THE DEVELOPMENT OF IMMUNOLOGICAL METHODS FOR THE CONTROL OF TICKS ON RABBITS AND CATTLE

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CHAPTER I

INTRODUCTION

Ticks are known to be vectors of disease and an important cause of economic loss to livestock producers. The possibility of controlling and regulating tick populations has been the goal of intensive research in several countries for many years. Graham and Hourrigan (1977) reviewed eradication programs for the control of ticks on livestock and described a variety of methods for control of this ectoparasite. These control methods include cultural, chemical and habitat alteration.

One of the oldest cultural methods is host manipulation or pasture spelling. Cultural control also includes the breeding of tick resistance into the host. Chemical controls include: dipping and/or spraying of cattle and sheep with aqueous pesticides, self-treatment devices and the use of slow-release devices such as cattle ear tags and boluses which are impregnated with pesticides. Habitat modification has generally involved controlled burning, brush removal and overstory reduction in woodlots. Tick control however generally involves laborious effort and time, and no technique has proven effective against all tick species. Therefore many livestock producers either ineffectively treat their animals or choose no prophylactic precautions at all.

In addition to the problems of time, labor and cost, another major dilemma has surfaced in the area of pest management: the confrontation between the need for pest control versus the protection of the consumer

and the environment. From this controversy came the present federal government policy dealing with pesticides. The Federal Environmental Pesticide Control Act of 1972 left the arsenal of registered pesticides currently cleared for tick control at a minimum; with some pesticides being no longer available to the consumer-producer.

It is therefore essential that new approaches to tick control on animal hosts and subsequent tick population suppression be investigated. Some recent experimental tick control methods have included the release of sterile male ticks into the wild population and the dissemination of tick predators. Also, the existence of tick assembly and sex pheromones has been demonstrated and further research is being conducted on the possibility of using these pheromones in control programs. Another approach which shows potential for use in an integrated control program may be found in the field of immunology.

The purpose of this study was to develop an immunological approach to the control of the Gulf Coast tick, Amblyomma maculatum Koch, and the lone star tick, Amblyomma americanum (L.); and to evaluate the possible applications of this work for the control of ticks on domestic livestock under natural conditions.

CHAPTER II

REVIEW OF THE LITERATURE

Several studies have been published in the area of immunological control of arthropods on animal hosts, and although some arthropod antigens have been isolated and characterized, not one has been structurally identified (Gregson 1970, Benjamini and Feingold 1970, Dickinson et al. 1976, Willadsen and Williams 1976, Nelson et al. 1977, Wikel et al. 1978).

Dubin et al. (1948) found that Anopheles quadrimaculatus, fed on rabbits which had been injected with whole mosquito homogenate, were not prevented from biting and the homogenate made the animals hypersensitive to mosquito bites. Alger and Cabrera (1972) found that Anopheles stephensi fed on rabbits injected with mosquito midgut antigen had higher death rates than those fed on control rabbits or on rabbits injected with the soluble or insoluble fractions of whole mosquito antigen. Sutherland and Ewen (1974) observed that Aedes aegypti females fed on sensitized rabbits and guinea pigs showed substantially reduced fecundity, while fecundity of Culex tarsalis females fed on the same mammals was not reduced.

Preparations of whole flea homogenate were made for use as antigenic material by Benjamini et al. (1960). Sensitivity of guinea pigs to flea bites was induced by intradermal injections of the homogenates suspended in saline. Guinea pigs injected intradermally with whole flea homogenate reacted to flea bites. Conversely, when sensitized by flea bites,

the animals reacted to challenge with the homogenate. The host reactions were skin reactions and apparently there was no effect on the fleas themselves as the authors reported no changes in flea fecundity or death rate.

Benjamini et al. (1963) reported that injection of flea oral secretion suspended in saline did not induce sensitivity to flea bites, nor to injections of whole flea extract or oral secretion. Sensitivity was induced only when oral secretion was inoculated in combination with Freund's complete adjuvant.

Schlein and Lewis (1976) fed Stomoxys calcitrans on rabbits previously immunized with various tissues of the fly. Mortality after 15 days of feeding on rabbits immunized with flight muscle proteins was twice that of the control flies fed on an untreated rabbit. The authors also reported other effects in flies exposed to immunized rabbits including paralysis of the legs, unequal deposition of the endocuticle and reduced post-emergence growth.

Relatively few studies have been published in the area of immunological control of ticks on animal hosts. Researchers in Cuba have reportedly developed a vaccine for canines, but the results of the study have not yet been published. Other researchers are studying the feasibility of immunizing cattle against tick growth hormones (Galun 1975).

The interactions between the tick and its host have been the subject of many studies (McGowan and Barker 1980). The area of tick-host interactions can be divided into two major parts: studies of host resistance produced by tick infestation, or studies in which the host animal is immunized with various tick antigens.

The acquisition of resistance to tick feeding by both laboratory and domestic animals is well-documented by a large number of authors

(Musatov 1957, 1966a, b, 1970a, b, 1973, Riek 1954, 1958, 1959b, Boese 1969, 1974, Francis 1966, Roberts 1968a, b, Brossard 1976, Wikel and Allen 1976a, b, 1977, Fujisaki 1978, and others). These studies have determined that resistance could be assessed by comparisons made between initial number or weight of engorging ticks and the results from subsequent infestations. Hosts were deemed resistant when numbers or weights of engorged ticks were significantly lowered.

