation agar: 0.1% agar and 0.05% glycerine in distilled water; (4) rinsing solution methyl alcohol, glacial acetic acid and water in the proportions 45:10:45; (5) staining solution: 0.60% Amido Black 10B dissolved in rinsing the solution.

All glass slides were first cleaned in dichromate-sulfuric acid and stored in ethyl alcohol. These were removed, dried and dipped in hot impregnation agar solution before placing them in the frames.

This step improved the adhesion of the gel to the slide.

Wells were cut 3.6 mm from a center trough 2 mm in width.

Rayon wicks were soaked in electrolyte for 15 minutes before use to insure a good electrical circuit.

Electrophoretic runs were carried out for one hour at 250 volts and 8-12 ma. This voltage was equivalent to 6 volts per cm of wick-gel distance. Antiserum reaction was allowed to progress for 24 hours.

Processing of slides followed the pattern given in Figure 1.

During the agar drying period wetted "lintless" blotter paper was applied to the gel layer to hold the gel in place.

After processing and cleaning, a thin layer of microscope immersion oil was applied to the agar surface. This aided greatly in elimination of scratches on the dried agar surface and allowed for easier cleaning in case of dust or dirt accumulations.

Experiment 2a

Fresh exsheathment fluid from 1×10^5 larvae/ml of saline was subjected to IE by two methods. The first method involved electrophoresis of the fluid and then reaction against positive and negative serum samples. The second method reversed the process and serum was subjected to electrophoresis in an attempt to identify the serum fractions containing anti-H. contortus antibody. The

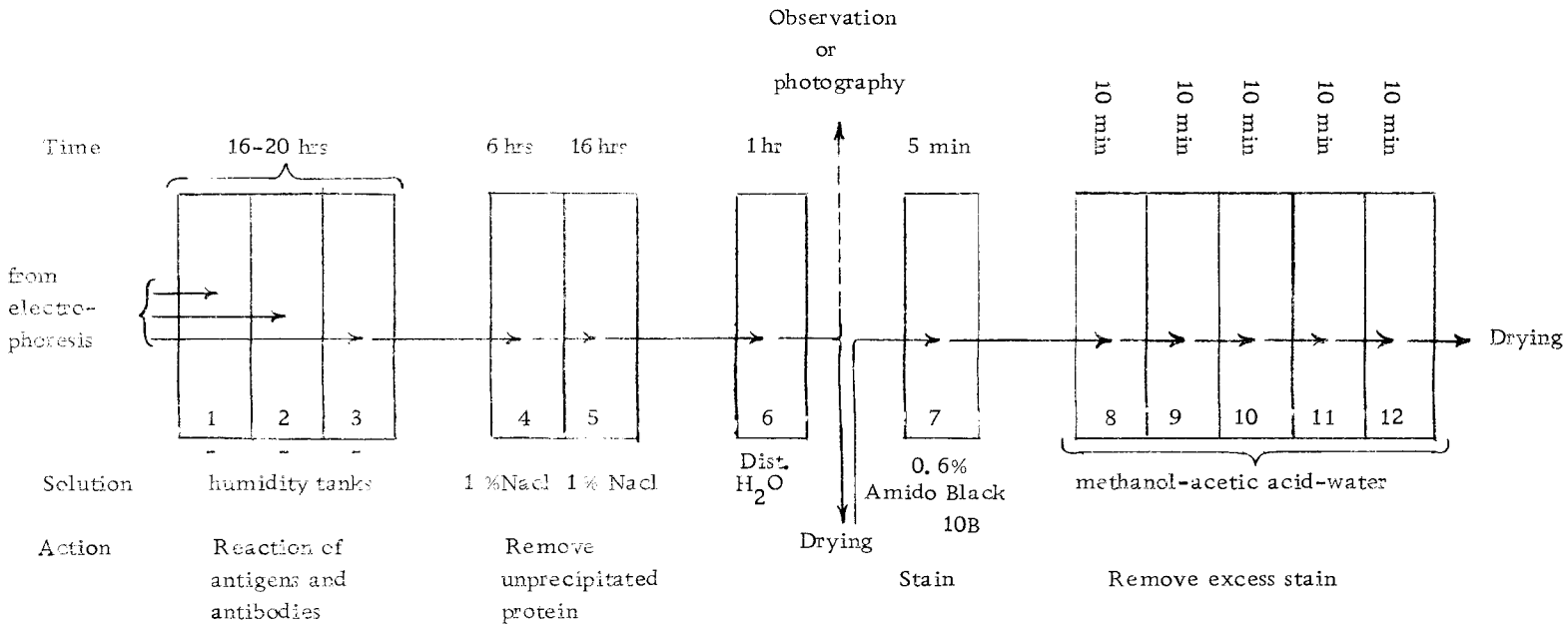

Figure 1. Arrangement of tanks for processing of immunoelectrophoresis slides.

antigen troughs were recharged every day for 3 consecutive days.

Experiment 2b

In this experiment, ground larval antigen at 1 x 10^5 LEQ/ml, prepared as described in Experiment 1, was subjected to analysis in the same manner as in Experiment 2a.

Immuno-diffusion

Immuno-diffusion studies were attempted using a gel formula consisting of one percent Noble Agar[6], 12 percent sodium chloride and 1:10,000 merthiolate as a preservative. The gel solution was heated and poured to a depth of four mm onto 2.5 x 10.5 inch glass plates with taped edges.

Using a steel template and a number five cork borer, wells were cut four mm apart using a longitudinal pattern for precipitin arc intersection and comparison. Vacuum was used to remove agar from the wells. The capacity of each well was 0.3 ml.

Experiment 3

Fresh exsheathment fluid, at a concentration of 1 x 10^5 LEQ/ml, was used as antigen and was placed in the center longitudinal wells.

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[6] Difco Laboratories, Inc., Detroit, Michigan.

Test sera from final harvest samples of group I animals were placed in the outside wells. The wells were refilled every 24 hours for three days and the plates were incubated at room temperature for 14 days in a high humidity chamber.

Pre-exposure sera was not analyzed in this experiment.

Experiment 3a

Ground larval antigens containing (1) 1×10^4 LEQ/ml, (2) 5×10^4 LEQ/ml and (3) 1×10^5 LEQ/ml were tested against the same serum samples as used in Experiment 3. Antigen at 1×10^5 LEQ/ml was also tested against pre-exposure serum from group I animals. Final observations were made 16 days after the last filling of the reaction wells.

RESULTS AND DISCUSSION

Experiment 1--Indirect Hemagglutination Test (IHA) of Serum Samples From All Sheep on Experiment II (Knapp) (Table I)

The IHA test was successful in detecting antibodies in this experiment. A tabulation of antibody titers of each animal at weekly intervals is presented in Table 3. Graphs of antibody titers for individual animals in groups I and V are shown in Figs. 2 through 6. Microhematocrits are also included in each graph as an index of anemia since H. contortus is a hematotropozoic parasite.

The test, as routinely used, did not detect high antibody titers in H. contortus infected sheep. Much higher titers than those found in this experiment have been reported by other workers (99, 107, 113). The experiments described by them did not use X-irradiated larvae for the sensitizing antigen. In general they used much higher sensitizing doses of normal larvae and data presented were based upon results obtained from tests involving animals infected periodically.

The highest IHA titer detected with ground larval antigen in this experiment was 1:320. Soulsby and Stewart (113) describe titers as high as 1:5,000 in their experiments. Knapp's method of infection and the experimental procedure are different from theirs and a direct comparison of titer data between my experiments

Table 3. Experiment 1--The antibody titers of serum samples from vaccinated and control animals. Samples tested against ground H. contortus larval antigen.

Experimental Group	Animal Number	Week 0	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	47	0[a]	10	20	10	10	0	0	0	0	20	20	20	0	10	20	40	40	(80)[g]	40	40
	50	0	0	160	10	0	0	0	0	80	0	40	80	10	10	(80)	80	80	40	10	80
	54	10	0	0	0	10	10	0	0	0	40	20	20	10	0	(80)	40	20	10	0	10
I[b]	61	0	0	10	0	10	10	0	10	0	20	20	20	10	0	20	20	20	10	(40)	40
Double vaccinate group	62	0	0	0	0	0	0	0	0	0	20	10	10	0	0	40	40	20	20	40	(80)
with irradiated larvae	67	0	10	40	0	0	0	0	10	40	40	80	160	10	0	(40)	40	40	40	40	40
	68	0	0	0	0	0	0	20	10	0	20	20	80	0	0	0	(40)	0	20	10	10
	70	320	10	20	20	10	10	40	10	40	80	20	20	10	10	40	20	40	160	80	(320)
	72	10	0	20	0	10	0	0	10	10	40	40	40	10	10	20	(80)	40	10	10	20
	79	0	40	20	0	0	0	0	0	0	20	10	20	10	10	0	0	40	160	10	(320)
II[c]	41	0	0	10	0	10	0	10	0	10	20	20	10	--							
Second vaccinate	74	40	40	20	0	0	0	10	0	0	10	20	10	--							
control group	256	0	0	0	10	10	0	0	0	0	20	10	10	--							
	16	10	0	10	0	0	10	20	80	4	40	20	40	--							
III[d]	39	0	0	20	0	10	0	0	0	--[h]	--	--	--	--							
Double vaccinate group	48	0	0	80	0	20	10	40	0	160	20	20	40	--							
with normal larvae	66	10	20	20	0	10	80	40	10	40	40	40	10	--							
	98	40	10	40	10	--	--	--	--	--	--	--	--	--							
IV[e]	38	10	0	0	--	--	--	--	--	--	--	--	--	--							
First vaccinate	97	0	20	20	--	--	--	--	--	--	--	--	--	--							
control group	100	20	40	40	--	--	--	--	--	--	--	--	--	--							
	34	0	0	20	0	10	10	40	20	20	20	20	80	0	10	0	0	0	10	10	(20)
	35	0	10	0	0	0	0	0	0	10	10	10	20	0	0	0	0	0	0	(20)	20
V[f]	46	10	40	20	10	0	10	0	0	20	0	10	40	10	10	20	20	40	(80)	20	80
Challenge control	51	0	0	0	0	10	10	0	0	0	20	20	20	0	(20)	20	10	20	--	--	--
group	55	40	0	40	0	0	0	0	0	0	20	80	20	10	20	40	20	10	0	10	(160)
	65	0	0	0	0	0	0	0	0	0	40	10	10	10	20	20	20	0	10	(40)	20
	69	80	160	80	80	80	160	160	40	40	80	80	80	20	40	80	40	(160)	160	160	80
	80	0	0	40	10	0	10	0	0	0	40	20	20	10	10	(40)	40	0	0	20	10
	98	0	0	0	0	0	0	0	0	0	20	10	10	0	0	(20)	20	0	10	10	20
	255	0	0	0	0	0	0	0	0	0	40	10	0	0	0	(10)	10	0	--	--	--

[a] Titers are presented as reciprocal of dilution

[b] Group I received 1×10^4 X-irradiated H. contortus third stage larvae on day 1 and day 30 and received 1×10^4 normal larvae on days 94, 95, 96, 97 and 98

[c] Group II received 1×10^4 normal larvae on day 30

[d] Group III received 3×10^4 normal larvae on day 1 and 1×10^4 normal larvae on day 30