There are however relatively few papers dealing with the immunization of host animals. One of the earliest published reports of the immunization of tick hosts was made by Hull (1912). Hull reported that injecting cattle with the lymph taken from tiny vesicles on the escutcheon and dew-lap of a tick-resistant cow afforded complete protection from ticks. There followed a number of conflicting reports concerning Hull's claims (Johnston and Bancroft, 1919) and the entire series of studies apparently ended with a paper by Johnston and Bancroft (1920).

Trager (1939a) reported that guinea pigs immunized with an extract of larval Dermacentor variabilis developed antibodies. Trager also sensitized animals by vaccinating them with homogenized tick salivary glands. Blood sera of rabbits immunized with the salivary glands gave a specific complement fixation reaction. Trager hypothesized that these host reactions made it impossible for subsequent ticks of the same species to engorge normally.

Musatov (1957) reported that the subcutaneous injection into rabbits of an emulsion of salivary glands from unfed male and female Rhipicephalus bursa decreased subsequent larval tick feeding. In later studies Musatov (1967a, b, 1976) reported on the use of tick salivary gland antigens made from male and female R. bursa and Hyalomma plumbeum.

In all of these studies, rabbits or sheep were immunized and subsequently infested with ticks. Significant decreases in numbers of ticks successfully engorging and changes in the ticks' physical parameters were reported. Musatov concluded that immunized hosts were producing anti-tick antibodies which were affecting tick feeding.

Riek (1958) found that precipitating and skin-sensitizing antibodies were produced by animals injected with an extract made from eggs of Haemaphysalis bispinosa. He concluded that precipitating antibodies protected the animals against toxic egg extracts, but tick resistance was associated with skin-sensitizing antibodies.

Garin and Grabarev (1972) reported the presence of resistance to R. sanquineus following injections of tick salivary gland antigen. Allen and Humphreys (1979) have reported the effects in ticks due to feeding on hosts immunized with antigens extracted from gut tissue and other internal organs of ticks fed for five days prior to dissection and antigen extraction.

CHAPTER III

METHODS AND MATERIALS

Antigen Preparation

Antigen was prepared by homogenizing unfed adult ticks in sterile water using an Omni-mixer (Sorvall Inc., Newton, Connecticut). The homogenate was centrifuged on a Beckman Ultracentrifuge (Model No. J-21B) at 13,000 rpm for 1 h. The supernatant was drawn off, lypholized and stored at -26°C. Protein concentration of the reconstituted homogenate was assayed according to the method of Lowry et al. (1951).

Experiment 1

One thousand live, male A. maculatum were homogenized for 5 min in 16 ml of sterile water. Proteins in the extract were separated and scanned as outlined below.

Experiment 2

An extract from a homogenate of 16,000 live, male and female A. americanum was made and protein separation was assayed as outlined below.

Experiment 3

A similar A. americanum extract (Experiment 2) was made with the exception that 100,000 ticks were homogenized. Protein separation was assayed as described below.

Disc Gel Electrophoresis

Proteins from the homogenates were separated according to Ornstein (1964) in 7.5% polyacrylamide gels (pH 8.9). Tube dimensions of 5 mm ID x 125 mm in length were the only technique modification. Stacking gels measured 2 cm, while separating gels were 9.5 cm in length. Samples buffered at pH 8.9, contained 10% sucrose and tracking dye. Electrophoresis was at room temperature at 2 mA/tube until the tracking dye was 1-3 cm from the bottom of the gel. Gels were stained with 0.5% aniline blue-black for approximately 1.5 h and later destained for 24 h in 7% acetic acid. A Beckman DU spectrophotometer with a Gilford Model 2410 scanning attachment was used to scan stained gels at 550 nm and a slit width of 0.2 mm. Molecular weights of the male A. maculatum homogenate were determined by SDS gel electrophoresis according to the method of Shapiro et al. (1967).

Experimental Animals

Experiment 1

Three-month old, healthy New Zealand white rabbits (Oryctolagus cuniculi) that weighted 1.8-2.2 kg were used in the experiment.

Experiment 2

A group of Jersey bull calves weighing between 70-115 kg was used for this experiment. All calves were wormed and checked for ticks before being employed in the test.

Experiment 3

A group of Hereford bull calves weighting ca. 215 kg each was used for this experiment. Calves were checked for tick infestation and wormed before the test.

Ticks

Adult and nymphal A. maculatum and A. americanum used to prepare the antigens and infest the host animals were reared at our laboratory. Ticks for all experiments and of all life stages were stored at 25°C and 90% RH in cardboard containers with plastic wrap serving as lids. The relative humidity was maintained by keeping the containers in sealed Plexiglas® hydrating chambers containing a saturated K_2SO_4 solution (Winston and Bates 1960).

Immunization

Experiment 1

Treated rabbits received a total of 1.5 mg/kg of antigen per injection. The immunogen was injected into the footpads of both hind legs as outlined by Campbell et al. (1970).

For each series of injections, the immunogen was thawed, rehydrated in 2.25 ml of sterile water and then emulsified with an equal volume of Freund's complete adjuvant. Freund's incomplete adjuvant was used in the injection series on day 22. Each test rabbit was injected on days one and 22 and bled by ear vein puncture every seven days for serological tests.

Experiment 2

Treated calves received a total of 3 mg/kg of antigen in a single injection on day one. The immunogen and Freund's complete adjuvant were injected subcutaneously into the side of the calf's neck. The calves were bled every seven days for serological tests.

Experiment 3

Treated calves received 6 mg/kg of antigen on days 1, 15 and 28 for a total of 18 mg/kg injected over the life of the test. The immunogen and Freund's incomplete adjuvant were injected subcutaneously into the animal's neck. Calves were bled every seven days for serological tests.

Infestation and Experimental Design

Experiment 1

Eight rabbits were randomly separated into treatment and control groups consisting of four rabbits each. All rabbits were infested with nymphal or adult A. maculatum contained in two, round plastic cells 2.5 cm x 2.5 cm glued to the dorsal surface of the hindquarters (Teel et al. 1977). Plastic collars prevented the animals from disturbing the cells (Watts et al. 1972). Rabbits were infested with 25 nymphs per cell on day 36 of testing. Ticks that successfully engorged were collected and mean weights and number of successful molts were determined.

Each rabbit was infested with eight pairs of adult ticks on day 49. Four pairs of colony orgin ticks served as controls and were placed in a cell on the rabbits' left flanks. The remaining four pairs were placed in a cell on the right flanks; these ticks had previously been fed as

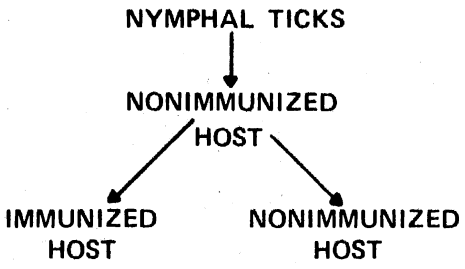
nymphs on rabbits immunized with A. maculatum extract, and thus, permitted observations of a second exposure effect on adult ticks.

Four designs were used to observe the results of feeding ticks on immunized and nonimmunized rabbits. These designs are summarized in Figure 1. In the first design nymphal ticks were fed on nonimmunized rabbits and later fed as adults on immunized and nonimmunized rabbits. In the second design the nymphal ticks were fed on immunized and nonimmunized rabbits and after eclosion to adults, they were fed on nonimmunized rabbits. The third design was similar to the second except that the nymphal ticks which had previously fed on the immunized and nonimmunized rabbits were later fed as adults on immunized rabbits. Design four consisted of feeding nymphal ticks on immunized rabbits and then feeding the subsequent adult ticks on immunized and nonimmunized rabbits.

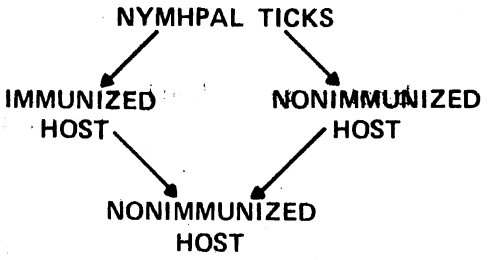
Experiment 2

Six Jersey bull calves were randomly separated into treatment and control groups consisting of three calves each. On day 42, following the initial antigen injection, all calves were placed in outdoor stanchions and infested with 50 pairs of adult A. americanum. The ticks were placed in orthopedic stocking cells glued to the calves' backs using Formica® contact cement. Each calf had two cells with 25 pairs of adults per cell. The total number of ticks that successfully engorged and the mean engorgement weight were recorded. The total number of egg masses and mean egg mass weight were calculated. Percent conversion of blood meal to eggs was determined by dividing each egg mass weight by the engorgement weight of its respective female and multiplying by 100. The mean was then calculated for all conversions and recorded.

Figure 1. The Four Designs Used to Observe the Performance of Feeding Ticks on Immunized and Nonimmunized Rabbits in Experiment 1.



Design 1



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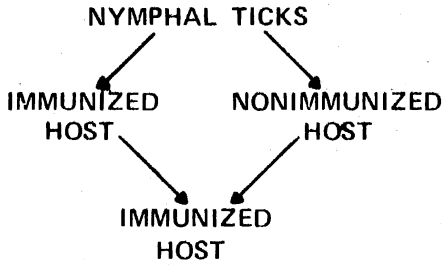

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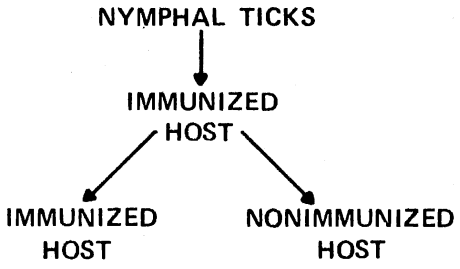


Design 4

Calves were reinfested with 50 pairs of adult ticks on day 70 of the test. Numbers of engorged females, mean engorgement weight and egg mass weight were recorded.

Experiment 3

Four Hereford bull calves were randomly separated into treatment and control groups consisting of two calves each. On day 56, these calves were placed in indoor stanchions and infested with 50 pairs of A. americanum as outlined above. All tick feeding parameters were measured as described previously. Additionally, the number of larvae hatched from each egg mass was determined as follows. Using repeated weighings, the mean weight of 10 groups of 100 A. americanum larvae was calculated as equaling 4.7 mg. It was further calculated that empty egg cases accounted for ca. 6% of the total weight of larvae and egg cases. Therefore vials containing larvae and empty egg cases were weighed and ca. 6% of the weight of the contents and the vial weight itself were subtracted from the total. The remaining value equaled the weight of the larvae. This weight was multiplied by 100 and the product represented the number of larvae. This procedure was used to calculate the number of larvae from each egg mass. From these values, the mean number of larvae per egg mass was determined.