[e] Group IV received 1×10^4 normal larvae on day 1

[f] Group V received 1×10^4 normal larvae on days 94, 95, 96, 97 and 98

[g] Circled entries denote highest titer for that animal following challenge

[h] Dead

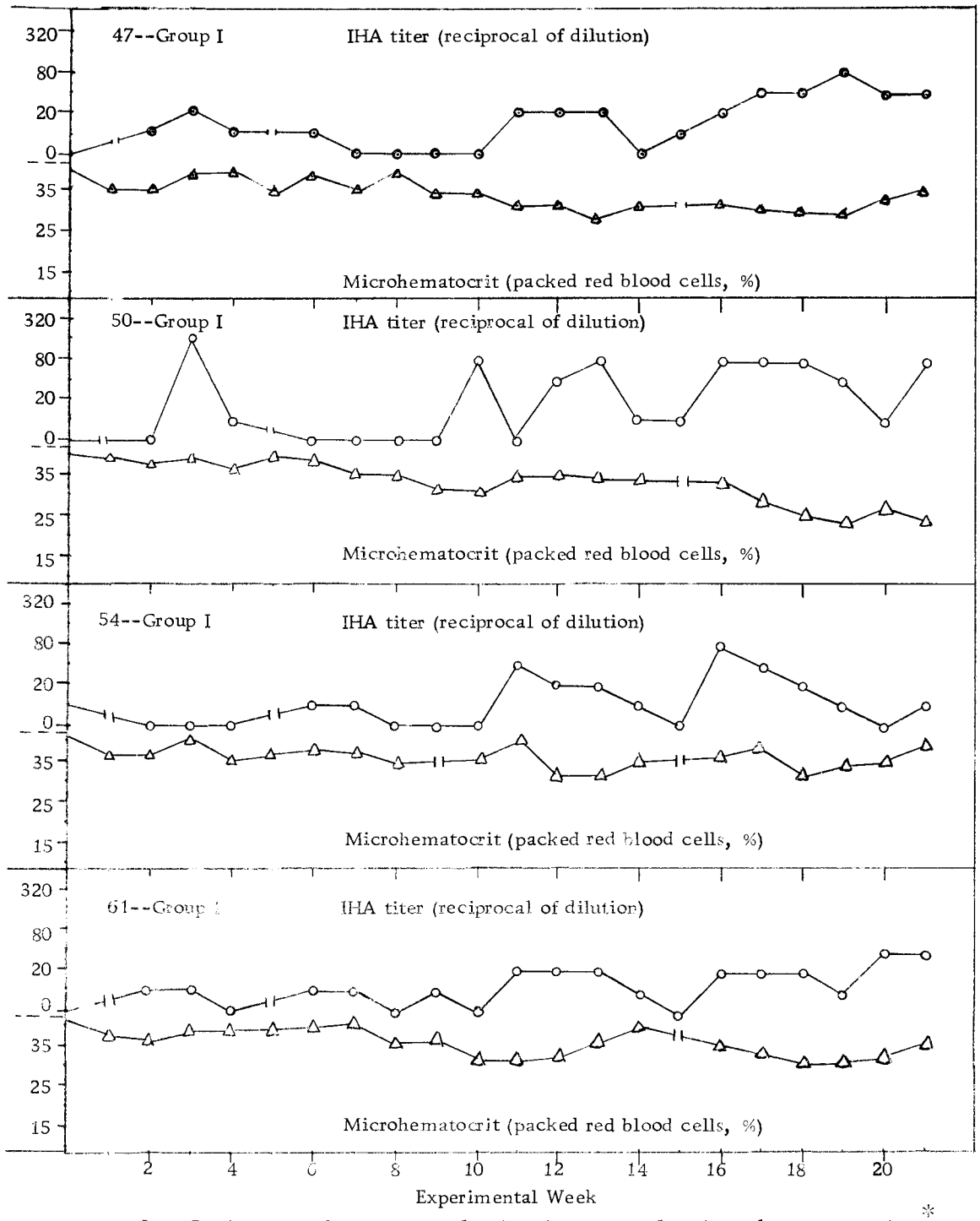


Figure 2. IHA circulating antibody titers and microhematocrits* of sheep no. 47, 50, 54 and 61. All sheep vaccinated with 10,000 X-irradiated infectious H. contortus larvae in weeks 1 and 4 and challenged in week 14 with 50,000 normal infective larvae.

*Data compiled from Knapp (54)

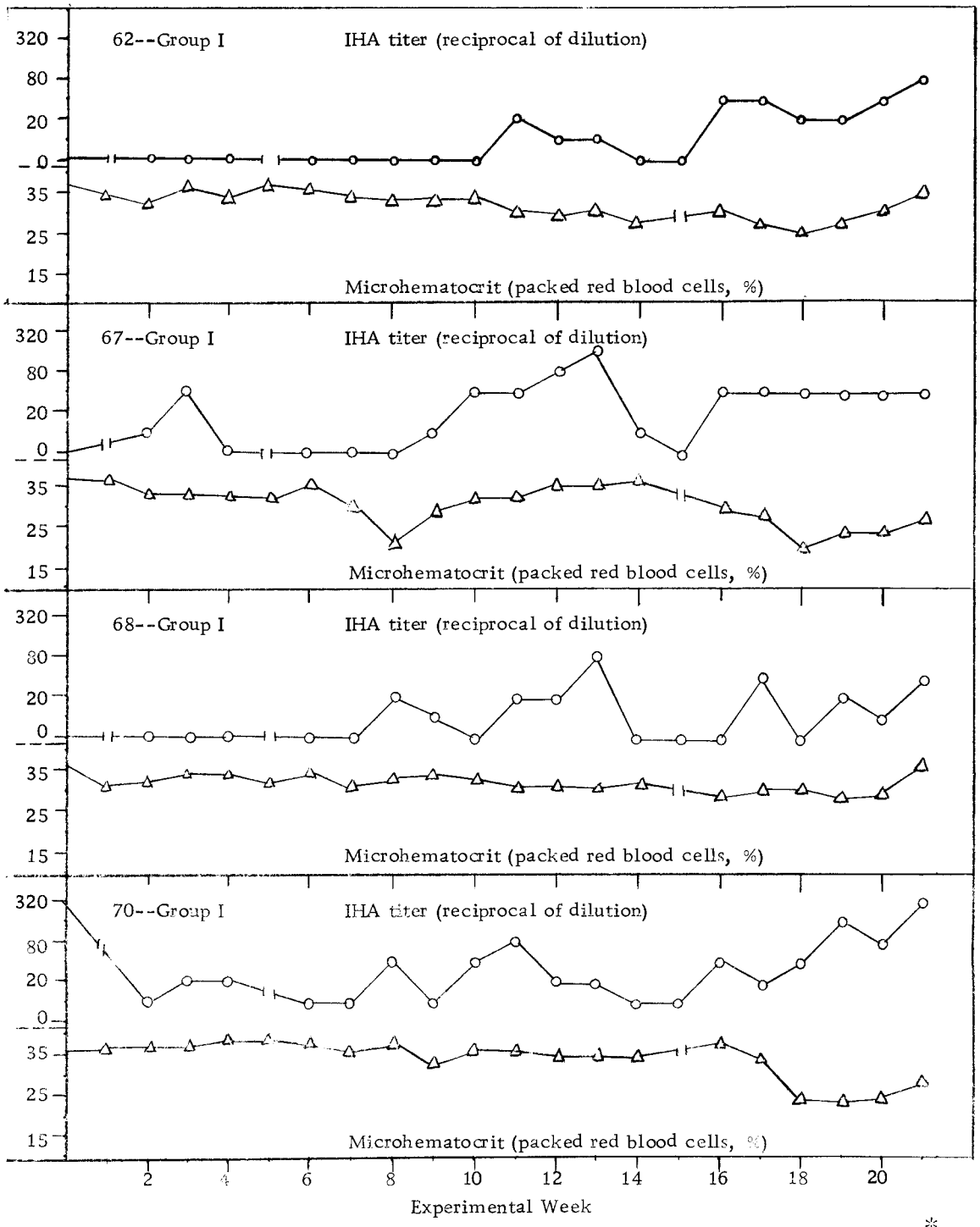


Figure 3. IHA circulating antibody titers and microhematocrits* of sheep no. 62, 67, 68 and 70. All sheep vaccinated with 10,000 X-irradiated infectious H. contortus larvae in weeks 1 and 4 and challenged in week 14 with 50,000 normal infective larvae.

*Data compiled from Knapp (54)

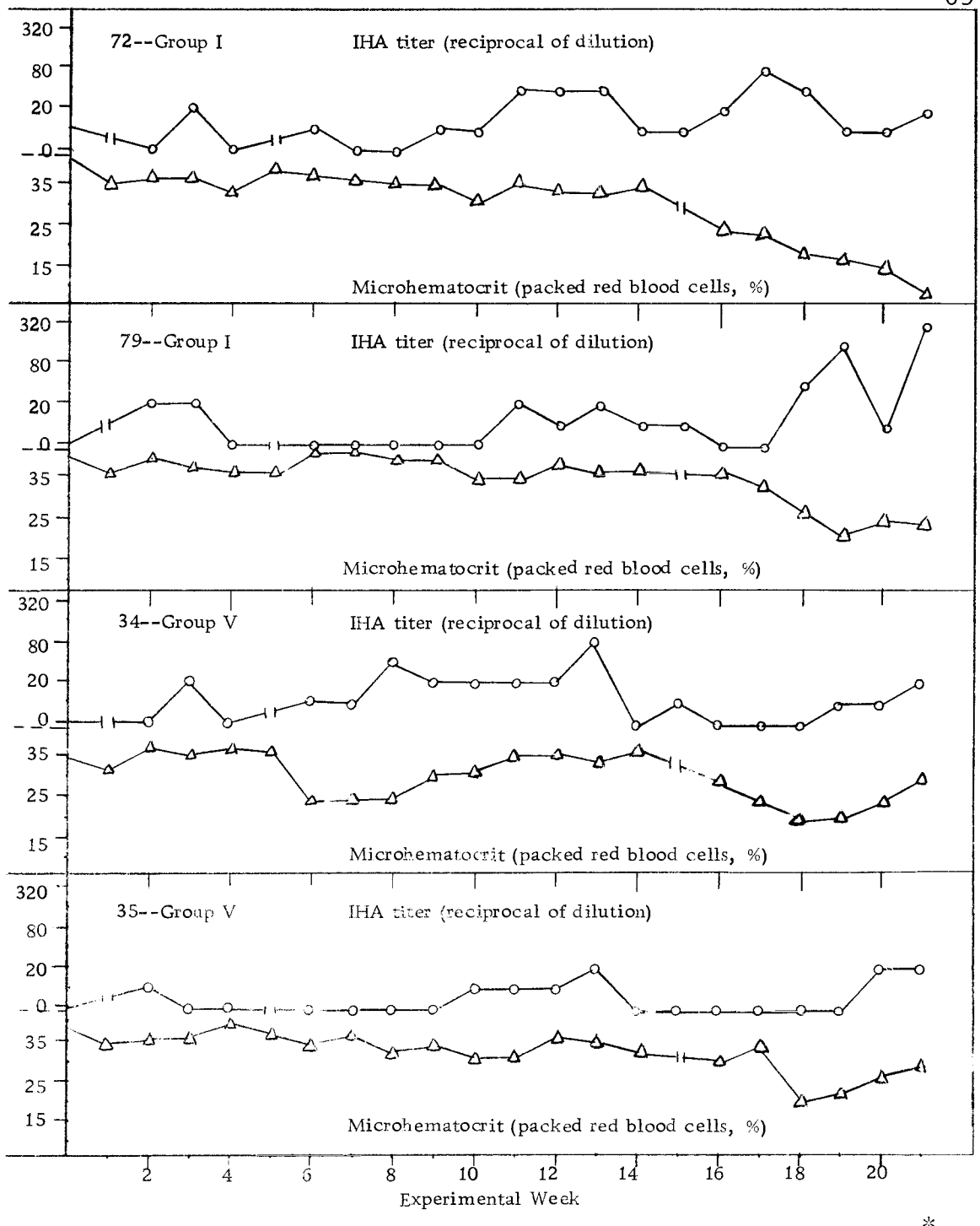


Figure 4. IHA circulating antibody titers and microhematocrits* of sheep no. 72, 79, 34 and 35. No. 72 and 79 vaccinated with 10,000 X-irradiated infectious larvae during weeks 1 and 4 and challenged in week 14 with 50,000 normal infectious larvae. No. 34 and 35 challenged in week 14 with 10,000 normal infective larvae.

*Data compiled from Knapp (54).

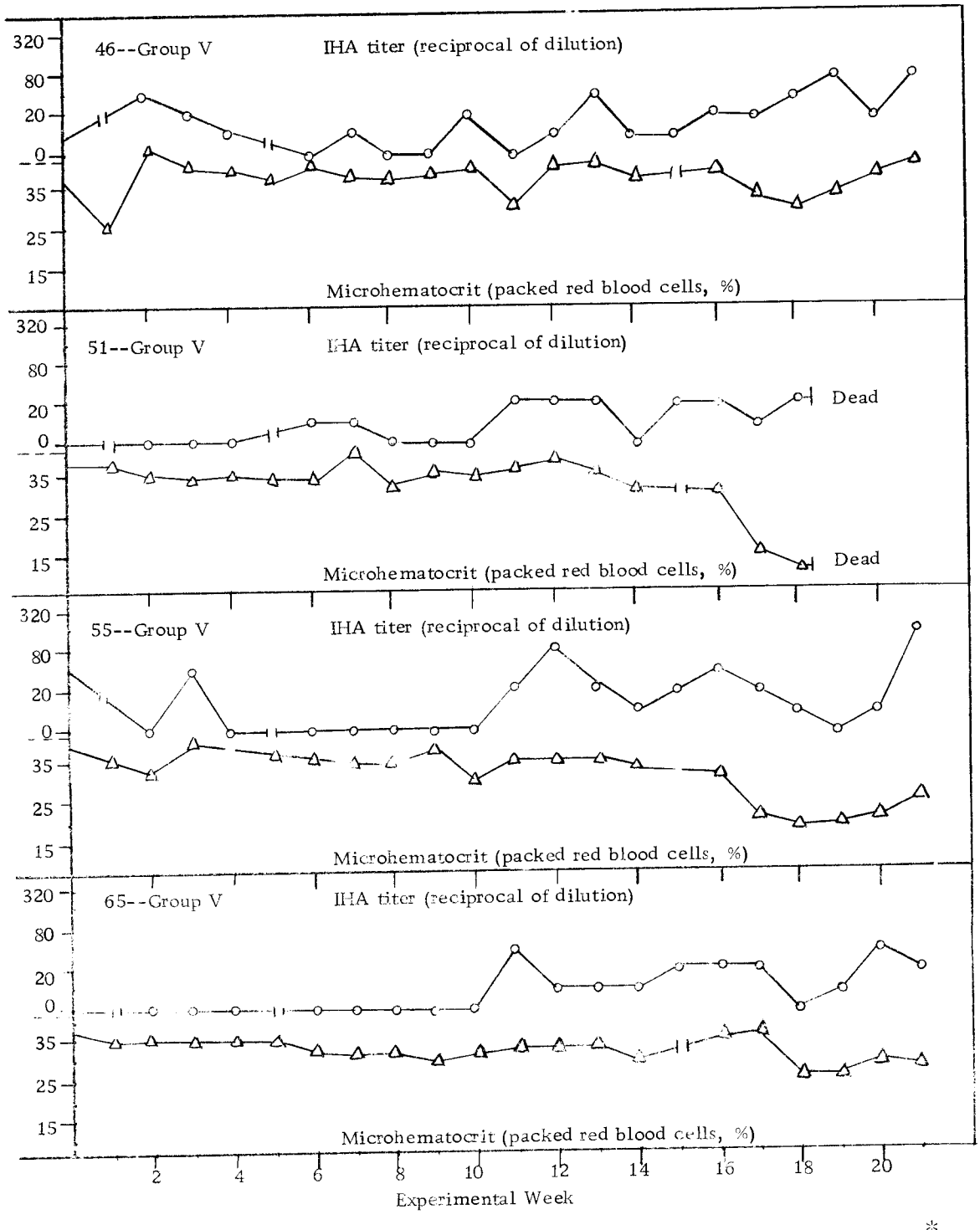


Figure 5. IHA circulating antibody titers and microhematocrits* of sheep no. 46, 51, 55 and 65. All sheep challenged with 50 000 normal H. contortus infective larvae in week 14.