Statistical Analysis

Data from design 1 (Experiment 1) and from both cattle trials were subjected to an analysis of variance for a completely randomized design. Data from designs 2-4 (Experiment 1) were subjected to an analysis of variance for a split plot design.

Serologic Tests

Passive hemagglutination (PHA)

A passive hemagglutination technique (PHA) was used to measure antibody titers. Antigen (3 mg/ml) was coupled to host red blood cells with bisdiazobenzidine (BDB) as outlined by Campbell et al. (1970). Titration of antibody using twofold serial dilutions was performed in disposable trays using a microtiter system. Normal host sera were used as controls.

Fluorescent antibody (FA)

Nymphal and adult A. maculatum were fed on immunized and nonimmunized rabbits. Upon repletion, nymphal ticks were frozen and cryostat cross-sections 8μ thick were made near the center of the body. Replete adult female ticks were collected, fixed in 10% buffered formalin, embedded in paraffin and sectioned (7-10μ). Phosphate buffered saline (PBS) was prepared as outlined by Campbell et al. (1970).

The procedure for clearing the adult tick sections was as follows: xylene, 4 min; alcohol series, 6 min; washed in tap water, 10 min; PBS, 10 min; and briefly air dried.

Goat-anti-rabbit fluorescent antibody was obtained from Cappell Laboratories, Inc., Cochranville, Pennsylvania. All slides were stained by placing several drops of a 1:6 (FA:PBS) solution on each tick section. All sections were then incubated at 38°C for 25 min. Slides were then rinsed with PBS and blotted dry. A drop of glycerol mounting medium (9 parts glycerol:1 part PBS, pH 7.5) and a cover slip were placed on each slide.

Slides were viewed and photographed on a Zeiss compound microscope

With fluorescent optics.

Immunodiffusion

The basic design for all combined polyacrylamide gel-agar gel immunodiffusion tests was the same. Disc gel electrophoresis was performed on the tick extracts. The polyacrylamide gels were then extruded and embedded in a 1% agarose in PBS gel as described by Wright et al. (1971). Troughs were cut alongside the gel columns and were charged with sera from test animals. All tests were run at room temperature for 24-48 h.

CHAPTER IV

RESULTS

Experiment 1

Electrophoretic studies revealed that protein content of nonengorged female and male A. maculatum were very similar (Figure 2). Therefore, it was hoped that alterations in female tick development attributed to host response to the injected male antigen would demonstrate cross-reactivity between tick sexes due to a common antigen(s).

The reconstituted tick homogenate yielded approximately 144 mg of material of which 43.2 mg was protein. The whole-male homogenate contained a minimum of 22-24 proteins or protein subunits of varying concentrations. Molecular weights of the proteins and protein subunits ranged from 6,000-340,000 daltons (Figure 3).

Results of the PHA test indicated that the four rabbits in the treatment group developed antibody titers within seven days which increased to a mean titer of 12 ($\log_2$) within 28 days in response to the injection of tick immunogen (Figure 4). Titers of control rabbits were always zero. The second injection elicited only a slight increase in titers.

Differences in skin responses between treated and control rabbits were also evident. During nymphal infestations on the treated rabbits, lesions appeared within 8-12 h postattachment and reached maximum diameters of approximately 3-4 mm (measured at right angles) by 24-36 h. Lesions on control rabbits measured approximately 1-1.5 mm during the

Figure 2. Disc Polyacrylamide Gel (7.5%) Electrophoresis of Whole Adult *Amblyomma maculatum* Extracts. A. Male Scan. B. Female Scan. (pH 8.9, 2 mA/tube).