*Data compiled from Knapp (54)

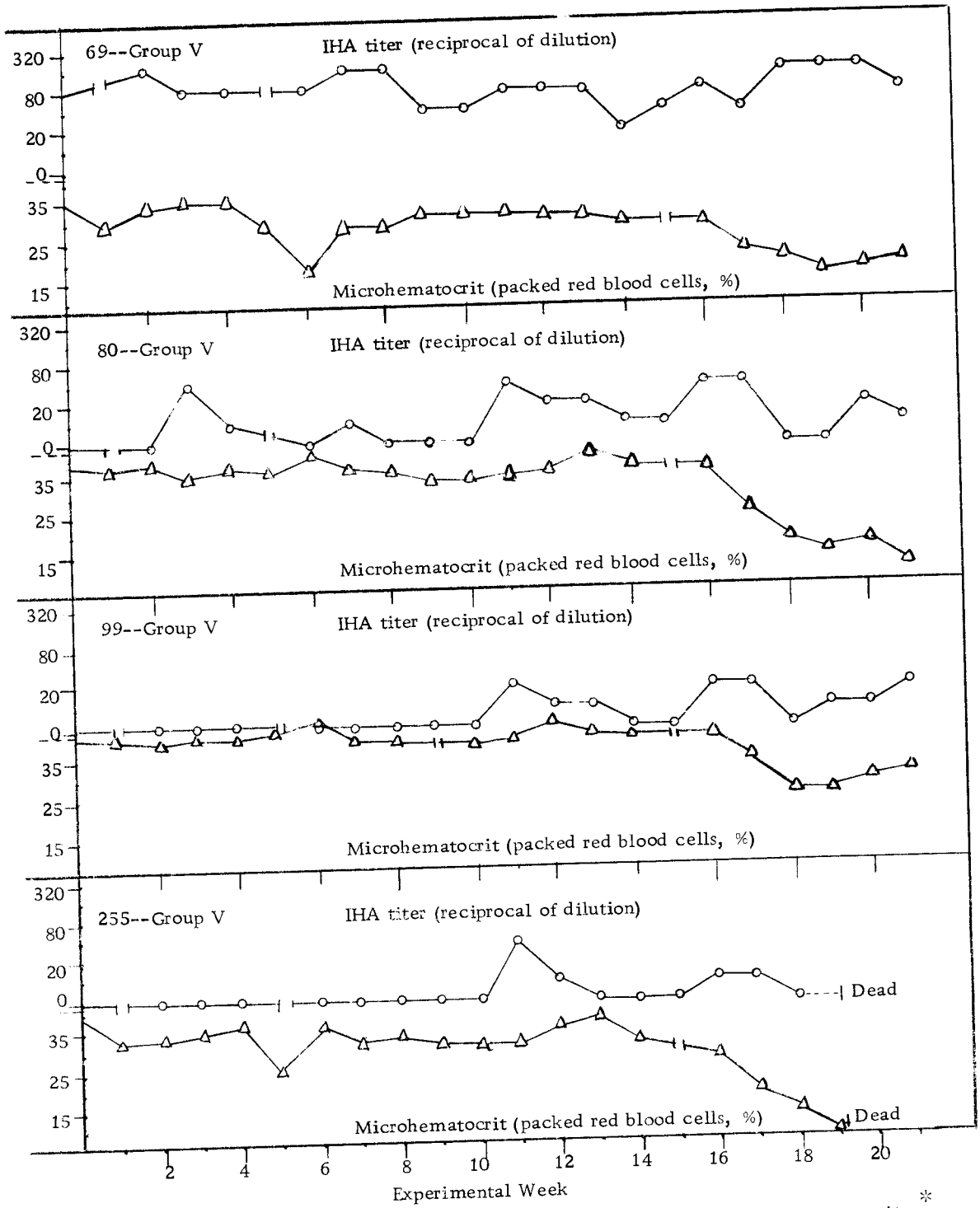


Figure 6. IHA circulating antibody titers and microhematocrits* of sheep no. 69, 80, 99 and 255. All sheep challenged with 50,000 normal H. contortus infective larvae in week 14.

*Data compiled from Knapp (54)

and those of Soulsby and Stewart cannot be made.

Group Geometric Mean Titer (GMT) data are presented in Table 4 and a graph of these results from groups I and V is presented for comparison in Figure 7. Both groups had high GMT values at weeks 3 and 13. At week 13 GMT values for these groups were 33.0 and 26.0 respectively. GMT values after challenge, were highest at week 21. These values were 57.0 and 20.0 for groups I and V respectively. The data for group V are from eight animals since two animals died from infection during week 18.

Average group microhematocrits for groups I and V are included in Figure 7. Values of both groups prior to challenge were lowest at week 10 and highest at week 12. Microhematocrit values of group I and V after challenge were lowest at weeks 18 and 19 respectively. The lowest value, 21.4, was found in group V at week 18.

Graphs of net weight gains and average fecal egg counts (EPG) are presented in Figures 8 and 9.

Data from control groups II, III, and IV are not represented since those animals were used for larvae viability checks and a control involving double vaccination with normal larvae.

No H. contortus eggs were found in feces of group I animals until the fourth week when the egg count was 1×10^3 EPG. This group count remained near this value until week 17 when a count of

Table 4. Experiment 1--Geometric mean antibody titers (GMT) of IHA tested serum samples from vaccinated and control animals. Samples tested against ground H. contortus larval antigen.

Group	Experimental Week																			
	0	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1[b]	14.0	11.5	20.0	10.0	10.0	10.0	12.3	10.0	26.0	23.0	23.0	35.0	10.0	10.0	28.0	37.0	30.0	33.0	21.0	57.0
2[c]	15.0	15.0	12.3	10.0	10.0	10.0	10.0	10.0	15.0	15.0	15.0	15.0	15.0	--	--	--	--	--	--	--
3[d]	13.0	11.5	26.0	10.0	11.9 -1[a]	16.5 -1	24.0 -1	16.5 -1	63.6 -2	20.0 -2	25.3 -2	25.3 -2	--	--	--	--	--	--	--	--
4[e]	12.5	20.0	20.0	12.5	--[g]	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
5[f]	14.0	15.0	19.0	12.3	12.3	13.0	15.0	12.3	14.0	25.0	15.0	26.0	10.7	14.0	20.0	17.0	16.0	16.0	19.0	26.0

[a] Denotes number of animals dead at date indicated

[b] Group 1 received 1 x 10^4 X-irradiated H. contortus third stage larvae on day 1 and day 30 and received 1 x 10^4 normal larvae on days 94, 95, 96, 97 and 98

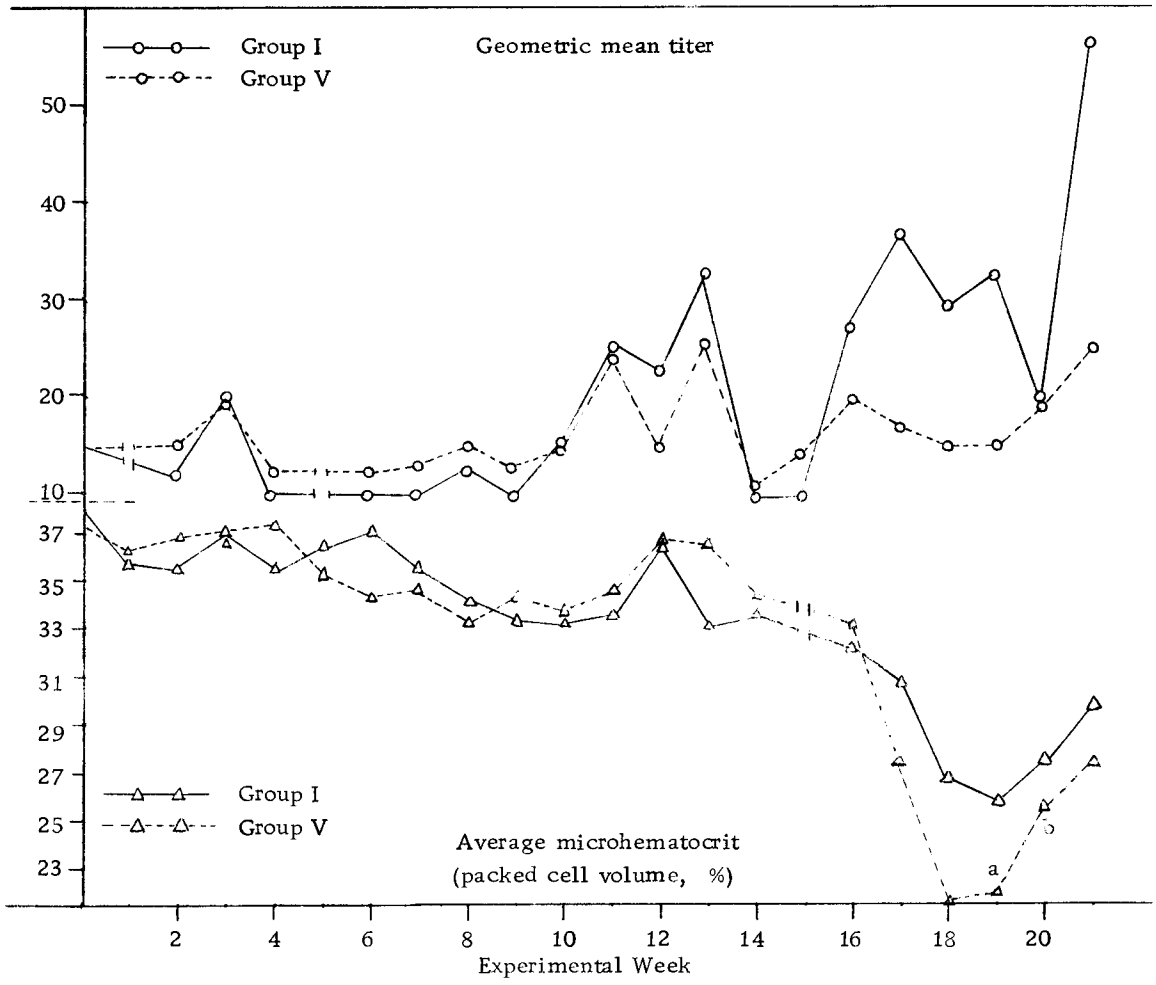
[c] Group 2 received 1 x 10^4 normal larvae on day 30

[d] Group 3 received 3 x 10^4 normal larvae on day 1 and 1 x 10^4 normal larvae on day 30

[e] Group 4 received 1 x 10^4 normal larvae on day 1

[f] Group 5 received 1 x 10^4 normal larvae on days 94, 95, 96, 97 and 98

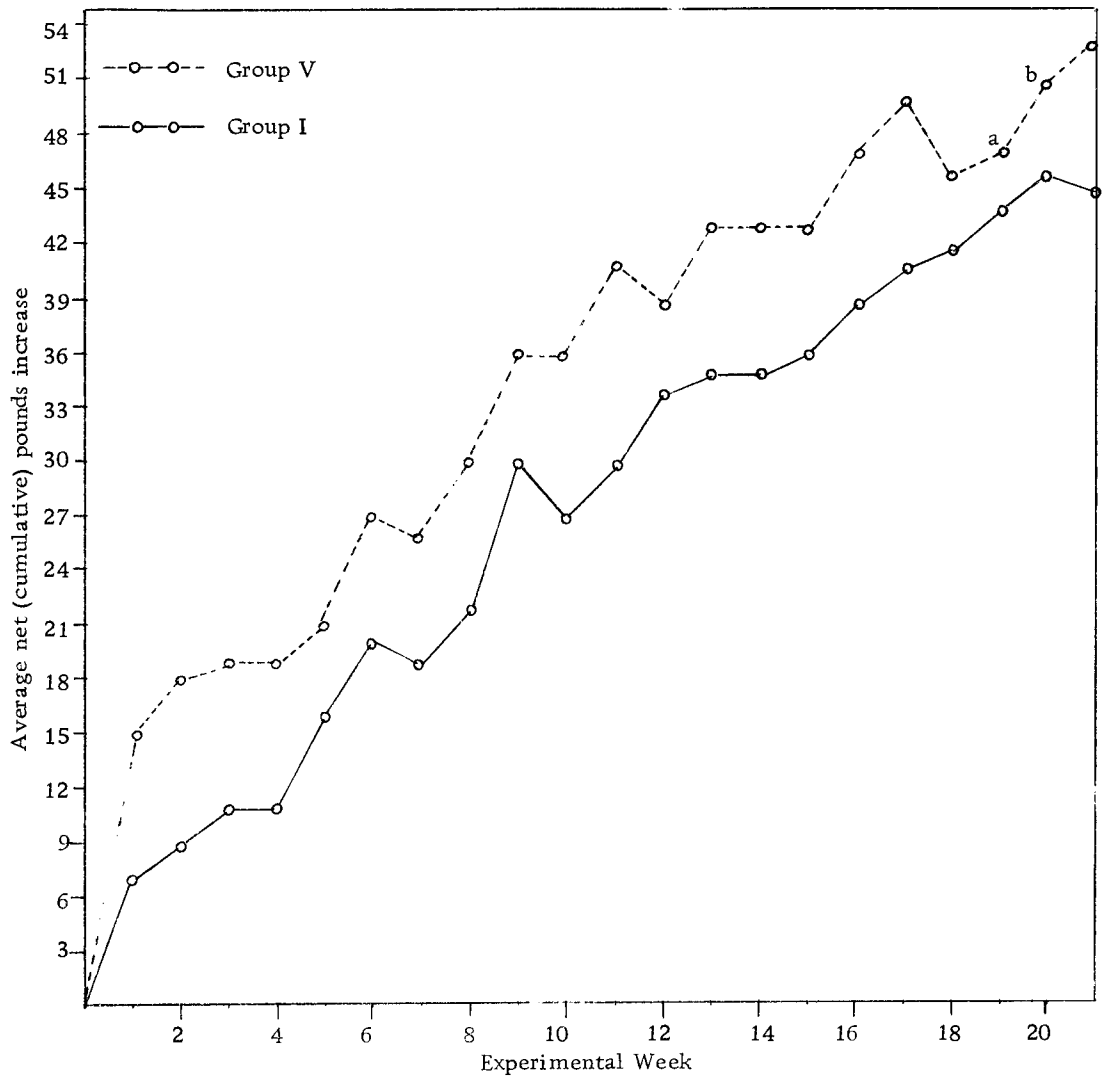
[g] Dead



[a]Average for 9 lambs; no. 51 died of Haemonchosis
[b]Average for 8 lambs; no. 255 died of Haemonchosis

Figure 7. Geometric Mean IHA Titers and average microhematocrits* of sheep in Group I and Group V. Group I given 2 doses X-irradiated H. contortus vaccine and challenged with 50,000 infective larvae in week 14. Group V given 50,000 infective larvae in week 14.

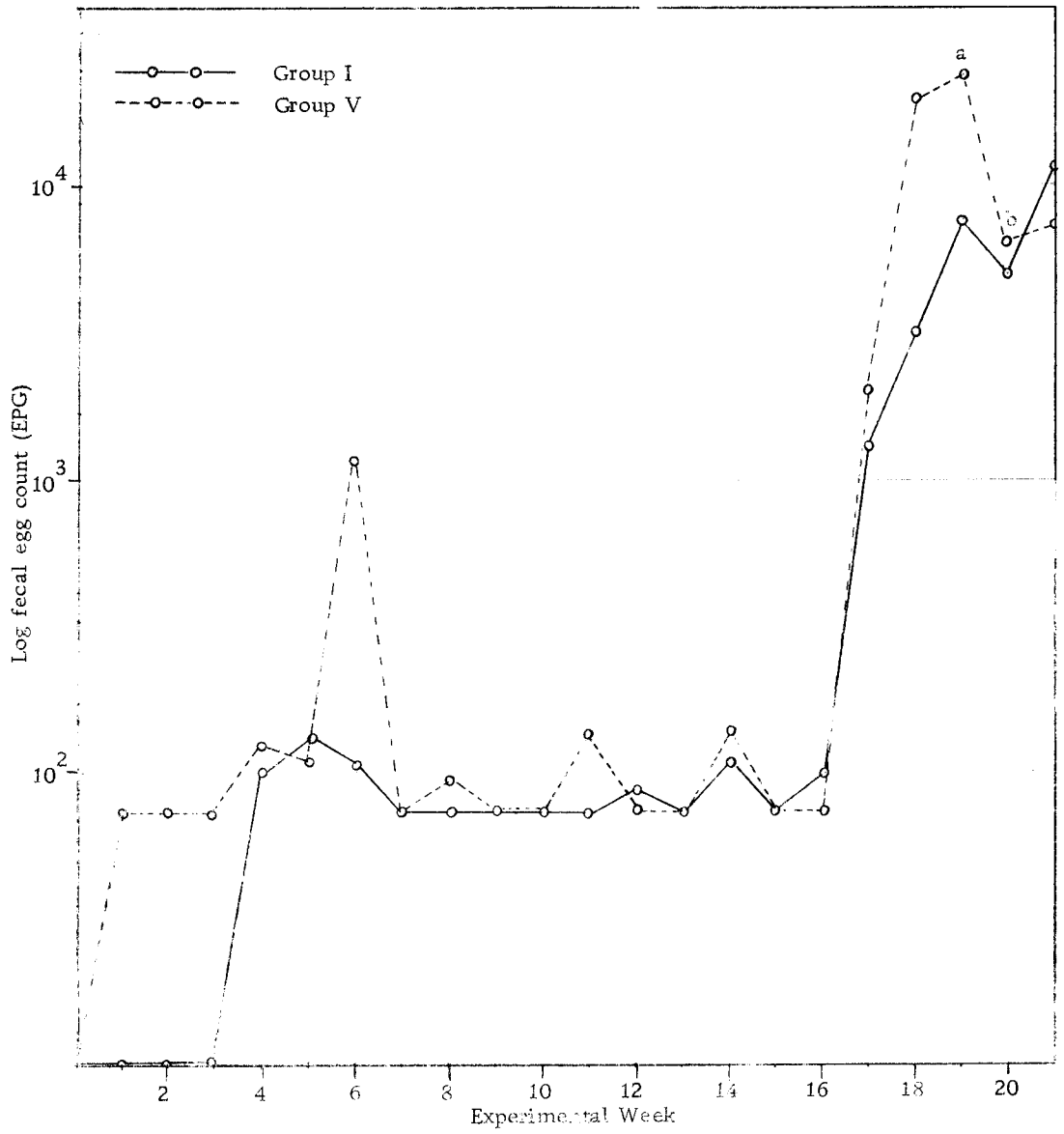
*Compiled from Knapp (54)



[a]Average for 9 lambs; no. 51 died of Haemonchosis
[b]Average for 8 lambs; no. 255 died of Haemonchosis

Figure 8. Average (cumulative) weight gains* of sheep in Groups I and V. Group I sheep given 10,000 X-irradiated H. contortus infective larvae in weeks 1 and 4 and challenged with 50,000 normal larvae in week 14. Group V sheep received 50,000 infective larvae in week 14. Weights measured in pounds.

*Data compiled from Knapp (54)



[a]Average for 9 lambs; no. 51 died of Haemonchosis
[b]Average for 8 lambs; no. 255 died of Haemonchosis

Figure 9. Average fecal egg count (EPG)* of sheep in Groups I and V. Group I received 10,000 X-irradiated H. contortus larvae in week 1 and 4 and 50,000 normal larvae in week 14. Group V received 50,000 normal H. contortus larvae in week 14.

*Compiled from Knapp (54).

near 1.5×10^3 EPG was found. Counts less than that were not found after that week. A count of 1.3×10^4 EPG was found just prior to termination of the experiment. The average egg count of group V animals was 75 at week one. A high count of 1.35×10^3 EPG was found at week six. From week seven to week sixteen, the average count was near 75. A high egg count of 2.9×10^4 EPG was found at week 17 and the final average egg count was 7.5×10^3 EPG. Except for the higher egg counts in group V during weeks 6 and 19, the average counts from the two groups were parallel.

Total counts of H. contortus worms found in the intestine of each animal at necropsy are presented in Table 5. Five vaccinated animals had worm burdens of less than 2, 500 with three of these animals having burdens less than 700. Worm burdens of the other five vaccinated animals were above 12,000. Only one group V control animal had a count of less than 2, 500 while seven animals had counts of above 11,000. Average worm burdens at necropsy of animals in groups I and V were 8,475 and 13, 727 respectively. The difference in average counts is significant and shows that a level of resistance to H. contortus did develop in the vaccinated animals.

In general, the group GMT levels of sheep on this experiment correlate with comparative date, i.e., microhematocrits, egg counts, et cetera, as supplied by Knapp (54). Some of these

Table 5. Experiment 1--Total intestinal adult H. contortus worms found at necropsy of animals in Groups I and V.*

Group	Animal Number	Total Adult Worm Count at Necropsy	Remarks
I	47	430	-
	50	12,350	-
	54	1,040	-
	61	2,430	-
	62	670	-
	67	18,260	-
	68	310	-
	70	16,740	-
	72	18,200	-
	79	14,320	-
Group Average		8,475	
V	34	4,900	-
	35	12,030	-
	46	1,170	-
	51	29,460	died during week 19
	55	4,890	-
	65	11,860	-
	69	14,300	-
	80	21,140	-
	99	11,800	-
	255	25,720	killed during week 19
Group Average		13,727	

*Compiled from: Knapp, S. E. Immune responses of domestic ruminants to irradiated gastrointestinal nematodes. Sept. 1964. 44 numb. leaves. (Oregon State University, Department of Veterinary Medicine, Corvallis, Oregon. Termination report to United States Atomic Energy Commission, contract AT(45-1) 13-76).

correlations are negative. The high GMT levels during weeks 10 to 13 in groups I and V correlate with low group hematocrit values which were found in previous weeks. Before challenge, the highest antibody level was detected at week 13 while the highest hematocrit values were at week 12. Apparently the immune response of the animals exerted an effect upon the worms to the extent that feeding activities of the worms were reduced. This apparent increased resistance was reflected by increased antibody titer.

The EPG data shows that sexually mature female H. contortus worms were present during this pre-challenge period in both the non-vaccinated control and vaccinated animals. Some of the X-irradiated larvae given to animals in group I might have survived the effects of radiation. This survival could allow sexual development of some of the females. Since feces from animals in group V also contained H. contortus eggs during these early experimental weeks, accidental exposure of all animals to H. contortus infective larvae might have occurred soon after the experiment was initiated. No evidence to support or reject this conclusion is available.

The general non-specificity of nematode antigens has been established by others (48, 78, 92, 99, 116) and other antigens might have been a factor in this experiment. Plant nematodes, or other gastro-intestinal nematodes such as Nematodirus spp., might have caused this antibody stimulation.

These animals were accidentally exposed to and subsequently vaccinated against contagious ecthyma during the eighth experimental week. This vaccination could have stimulated higher general resistance which resulted in a functional immunity against H. contortus. This explanation is in conflict with Soulsby (111) who reports that functional immunity appears to be specific, at least at the nematode generic level. But since my data show that a higher resistance did develop and antibodies were detected, I believe that this vaccination helped stimulate the response.

Group GMT data show that higher antibody titers were detected in the vaccinated animals. In group I, five animals (no. 47, 54, 61, 62 and 68) were shown to be partially immune as demonstrated by the total adult worm burden at necropsy.[7] None of these animals had IHA titers above 1:80. The high IHA titer in these animals occurred during or just prior to the period of lowest hematocrit. Later, the IHA titer value either dropped or remained constant while the hematocrits were higher. In each of these five animals, except no. 62, higher IHA titers were found prior to the period of highest EPG. In no. 62 the highest antibody titer was detected three weeks after the highest EPG. The EPG of no. 61 was

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[7] These animals will be considered to be successfully, although only partially, immune to H. contortus reinfection in the discussion and summary of this thesis.

low during the six week period prior to necropsy and high at necropsy. This animal apparently developed some resistance to the challenge infection. This resistance was not reflected by high antibody titer. No correlation between IHA results and other comparative data was found for animals no. 50, 67, 70, 72, and 79. The highest IHA titers detected in group I samples were in non-immune animals no. 70 and 79 during the final week. Three animals in group V developed partial resistance to the challenge infection.

The group GMT values, after challenge, show an inverse relationship to the hematocrit averages until the final week when there was a positiv e and direct correlation. As explained, this relationship is not found when data is analyzed by individual animals. During the post-challenge period, there is a direct and positive correlation between antibody titer and EPG. This relationship has been found by Soulsby (107) who states that the rise in titer might be a measure of antibodies which are not at that time concerned with the protection of the animal and may, in fact, be a reflection of the activity of the parasite.