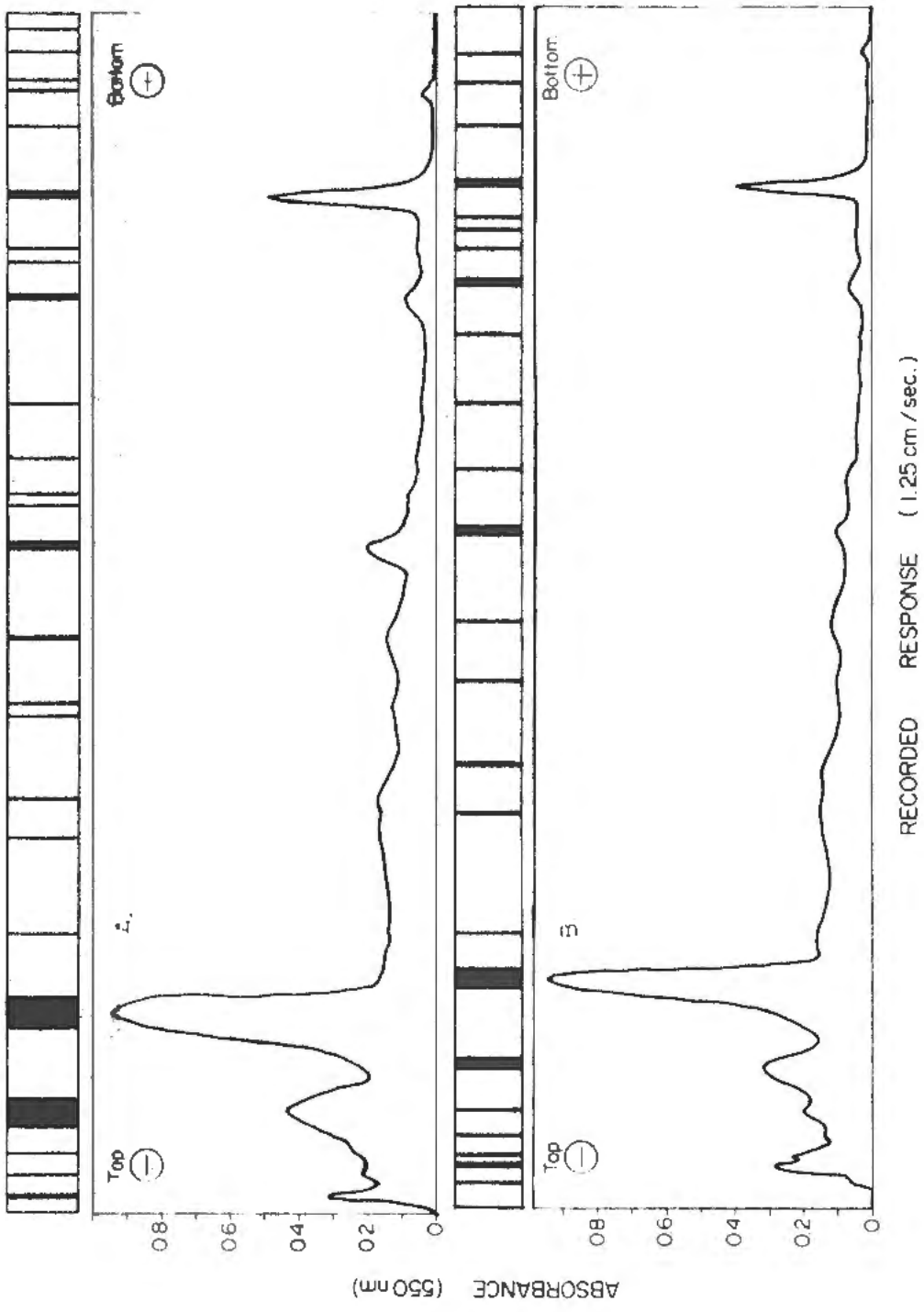
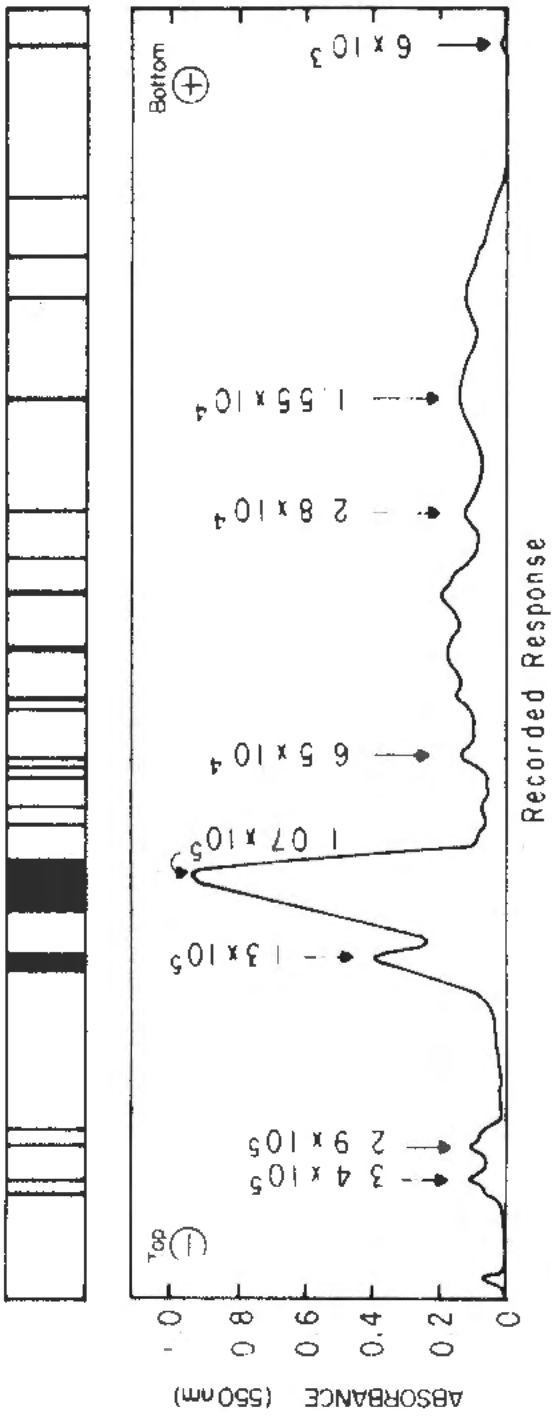

Top
Bottom
0.8
0.6
0.4
0.2
0
ABSORBANCE (550 nm)
RECORDED RESPONSE (1.25 cm / sec.)

Figure 3. Spectrophotometric Scan of SDS Polyacrylamide Gel (7.5%) Electrophoresis of Whole Unfed Male Adult Amblyomma maculatum Extract Used in Experiment 1 Depicting Protein Peaks and Ranges of Molecular Weights (Daltons).


Top
Bottom
6 x 10³
1.55 x 10⁴
2.8 x 10⁴
6.5 x 10⁴
1.07 x 10⁵
1.3 x 10⁵
2.9 x 10⁵
3.4 x 10⁵
0
0.2
0.4
0.6
0.8
1.0
ABSORBANCE (550 nm)
Recorded Response

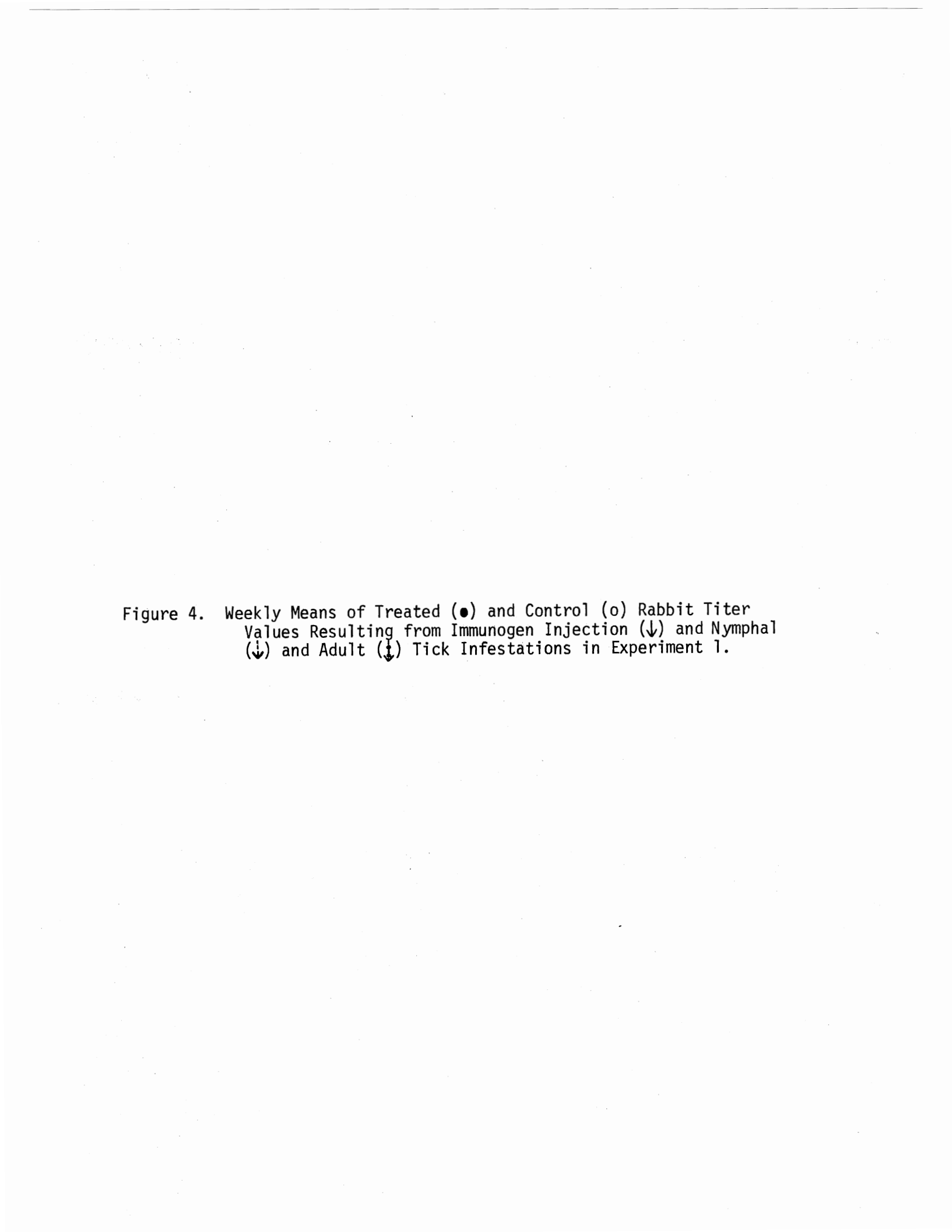
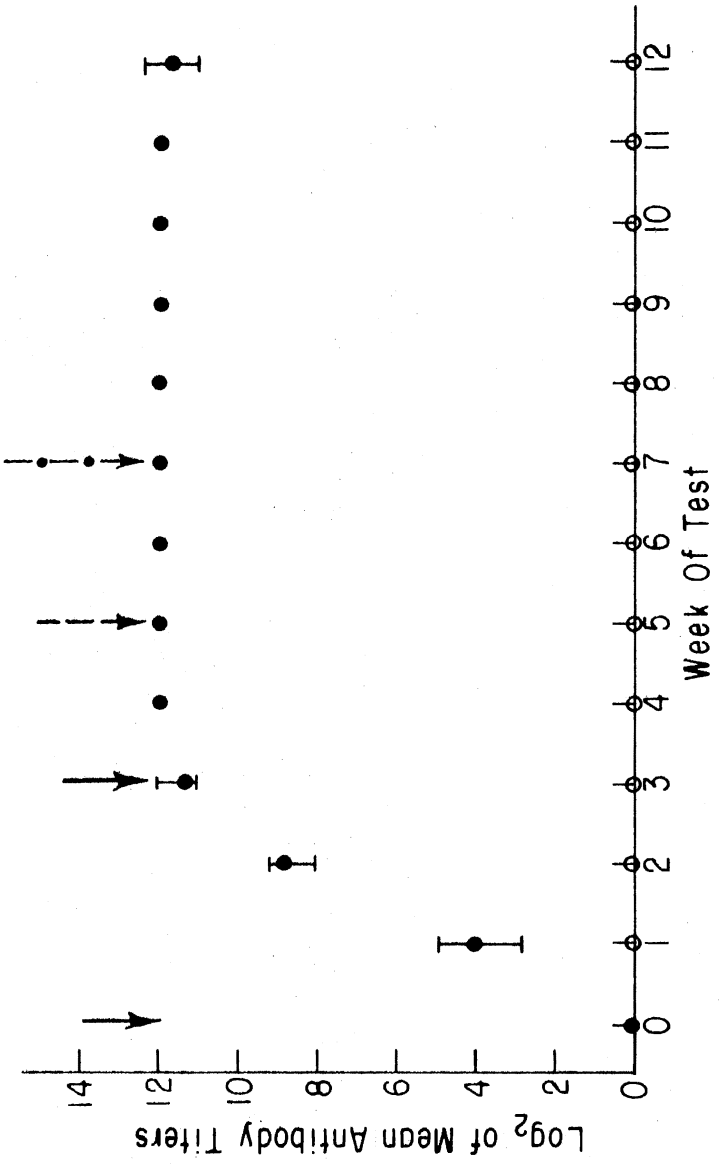