I found no constant relationship between individual IHA titers and resistance. This finding partially supports results of others (74, 99, 113) who conclude that although resistance trends may be indicated by the IHA test, it does not give any indication of the ability

of the animal to resist the nematode infection. Since higher Geometric Mean Titers were found in the vaccinated animals, of which five were immune, the GMT seemed to relate to group resistance.

Experiment 1a--Larvae Grinding Efficiency of Potter-Elvehjem Grinder

The numbers of intact larvae counted are shown in Table 6. The larval sheaths remained intact at completion of all grinding periods. At the end of ten minutes, much of the cuticle and internal structures of the larvae remained intact. Less of this non-disrupted material remained after grinding for 20 minutes. Very little particulate matter, other than the pieces of sheaths, remained after the 30-minute grinding period.

Table 6. Experiment 1a--Efficiency of Potter-Elvehjem grinder in disruption of H. contortus third stage larvae.

Lot no.	Intact H. contortus third stage larvae/ml after grinding periods of:			
	0 minutes	10 minutes	20 minutes	30 minutes
1	9.85×10^4	4.6×10^3	40	20
2	1.1×10^5	1.65×10^3	125	110
3	1.15×10^5	9×10^2	78	10

It was assumed that finely ground larvae were needed for satisfactory antigen. The results of this experiment showed that larvae were ground fine after 20 minutes and this period was selected for routine preparations of antigens. No IHA analyses were made of these larvae preparations. They should have been tested since satisfactory antigens might have existed in preparations from shorter grinding periods.

Experiment 1b--Optimum Concentration of Ground Larval Antigen Needed to Coat Tanned Cells

Only antigen-coated cells containing 1×10^4 LEQ/ml and 2.5×10^4 LEQ/ml were satisfactory for test although the second antigen had a small fringe of autoagglutinating cells around the central cell button in the control tube. Antigens three and four autoagglutinated completely. Additional washing of these antigens with serumized VB did not alter the result. Suspensions of 5×10^5 LEQ/ml or less were satisfactory for coating tanned cells if they were diluted to contain 1×10^4 LEQ/ml before adsorption. These findings support the results of others (32, 49, 92, 99). The autoagglutinating antigens found at higher larval equivalent concentrations may result from an accumulative effect of reacting antigen. These heavy concentrations may have a tendency to overload the system and produce a reaction similar to a prozone as seen in other

immunological tests. This explanation is not supported by results reported by Silverman (92) since he found that antigens at 1×10^4 LEQ/ml could be used several times to coat tanned cells. His results indicate that the tanned cells have a maximum antigen adsorption capacity.

Experiments 1c and 1d--Tests of Different Diluents for Larvae Suspension During the Grinding Process and Test of Various Time Periods for Antigen Extraction

All diluents tested in Experiment 1c were found to be satisfactory. The antigen prepared from larvae ground in saline was the most sensitive. In Experiment 1b, suspensions of ground larvae extracted for less than 12 hours caused autoagglutination of tanned cells and this autoagglutination could not be eliminated by repeated washing of the cells. Antigens prepared from lots four and five were equal in sensitivity. Antigen-coated cells prepared from lot three werelow in sensitivity. Only lots four (16 hour extraction) and five (24 hour extraction) yielded satisfactory antigens for test.

The results of these experiments support results obtained by Silverman (92). He found that antigens extracted for 16 hours in water and normal saline were more satisfactory than those extracted in buffered saline. He related his results directly to soluble nitrogen from disrupted worm material. I related antigens in my experiments to sensitivity and found saline extracts were the most

sensitive.

I think that proteins in disrupted worm material may be stabilized when buffers are present and thus the release of soluble nitrogen may be impaired. If this relationship is true, and antigen sensitivity is dependent upon soluble nitrogen, then antigens prepared in buffers would be low in sensitivity. My results support this relationship.

The autoagglutinating antigen coated cells found in Experiment 1d may also be related to the soluble nitrogen content of the suspensions. If the soluble nitrogenous compounds were to be of low molecular weight, then cells coated with those compounds should remain in stabile suspensions because fewer chemical or physical reactions should occur between these captured compounds. If sensitized cells were placed in solutions low in soluble nitrogenous compounds, but high in stabile, high molecular weight compounds, the cells would adsorb a high percentage of the latter. The compounds adsorbed might have more reactive groups exposed and physical and chemical reactions may readily occur between antigen coated cells, resulting in autoagglutination of these cells. This hypothesis also depends upon low soluble nitrogen content of antigens extracted for short periods. Those low nitrogen levels have been shown by Silverman.

Experiment 1e--A Test on the Use of Dextrose-Gelatin-Veronal Buffer (DGVB) as a Replacement for Serumized Veronal Buffer in the IHA Test

DGVB was shown to be a completely satisfactory replacement for serumized VB when tested against antigens prepared as described in Experiment 1. The successful use of this buffer indicates that the protective effect exerted by these buffers upon coated cells is due to macromolecular protein. Further research is needed to satisfy this hypothesis.

Experiment 1f--A Test of the Use of Exsheathment Fluid as Antigen in the IHA System

Exsheathment fluid was antigenic when tested in the IHA system. Antigen coated cells prepared from exsheathment fluid dilutions containing 1.36×10^5 and 5×10^4 LEQ/ml autoagglutinated. Cells prepared from dilutions one (1×10^4 LEQ/ml) and two (2.5×10^4 LEQ/ml) were satisfactory as antigens but were found to detect titers no higher than 1:40 in positive serum samples. Repeated testing of other lots of exsheathment fluid did not alter the results.

The low sensitivity of antigen coated cells found in this experiment could be due to inactivation of the exsheathment fluid prior to coating. This inactivation may have been by reaction of the fluid with magnesium ions which were present in the VB. That reaction has been shown by Rogers and Sommerville (78). This explanation

may not be valid since Soulsby (107) successfully used exsheathment fluid coated cells in the IHA system. But, since he did not report the formula of his VB, it might be assumed that he may not have used added cations in the buffer.

Another explanation for the low sensitivity found could be that the samples tested were actually very low in antibody content. This has been discussed in Experiment 1. Also the method of exsheathment fluid preparation may have been unsatisfactory. This is unlikely since the satisfactory methods of others (112, 125) were closely followed and they found that exsheathment fluid was quite stabile. But activity of this fluid may not be related to antigenic sensitivity and the sensitivity may be easily reduced.

Experiment 1g--A Test of the Use of Metabolic and "Captured" Exsheathment Fluid Antigens in the IHA Test

The metabolic antigens (A and B) were satisfactory for test. Results of IHA tests with these antigens compared to the routine ground larval antigen (C) and the exsheathment antigen (D) are given in Table 7. Of 18 positive serum samples tested, the metabolic antigens detected the same titer in six; antigen A detected a two-fold higher titer in two, three-fold higher titers in two and a four-fold higher titer in one sample; antigen B detected two-fold higher titers in seven samples. The widest range of differences detected using antigen A, as seen in Table 7, were in challenged

Table 7. Experiment 1g--IHA antibody titers of selected serum samples tested against experimental metabolic and captured exsheathment fluid antigens.

Group	Serum Sample	Sample Date	Antigen A[a]	Serum Control	Antigen B[b]	Serum Control	Antigen C[c]	Serum Control	Antigen D[d]	Serum Control
neg	32	3-66	1:20	-0-	<1:20	-0-	1:20	-0-	<1:20	-0-
controls	37	3-66	<1:20	-0-	<1:20	-0-	<1:20	-0-	<1:20	-0-
	39	3-66	1:20	-0-	<1:20	-0-	1:40	-0-	<1:20	-0-
	47	3-28-63	1:80	-0-	1:80	-0-	1:40	-0-	1:320	-0-
	54	3-28-63	1:40	-0-	1:40	-0-	<1:20	-0-	1:80	-0-
	61	3-28-63	<1:20	-0-	1:40	-0-	1:40	-0-	1:640	-0-
I	62	3-28-63	1:160	-0-	1:160	-0-	1:80	-0-	1:1280	-0-
	67	3-28-63	1:80	-0-	1:40	-0-	1:40	-0-	1:320	-0-
	68	3-28-63	<1:20	-0-	1:40	-0-	1:40	-0-	1:320	-0-
	70	3-28-63	1:80	-0-	1:160	-0-	1:320	-0-	1:5120	-0-
	72	3-28-63	1:20	-0-	1:40	-0-	1:20	-0-	NT[e]	NT
	79	3-28-63	1:20	-0-	1:40	-0-	1:320	-0-	1:1280	-0-
	34	3-28-63	1:60	-0-	1:20	-0-	1:20	-0-	1:80	-0-
	35	3-28-63	1:80	-0-	1:160	-0-	1:20	-0-	1:40	-0-
	46	3-28-63	1:160	-0-	1:160	-0-	1:80	-0-	1:1280	-0-
	51	3-7-63	1:320	-0-	1:80	-0-	1:20	-0-	1:2560	-0-
V	55	3-28-63	1:80	-0-	1:80	-0-	1:160	-0-	1:1280	-0-
	65	3-28-63	1:20	-0-	1:40	-0-	1:20	-0-	1:40	-0-
	69	3-28-63	1:160	3^{+f}	1:40	2^{+}	1:80	3^{+}	1:320	4^{+}
	80	3-28-63	1:80	-0-	1:40	-0-	<1:20	-0-	NT	NT
	99	3-28-63	<1:20	-0-	<1:20	-0-	1:20	-0-	1:20	-0-
	Antigen control		-0-		-0-		-0-		-0-	
	GMT		54.6		54.6		41.5 18 samples		-	
	GMT		-		-		49.5 16 samples		347.5 16 samples	

[a] Antigen A--30-day-extracted, fourth stage larval, metabolic antigen at 1.6×10^4 LEQ/ml.

[b] Antigen B--24-hour-extracted, fourth stage larval, metabolic antigen at 1.6×10^4 LEQ/ml.

[c] Antigen C--Ground third stage larval antigen at 1.0×10^4 LEQ/ml

[d] Antigen D--Captured exsheathment fluid antigen at 1.0×10^4 LEQ/ml

[e] NT--Not tested

[f] Denotes amount of agglutination based upon 1^+ to 4^+ classification with 4^+ being a complete agglutination of cells.

control animals and five of the seven higher titers detected using antigen B were in vaccinated animals. The GMT of samples tested with antigens A and B was 54.6.

When compared to the routine antigen (C), higher titers were detected using antigen A in ten serum samples. The greatest differences in titer were found in challenge control sheep samples. Antigen B detected higher titers in nine samples with the same differences found between control samples. Antigen C detected higher titers than antigen A in six samples and higher titers than antigen B in five. The greatest titer differences detected by antigen C in both instances were in samples from vaccinated sheep. The GMT of samples tested by antigen C was 41.5.

GMT analysis of samples split into vaccinates (group 1) and non-vaccinates (group V) is given in Table 8.

Table 8. Experiment 1g--Geometric mean titer analysis of IHA results from Table 7. Analysis made by serum sample group.

Antigen[a]	A		B		C		D	
Group	I	V	I	V	I	V	I	V
GMT	36.6	74.0	59.0	50.3	54.6	34.1	590.0	207.5

[a]Antigens refer to same antigens as shown in Table 7.

The use of metabolic antigens in the IHA test did not substantially increase its sensitivity although a significant increase was evident in the GMT of tested samples when compared to the GMT of samples tested by the routine antigen. The increase in sensitivity could be due to a loss of antigen during the grinding process in preparation of antigen C. This problem has been pointed out by Silverman (92). My results demonstrate slightly more sensitive metabolic antigens than those produced by Soulsby (107). Also, as shown in Table 7, the metabolic antigens were prepared from higher larval equivalent extracts. The GMT values for samples tested by both 24-hour and 30-day extracted antigens were the same, even though the individual titers of animals tested in each group were different. This data helps to support Silverman's (92) view that the tanned cell apparently has limited antigen adsorption potential. I assume that the 30-day metabolic antigen contained more antigen.

Groups I and V GMT values, when tested with antigens B and C, were similar. The high GMT value of 74.0 in group V samples, when tested with antigen A, was unexpected. This higher titer in the control samples was not found in other experiments and I cannot explain the difference.

During preparation of exsheathment fluid coated cells, a sample of the mixture of larvae and tanned cells was withdrawn and observed microscopically after one, two and four hours. The

following observations were made: (1) the larvae were tightly coiled at each observation period, (2) the red blood cells did not adhere to the cuticle of the larvae, (3) the red cells did not accumulate near body orifices of the larvae, (4) 85% exsheathment was realized and the addition of red blood cells did not noticeably affect the normal in vitro exsheathment rate, and (5) exsheathment was essentially complete at four hours.

The Buchner filtration method was completely satisfactory for separating the red cells from the spent sheaths and larvae which were retained on the filter paper. Some hemolysis of the red cells occurred during the coating and filtering phases as evidenced by the pink color of the supernatant solution after the first centrifugation.

IHA test results are presented in Table 7 under the column labeled Antigen "D." GMT of samples tested by this antigen was 347.5. When samples are analyzed according to groups, GMT values obtained are 590.0 and 207.5 for the selected samples from groups I and V respectively. Sixteen positive samples tested were duplicates of ones tested by Antigen C. The GMT of these samples tested by Antigen C was 47.5. The two antigens detected the same titers in only one sample, a challenge control. Antigen "D" detected higher titers in all other positive samples tested, of which two were two-fold higher titers, three were three-fold higher titers, five were four-fold higher titers, four were five-fold higher titers

and one sample, no. 70, a vaccinated sheep, was eight-fold higher in titer. Eleven samples tested by antigen "D" yielded titers of 1:320 or above as compared to two samples tested by antigen "C." The negative controls, selected from helminth-free sheep, yielded no antibody titers against antigen "D" but two had low titers when tested by antigen "C."

These highly significant results show that the tanned cells apparently adsorbed an antigenic substance which was inactive or not present in other exsheathment fluid experiments. This antigen could be so closely associated with the larvae or larval sheaths that exsheathment fluid harvest procedures, as routinely used, fail to extract this component. The high GMT value (347.5) obtained with this antigen as compared with a GMT of 49.5 with antigen C indicates this method of exsheathment fluid extraction could be very important and should be further studied. The GMT values of animals grouped into groups I and V showed a wider difference than previously found with other antigens. The highest titer found in samples tested by antigen D was 1:5,120 as compared to 1:320 using other antigens.

Soulsby (107) has reported individual high IHA titers in samples tested with exsheathment fluid coated cells but the titers were no more than two dilutions higher than those found with metabolic antigen. He also reports a few three-fold higher titers using exsheathment fluid antigen when compared to ground larval

antigen.

Fresh larvae were not available and this method of antigen preparation could not be tested further.

Experiment 2--Immunoelectrophoresis (IE) Study of Specific Serum Fraction Changes in Animals From Groups I and V

Satisfactory IE results were obtained with five microliter serum samples and 0.08 ml anti-sheep globulin samples. The best IE reaction (Figure 10) yielded 23 distinct arcs, of which 18 were identified by location only. Photographs of individual slides from principle vaccinate animals (Group I) appear in Figures 11 through 20. The precipitation arc designated by X in the photographs and in Table 9 could not be identified.

Results, as determined by observation of IE reaction slides from Groups I and V, are given in Table 9. Fractions other than those listed showed no changes. The entries in Table 9 are observations made of serum samples taken from the individual animals during the week of the highest IHA titer and at necropsy. In some instances, the highest IHA titer occurred during the final week. A single entry is made for that animal when these dates coincide. Except for reactions from the pre-exposure samples, observations were made from at least two IE slides. Some slides were prepared from diluted anti-sheep globulin to produce clearer reaction regions.

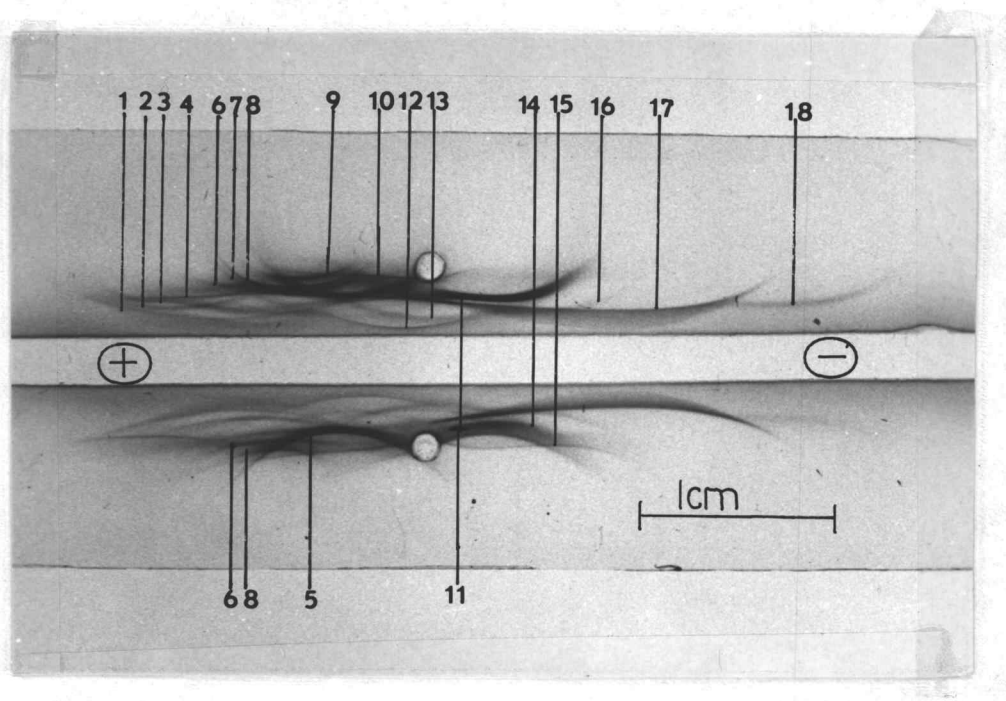

Figure 10. Immunoelectrophoresis of anti-H. contortus sheep serum against rabbit anti-sheep globulin. Fractions identified by location only according to Chordi and Kagan (10) and Silverstein et al. (97).

- denotes cathode end of slide
+ denotes anode end of slide

1. α_1-I
2. α_1-II
3. α_1-III
4. α_1-IV
5. α_2-I
6. α_2-II
7. α_2-III
8. α_2-IV
9. α_2-V
10. α_2-VI
11. β_1-I
12. β_1-II
13. β_1-III
14. β_2-I
15. β_2-III (19 S)
16. X (Possible β_2-V or γ_1-A)
17. Fast γ_1 (7 S)
18. Slow γ_2 (7 S)

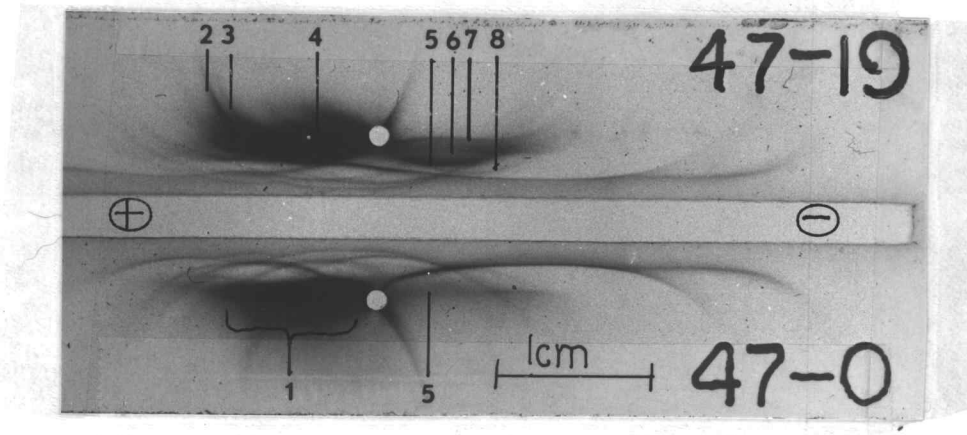

Figure 11. Immunoelectrophoretic patterns of sheep no. 47 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

19 denotes serum sample from week 19 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. smeared α_2 region
2. α_2-I
3. α_2-II
4. α_2-IV
5. β_1-I
6. β_2-I
7. β_2-III
8. X

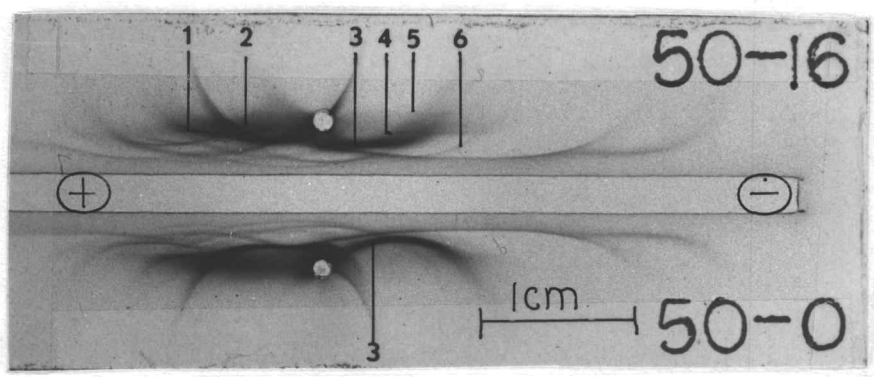

Figure 12. Immunoelectrophoretic patterns of sheep no. 50 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

16 denotes serum sample from week 16 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_2-II
2. α_2-IV
3. β_1-I
4. β_2-I
5. β_2-III
6. X

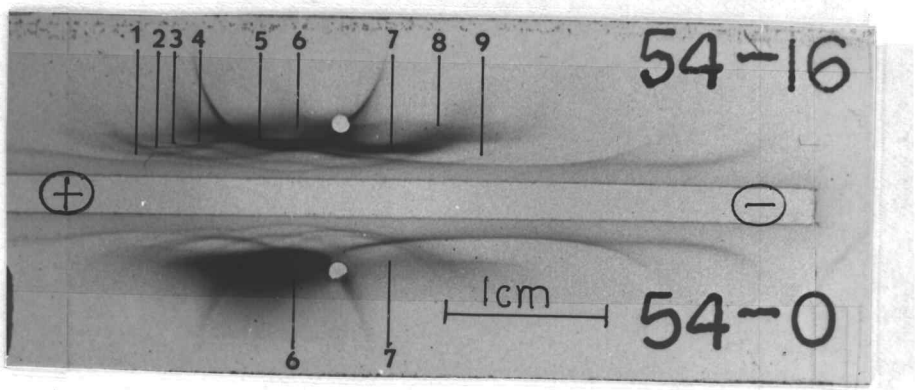

Figure 13. Immunoelectrophoretic patterns of sheep no. 54 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.
16 denotes serum sample from week 16 (week of highest IHA titer)
0 denotes pre-exposure serum sample
arcs indicated are ones that change in density

1. α_1-I
2. α_1-II
3. α_1-III
4. α_1-IV
5. α_2-II
6. α_2-IV
7. β_1-I
8. β_2-III
9. X

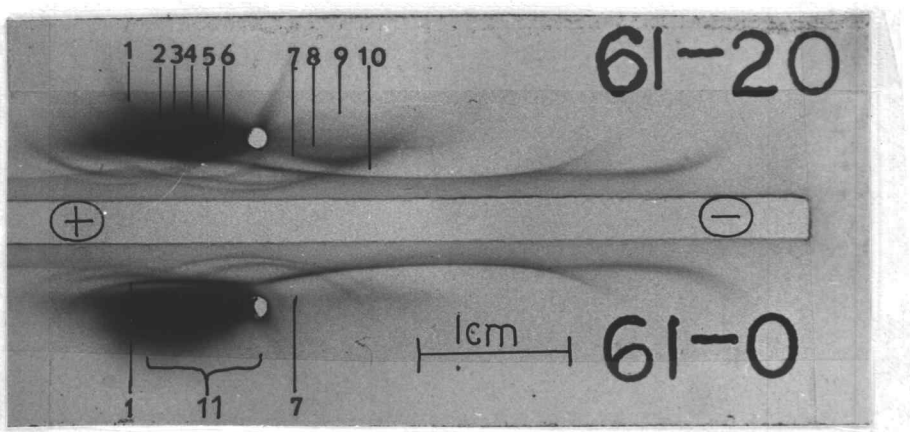

Figure 14. Immunoelectrophoretic patterns of sheep no. 61 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.
20 denotes serum sample from week 20 (week of highest IHA titer)
0 denotes pre-exposure serum sample
arcs indicated are ones that change in density

1. α_2-I
2. α_2-II
3. α_2-III
4. α_2-IV
5. α_2-V
6. α_2-VI
7. β_1-I
8. β_2-I
9. β_2-III
10. X
11. smeared α_2-region

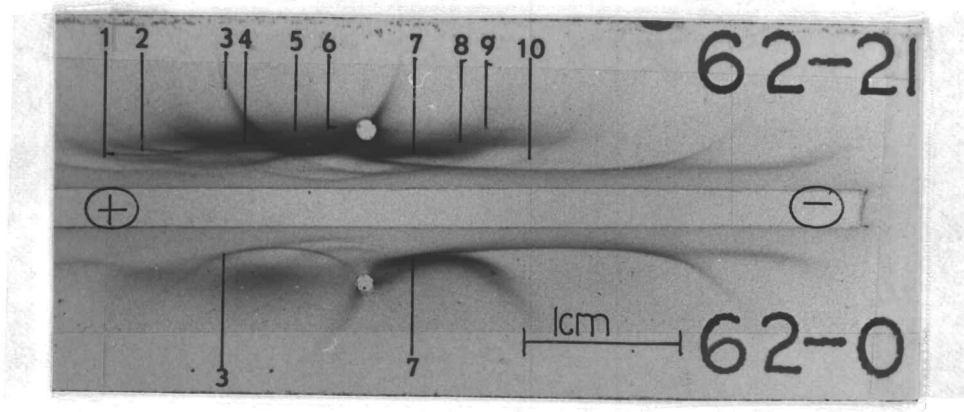

Figure 15. Immunoelectrophoretic patterns of sheep no. 62 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