Figure 4. Weekly Means of Treated (●) and Control (o) Rabbit Titer Values Resulting from Immunogen Injection (↓) and Nymphal (↓) and Adult (↕) Tick Infestations in Experiment 1.


Log2 of Mean Antibody Titers
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
8
9
10
11
12
Week Of Test

same infestation period. During adult tick infestations, lesions on treated rabbits appeared within 2-4 h postattachment and reached diameters of 4-5 mm. Control rabbits also exhibited lesions of 4-5 mm, but these appeared after 18-36 h. Lesions were still apparent at the conclusion of the experiment.

When nymphal ticks were fed on immunized or nonimmunized rabbits (designs 1,2,3, and 4), there were no significant differences ($P>0.05$) in nymphal engorgement weights or the percent eclosion to the adult life stage (Table I).

When adult ticks were fed on immunized and nonimmunized rabbits (design 1) the respective mean engorgement weight of ticks fed on immunized rabbits was significantly ($P<0.01$) less than ticks that were fed on nonimmunized rabbits (0.48, 0.83 g, Table II). A further significant ($P<0.01$) reduction in the mean engorgement weight (0.30 g) of female ticks resulted when both nymphal and ensuing adult ticks were fed on immunized rabbits (Table II). Also, the respective mean egg mass weight of female ticks fed on immunized rabbits was significantly ($P<0.05$) less compared to female ticks that fed on nonimmunized rabbits (0.14 vs 0.40 g, Table III). This decrease may have resulted from a long-term effect in the tick as a result of feeding on an immunized host. Long-term effects of host immunity on ticks were assessed as significant ($P<0.05$) differences between the mean egg mass weight of female ticks exposed only as adults to immunized rabbits compared to female ticks exposed as nymphs to immunized hosts and as adults to nonimmunized hosts (0.14 vs 0.26 g, Table III), respectively. However, this long-term effect of host immunity on the respective mean engorgement weights (0.48 vs 0.53 g, Table II) was not expressed in female ticks exposed only

TABLE I

MEAN ENGORGEMENT WEIGHTS AND PERCENT ECLOSION OF NYMPHAL A. MACULATUM FED ON IMMUNIZED AND NONIMMUNIZED RABBITS

Rabbit Treatment	No. Ticks Engorged [3]	Tick Wt. (mg) [4]	% Eclosion [4]
Immunized [1]	99	15 ± 1.7 a	92.5 b
Nonimmunized [2]	101	14 ± 0.9 a	94.6 b

[1] Data from 4 rabbits immunized with 3 mg/kg of male A. maculatum extract.

[2] Data from 4 nonimmunized rabbits.

[3] Each rabbit was infested with 50 A. maculatum nymphs.

[4] Means in the same column followed by a different letter are significantly different ($P<0.05$).

TABLE II

MEAN ENGORGEMENT WEIGHTS OF MATED FEMALE A. MACULATUM TICKS FED ON IMMUNIZED AND NONIMMUNIZED RABBITS

Rabbit Treatment	Tick Source	No. Ticks Engorged	Tick Wt.[5] (g)
Immunized[1]	I[3]	12	0.48 ± 0.15 a
	II[4]	14	0.30 ± 0.09 b
Nonimmunized[2]	I[3]	12	0.83 ± 0.23 c
	II[4]	11	0.53 ± 0.17 a

[1] Data from 4 rabbits immunized with 3 mg/kg of male A. maculatum extract.

[2] Data from 3 nonimmunized rabbits.

[3] No previous exposure as nymphs to immunized hosts (4 pr/rabbit).

[4] Previously exposed as nymphs to immunized hosts (4 pr/rabbit).

[5] Means followed by a different letter are significantly different ($P<0.01$).

TABLE III

MEAN EGG MASS WEIGHTS OF A. MACULATUM FEMALE TICKS FED ON IMMUNIZED AND NONIMMUNIZED RABBITS

Rabbit Treatment	Tick Source	No. Egg Masses	Egg Mass Wt.[5] (g)
Immunized [1]	I[3]	8	0.14 ± 0.07 a
	II[4]	10	0.17 ± 0.07 a
Nonimmunized[2]	I[3]	7	0.40 ± 0.09 b
	II[4]	7	0.26 ± 0.07 c

[1] Data from 4 rabbits immunized with 3 mg/kg of male A. maculatum extract.

[2] Data from 3 nonimmunized rabbits.

[3] No previous exposure as nymphs to immunized hosts (4 pr/rabbit).

[4] Previously exposed as nymphs to immunized hosts (4 pr/rabbit).