21 denotes serum sample from week 21 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_1-I
2. α_1-II
3. α_2-I
4. α_2-II
5. α_2-III
6. α_2-IV
7. β_1-I
8. β_2-I
9. β_2-III
10. X

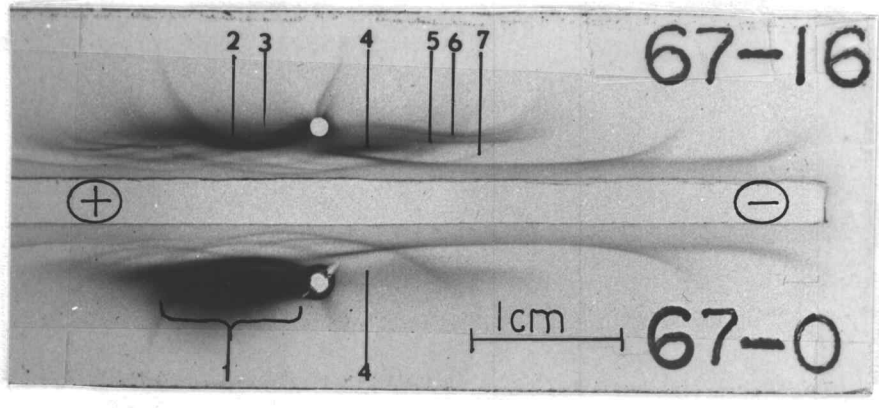

Figure 16. Immunoelectrophoretic patterns of sheep no. 61 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

16 denotes serum sample from week 16 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. smeared α region
2. α_2-II
3. α_2-III
4. β_1-I
5. β_2-I
6. β_2-III
7. X

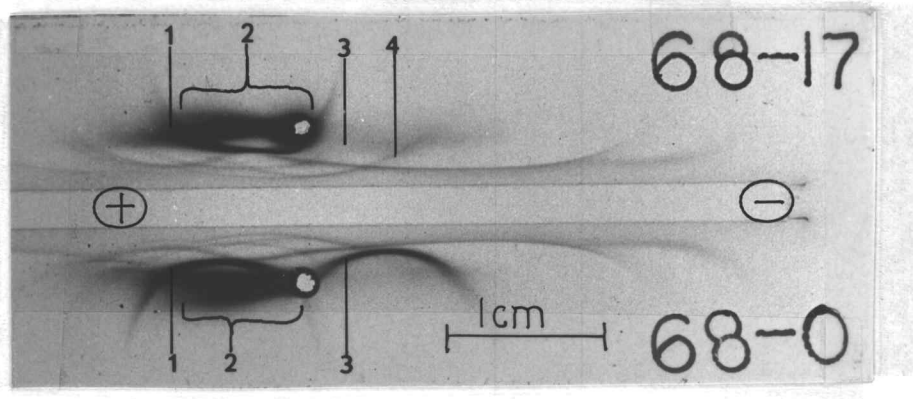

Figure 17. Immunoelectrophoretic patterns of sheep no. 68 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

17 denotes serum sample from week 17 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_2-I
2. smeared α_2 region
3. β_1-I
4. X

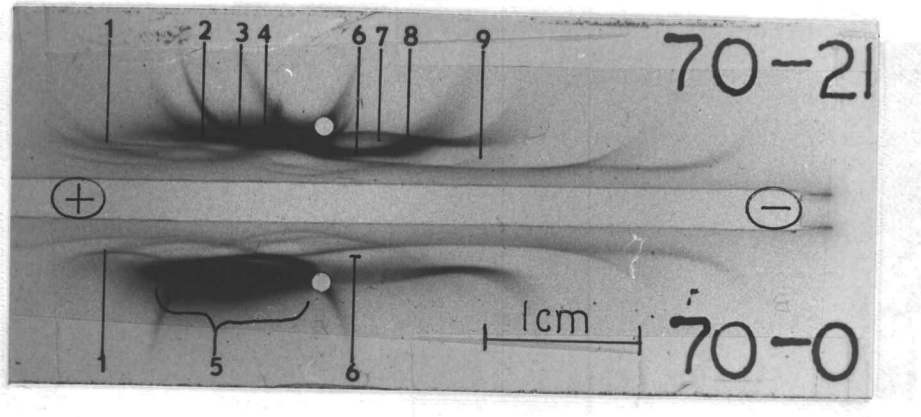

Figure 18. Immunoelectrophoretic patterns of sheep no. 70 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

21 denotes serum sample from week 21 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_1-I
2. α_2-II
3. α_2-III
4. α_2-IV
5. smeared α_2 region
6. β_1-I
7. β_2-I
8. β_2-III
9. X

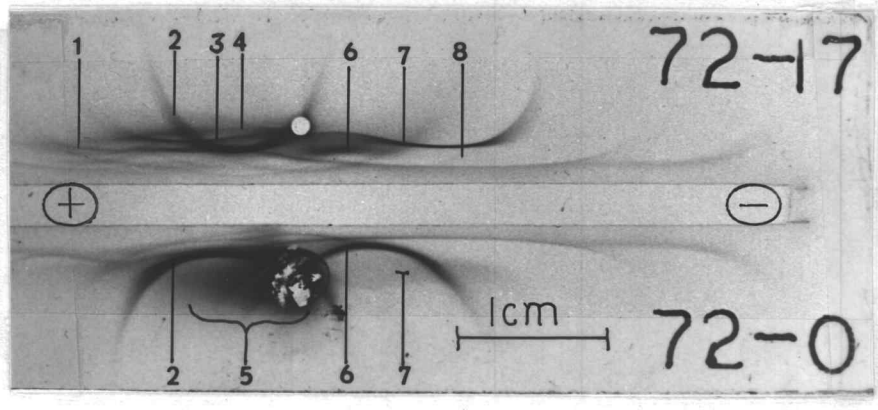

Figure 19. Immunoelectrophoretic patterns of sheep no. 72 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

17 denotes serum sample from week 17 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_2-II
2. α_2-I
3. α_2-II
4. α_2-IV
5. smeared α_2 region
6. β_1-I
7. β_2-III
8. X

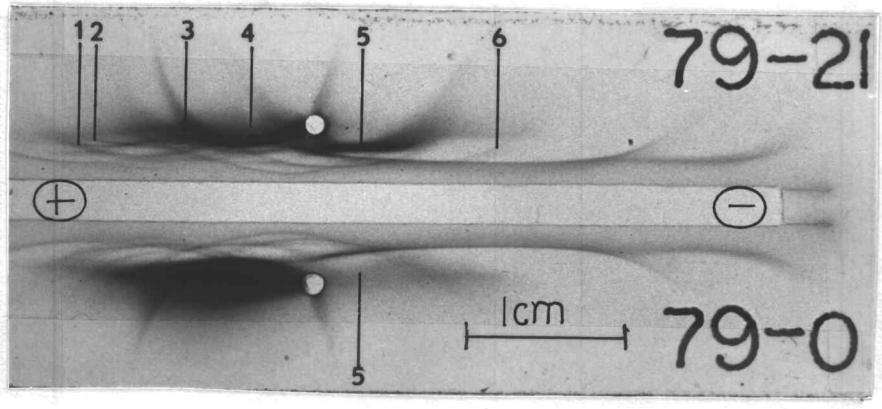

Figure 20. Immunoelectrophoretic patterns of sheep no. 79 developed against rabbit antisheep globulin. Animal vaccinated with 10,000 X-irradiated H. contortus infective larvae during weeks 1 and 4 and challenged with 50,000 normal infective larvae during week 14.

21 denotes serum sample from week 21 (week of highest IHA titer)

0 denotes pre-exposure serum sample

arcs indicated are ones that change in density

1. α_1-II
2. α_1-IV
3. α_2-II
4. α_2-IV
5. β_1-I
6. X

Table 9. Experiment 2--Observations of changes[a] in immunoelectrophoresis patterns on slides prepared from serum samples of H. contortus vaccinated (Group I) and non-vaccinated control (Group V) sheep. (Table 1) Rabbit anti-sheep globulin used as antibody.

Group	Animal Number	Sample[b] Week	Serum Fractions Representing Precipitation Arcs Undergoing Change													
			α_1-I	α_1-II	α_1-III	α_1-IV	α_2-I	α_2-II	α_2-III	α_2-IV	α_2-V	α_2-VI	β_1-I	β_2-I	β_2-III	X
I	47	0					⊢	smeared[c]				⊣	A	A	A	Q
I	47	19					I	I		I			I	I	I	P
I	47	21					I	I		I			MI	MI	MI	P
I	50	0												A		Q
I	50	16						I		SI			D	I	I	P
I	50	21						I		I			I	I	I	P
I	54	0											A			A
I	54	16	SI	SI	SI	SI		MI		MI			P		I	P
I	54	21						I	I	I	I		I		I	P
I	61	0					⊢	smeared				⊣	A			A
I	61	20					⊢	distinct				⊣	P	I	I	P
I	61	21						SI	MI	MI	SI					P
I	62	0					L	L	L	L						A
I	62	21	SI	SI			MI	MI	I	I			D	I	I	P
I	67	0						⊢	smeared			⊣	A	L		A
I	67	16						SI	SI				SI	MI	MI	P
I	67	21						I	MI	MI	I	I	I	A	A	P
I	68	0					di	⊢	smeared			⊣	P			A
I	68	17					E	⊢	smeared			⊣	A			Q
I	68	21						⊢	smeared			⊣	I	I	smeared	I P
I	70	0						⊢	smeared			⊣	L	P	P	A
I	70	21	D					MI	SI	MI			MI		SI	P
I	72	0						⊢	smeared			⊣	P			A
I	72	17		I			S, D	I		I			D		MI	P
I	72	21		I				I	I	I			A			P
I	79	0						⊢	smeared			⊣	L			A
I	79	21		I		I		I		I			I			P
V	34	0														A
V	34	21						E								P

Table 9. (Continued)

Group	Animal Number	Sample Week	Serum Fractions Representing Precipitation Arcs Undergoing Change													X
			α_1-I	α_1-II	α_1-III	α_1-IV	α_2-I	α_2-II	α_2-III	α_2-IV	α_2-V	α_2-VI	β_1-I	β_2-II	β_2-III	
V	35	0						di	⊢ smeared ———			——⊣				A
V	35	20						I	I	I						P
V	35	21														P
V	46	0											L			A
V	46	19											I	I		P
V	46	21											I			P
V	51	0				di		di					L	P		A
V	51	18				A		I	I	I	I	I	Sp	A		P
V	55	0											L			A
V	55	21						E	I	I			I			P
V	65	0											P			A
V	65	21														P
V	69	0														A
V	69	21					E	E	I		I	I	I			P
V	80	0											P			A
V	80	21					D	D		D			A			P
V	99	0						⊢ smeared ———				——⊣				A
V	99	21						D	3 distinct bands ⊢ widening of inter-arc area ——			——⊣	D			P
V	255	0														P
V	255	21						I	Sp, I	I						P

Note: A = absent D = decreased di = distinct E = elongated I = increased L = lengthened
MI = marked increase P = present Q = questionable presence SI = slight increase
Sp = split

[a] Changes refer to observed increases, decreases and inter-arc relationships of precipitation arcs.

[b] 0 refers to pre-exposure sample, other weeks listed are weeks of highest IHA titer and week of experiment termination. When the latter two dates coincide a single entry is made as week 21.

[c] Smeared denotes precipitation arcs present but individual arcs cannot be identified.

The results concerning increased or decreased densities of precipitation arcs are not to be directly interpreted as changes in corresponding serum fractions since biochemical fraction analysis was not performed.

The greatest increases in density occurred in fractions α_2-II, α_2-III, α_2-IV and β_2-III. These increases were most evident in samples from sheep no. 47, 50, 54, 61, 62 and 70 of which no. 47, 54, 61 and 62 were shown to be immune. Changes in the α_1 region were associated with samples (except for no. 54) from sheep which were not successfully immunized but were the most heavily infected with adult worms at necropsy. The most severe α_2-I changes occurred in the most heavily infected, apparently non-immune, animals. The change involved either a decrease in density and/or an elongation of the precipitation arc toward the cathode. Except for samples from no. 72, all β_2-III increases occurred in successfully immunized animals. Of the five immune, vaccinated sheep, only animal no. 68 did not show this change. In general, fraction β_2-I increases were also associated with successfully immunized animals.

Fraction β_1-I decreases and increases were directly related to the hematocrit values and the most anemic sheep had the least dense β_1-I fraction.

Increases in the β_2-I fraction were noted in control animals

no. 46, 55 and 69. As shown by the necropsy worm burdens, it is apparent that these animals underwent self-cure after challenge. Although no increase in density of arcs in the gamma region were observed, small changes might have been present.

There was no direct correlation between IHA antibody titer and specific serum fraction increases.

Fraction X appeared in all samples tested from infected sheep. It was present in only one sample of serum (no. 255) taken at week zero. This fraction appears in the same region as γ_1-A of human serum as described by Silverstein et al. (97).

The results obtained from this IE experiment, which indicated involvement of α_1, α_2 and β globulins, are in agreement with results of other H. contortus experiments as reported by Leland and Drudge (59) and studies of other nematodes (4, 32, 57, 62). Failure to demonstrate changes in gamma globulins is not in agreement with the results in the literature cited. The above listed reports were concerned only with electrophoretic analysis. I do not know of another report involving IE studies of H. contortus infections.

Experiment 2a--Test of Exsheathment Fluid as Antigen Against Electrophoretically Separated H. contortus Antisera

No precipitation arcs appeared on slides prepared in this experiment. Repeated recharging of the longitudinal trough with

fresh fluid or serum, depending upon the technique used, failed to yield positive results. This failure was probably due to the low antigen and antibody concentrations. This result is in agreement with work by Chordi and Kagan (10) who state that antisera with IHA titers of less than 1:3000 against hydatid fluid antigen do not yield IE bands.

Experiment 2b--Test of Ground Larval Antigen Against Electrophoretically separated *H. contortus* Antisera

Negative results were obtained when the ground antigen extract was subjected to electrophoresis before reaction with positive sera. When the process was reversed and serum samples of highest IHA titer from Group I were subjected to electrophoresis, precipitation arcs developed in IE slides of serum samples from animals no. 47, 54, 61 and 68. In each sample, a single arc appeared, beginning in the area of the anode end of the α_2-I globulin. This lateral sweeping arc extended toward the anode into and through the α_1 globulin region (refer to Figure 10). It ended lateral to the anode end of the center (antigen) trough.

These arcs were first noticed ten days following the third recharging of the antigen trough and density was greatest at 14 days. This arc "washed-out" during processing and did not stain with Amido Black 10B.

This experiment demonstrates a serum fraction containing antibody against H. contortus. The arc appeared in slides of all, except no. 62, serum samples taken from successfully immunized sheep. It did not appear in tested serum samples from other vaccinated animals or in pre-exposure samples from any of the animals.

The position of the arc places it in the fast α_2, α_1 or α_0 region. Since this arc did not visibly stain with Amido Black and it appeared to "wash out" during processing, I assume it was very low in protein content. The arc did not appear as dense as the arcs observed in experiment 2, and it is probable that either the antigen and/or the antibody contents were low. The position of the arc as shown in my experiment, is in accordance with an arc containing antibody against other nematodes as shown by Bird (4).

Experiment 3a--Immuno-diffusion With Exsheathment Fluid Antigen

In all positive serum samples tested, 11, two precipitation arcs were observed; one near the antigen well and one near the serum well. The most dense precipitation arcs were found in the control positive H. contortus antiserum. The most dense of these arcs appeared near the serum well. Little variation was seen among the reaction patterns of the individual samples. Limited amounts of serum were available from each animal and comparison

between pre-exposure and final samples could not be made.

Experiment 3b--Immuno-diffusion With Ground Larval Antigen

Antigens prepared from ground third stage larvae at 1×10^4 LEQ/ml and 5×10^4 LEQ/ml did not produce precipitation arcs. Precipitation arcs were found when ground larval antigen was used at 1×10^5 LEQ/ml. This experiment was repeated with pre-exposure serum samples and samples taken at the termination of the experiment from the principle vaccinate animals. A drawing of the reactions found in this part of the experiment is given in Figure 21.

Precipitation arcs appeared near the antigen well in pre-exposure serum samples no. 62 and 68. A precipitation arc was found completely surrounding the positive serum wells of all samples, except no. 61. The most dense arcs were found in reactions from animals no. 47, 50, 62, 67 and 70. A discontinuous arc extending toward the antigen well was present in sample no. 50 and the circular arc around serum sample no. 68 was not clear. A second single arc appeared near the antigen well in samples no. 54, 61, 62 and 72; the most dense arc was in sample no. 54. A distinct third arc extending laterally from in front of the well

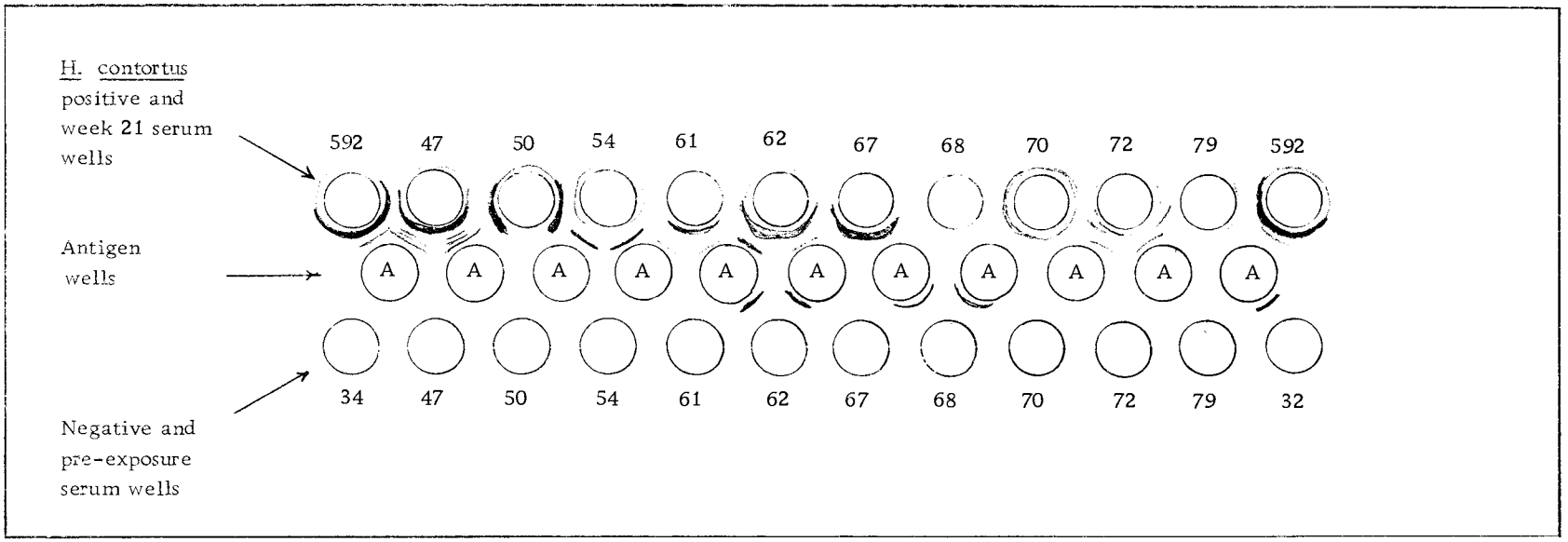

Figure 21. Graphic representation of gel-diffusion patterns of Group I sheep serum samples taken at necropsy (week 21) as compared to pre-exposure serum sample. Antigen prepared from ground H. contortus third stage larvae at a concentration of 1×10^5 LEQ/ml. Sample no. 592 = positive H. contortus antiserum. Samples 32 and 34 are negative serum controls from helminth free sheep. Diffusion plate analyzed 21 days after third charging of wells.

appeared in sample no. 72. This arc was close to the circular arc. Three separate arcs were present between the antigen and serum wells in sample no. 47.

Results obtained from these gel-diffusion studies were inconclusive. The two samples (no. 62 and 68) which demonstrated precipitation arcs in pre-exposure serum were from animals that apparently were immunized by the vaccination. These arcs could have resulted from prior sensitization to H. contortus or related antigens. All serum samples, except no. 72, which were found to have multiple precipitation arcs were from immune animals. Sheep no. 72 had a hematocrit of eight and an IHA titer of 20 at the time of necropsy and demonstrated no apparent resistance. The multiple precipitation patterns which developed in these samples, particularly the sample from sheep no. 47, were similar to those reported by Soulsby and Stewart (113) but were not of sufficient density to analyze critically.

SUMMARY AND CONCLUSIONS

Ten helminth-free sheep were given two doses of 10,000 X-irradiated H. contortus third stage larvae 30 days apart. These animals and a group of ten non-vaccinated sheep were challenged with 50,000 normal H. contortus third stage larvae 60 days following the second vaccination. Antibody levels were measured in both groups by the Indirect Hemagglutination Test (IHA) with ground larval antigen. Significant levels of antibodies were not found in vaccinated animals following administration of either dose of vaccine. Following challenge, the vaccinated group contained antibodies of significant high levels as compared to the control group. The highest antibody titers were found in vaccinated animals which did not develop a protective immunity of a level high enough to resist challenge; and resistance in the individual vaccinated animals was not related to individual antibody levels.

Antigens extracted from H. contortus third stage larvae, ground with a Potter-Elvehjem grinder, were satisfactory for the IHA test. Tanned red blood cells did not have a limited adsorption capacity for ground larval antigen. Larvae ground in normal saline were found to yield the most sensitive ground antigens. Dextrose-Gelatin-Veronal-Buffer was found to be a satisfactory replacement in the IHA test for veronal buffer with serum. Saline extracts from

metabolizing, early fourth stage H. contortus larvae were satisfactory as antigens in the IHA test.

Antigen of extremely high sensitivity was extracted from third stage larvae in the process of exsheathment. When tested against selected positive H. contortus antisera, this exsheathment antigen was seven times more sensitive than the routine ground larval antigen.

Immunoelectrophoresis (IE), with rabbit antisheep globulin as antibody, was used successfully to determine qualitative changes in specific fractions of serum samples taken from vaccinated and non-vaccinated sheep. An unidentified fraction, seen as a precipitation arc in the slow beta or fast gamma region, appeared in all reactions of serum samples taken from sheep exposed to H. contortus larvae. Increases in $alpha_2$-II, $alpha_2$-III, $alpha_2$-IV, $beta_2$-I and $beta_2$-III globulin fractions, as evidenced by increases in densities of precipitation arcs representing these fractions in reacted serum samples, were, in general, associated only with successfully immunized sheep. Of these fractions, $beta_2$-III globulin increases were found to be most closely associated with successfully immunized animals. Increases in $alpha_1$ globulins were seen to be limited to heavily infected, non-immune animals. No apparent changes in gamma globulin were observed in IE reactions of any serum sample. Increases and decreases in densities of the $beta_1$-I globulin fraction

were directly correlated with the anemic status of the animal and the arc representing this fraction was absent or very light in reactions from animals with severe anemia.

When H. contortus antisera were subjected to electrophoresis and subsequently reacted against ground H. contortus larval antigen placed in the antigen trough, a precipitation arc, apparently indicating antibody against H. contortus, developed in the alpha globulin region. This fraction was easily washed out during processing and could not be stained with Amido Black 10B. It could not be identified.

Immuno-diffusion studies of serum samples from vaccinated and non-vaccinated sheep, when tested against ground H. contortus larval antigen, were inconclusive. Multiple precipitation arcs which developed between antigen and serum wells were, in general, associated only with serum samples taken from sheep successfully immunized against H. contortus.

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APPENDIX H

Glossary

1. Amplifier - A device which can increase the power level of a signal.

2. Analog Signal - A continuous and stepless function without discontinuities or breaks.

3. Analog to Digital Encoder - A device which converts an analog signal to a digital signal by means of quantization.

4. Binary pulse - Momentary flow of energy of short time duration.

5. Digital Signal - A function consisting of a series of discrete pulses. A unique set of pulses is assigned to each number to be transmitted.

6. Frequency-shift-keying - A system in which the frequency being transmitted deviates plus or minus X Hertz from a nominal center frequency. A deviation of plus X Hertz may correspond to the presence of a pulse while the deviation of minus X Hertz would correspond to the absence of the pulse.

7. Hertz - One cycle per second equals one Hertz.

8. Interexchange Channel - A telephone circuit linking two central offices.

9. Interrogation Signal - A signal transmitted to a monitoring station to initiate the transmission of data.

10. Instrumentation - Equipment used to detect changes in the behavior of a parameter.

11. Line-Interrupt - A technique for the transmission of binary pulses by the interruption of a constant flow of current.

12. Line-Of-Sight - An uninterrupted view between two points.

13. Local Channel - A telephone circuit linking a central office with a monitoring station.

14. Main Stem of the Willamette - From river mile 185 at Eugene to the mouth of the river.

15. Parameter - A quantity whose behavior can be explained by the application of scientific laws. Table 2 lists parameters of interest to water-quality monitoring.

16. Repeater - A station which receives and relays a radio signal. Repeaters are commonly used to maintain line-of-sight transmission between distant radio stations.

17. Signal Conditioning- The process of converting an electrical signal into a more usable form.

18. Solid-State - Refers to the use of active semiconductor components. Germanium and silicon are semiconductor materials. Transistors and diodes are examples of semiconductor components.

19. Stage - Depth of water in a stream or river.

20. Subscriber Mileage - Telephone circuit mileage required to extend a local channel beyond the base rate area. The base rate area normally consists of the area containing the developed telephone system in and around a town.

21. Telemetry - "the process by which the quantity measured is transferred to a remote location to be recorded, displayed, actuate a process, . . ." (6, p. 277)

22. Telephone data communications - The transmission of data, usually in a digital form, over telephone circuits.

23. Very High Frequency Radio - Radio transmission in the 30 to 300 megahertz range.

24. Weston Standard Cell - An electrolytic cell yielding a precisely known e.m.f. of 1.0183 V at 25^{o} C. The Weston cell is based on the invariant activity of cadmium and mercurous ions in solution at a constant temperature.

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