[5] Means followed by a different letter are significantly different ($P<0.05$).

as adults to immunized rabbits compared to ticks exposed as nymphs to immunized hosts and as adults to nonimmunized hosts. It is interesting to note that the reduction in the mean egg mass weight of ticks fed only as adults on immunized hosts was not significant ($P>0.05$) compared to ticks fed on an immunized host during both the nymphal and adult stages (0.14 vs 0.17 g, Table III), respectively.

Results from the second design (Table II) showed a significant ($P<0.01$) decrease in the respective mean engorgement weight (0.53 vs 0.83 g) of female ticks fed as nymphs on immunized rabbits compared to females fed as nymphs on nonimmunized rabbits. In Table III, this decrease in engorgement weights of previously exposed female ticks was reflected in the significantly ($P<0.05$) smaller mean egg mass weight from these ticks than that of control ticks (0.26, 0.40 g), respectively.

In the third design, the respective mean engorgement weight of ticks previously fed as nymphs and then as adults on immunized rabbits was significantly ($P<0.01$) less than that of ticks which had previously fed only as adults on immunized rabbits (0.38, 0.48 g, Table II).

In design 4, the respective mean engorgement weight of female ticks exposed twice to immunized rabbits was significantly ($P<0.01$) less than that of ticks exposed once to immunized rabbits (0.30 vs 0.53 g, Table II). The respective mean egg mass weights from these female ticks (0.17, 0.26 g, Table III) were also significantly different ($P<0.05$).

Overall statistical analysis of the mean egg mass weights indicated the presence of an interaction ($P<0.01$) between rabbit treatment and the feeding history of adult ticks.

Experiment 2

The tick homogenate yielded approximately 660 mg of protein. A spectrophotometric scan of this immunogen is shown in Figure 5. The male-female homogenate contained a minimum of 20-24 proteins or protein subunits of varying concentrations.

Results of the PHA test indicated that no circulating antibodies were formed by the immunized calves.

Differences in skin responses between treated and control calves were evident. During the first infestation in treated calves, lesions appeared within 12 h postattachment and reached maximal diameters of approximately 4 mm (measured at right angles) by 36 h. Lesions on control calves measured approximately 2 mm during the same time period. These results reoccurred upon reinfestation, however the time of appearance of the lesions was accelerated.

When adult ticks were fed on immunized and nonimmunized calves in infestations 1 and 2, the observed significance level (OSL) between the mean engorgement weights was 0.058 (Table IV). Results from both infestations indicated that there were no significant ($P>0.05$) differences in numbers of engorged ticks and egg masses nor in egg mass weights or percent conversion of blood meal to eggs regardless of host treatment (Table IV).

Experiment 3

The tick homogenate yielded approximately 4.5 g of protein. A spectrophotometric scan of the immunogen was made to check reproducibility of the antigen preparation techniques. This scan was identical to the scan of the Experiment 2 antigen (Figure 5). The male-female homogenate

Figure 5. Disc Polyacrylamide Gel (7.5%) Electrophoresis of Whole Adult Male and Female Amblyomma americanum Used in Experiments 2 and 3. (pH 8.9, 2 mA/tube).

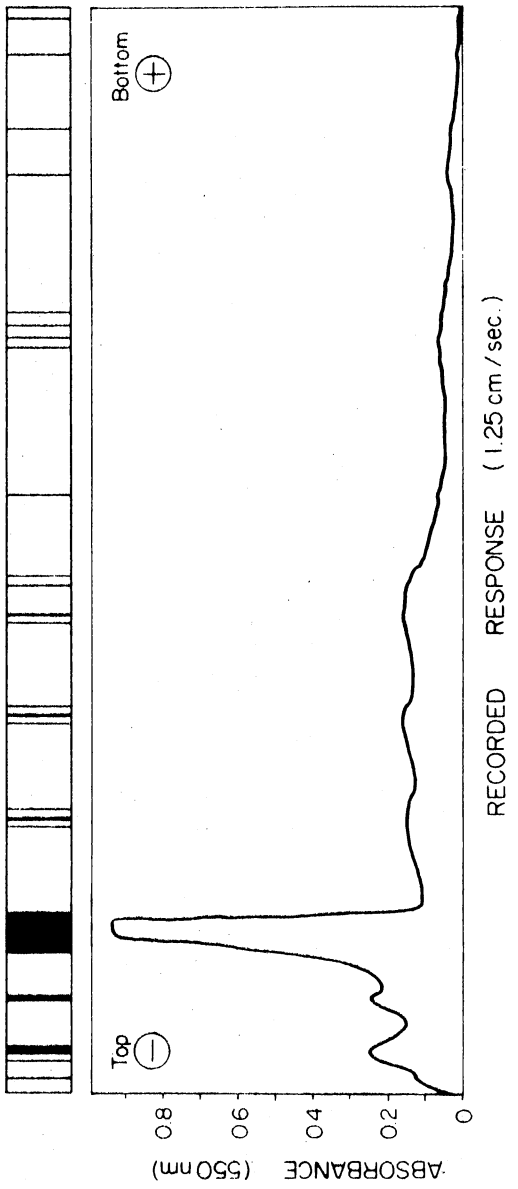

Top
Bottom
0
0.2
0.4
0.6
0.8
ABSORBANCE (550 nm)
RECORDED RESPONSE (1.25 cm / sec.)

TABLE IV

EFFECTS OF HOST IMMUNIZATION AND TICK REINFESTATION ON FEMALE *A. AMERICANUM* FED ON IMMUNIZED AND NONIMMUNIZED JERSEY CALVES[1]

Calf Treatment	No. Ticks[2] Engorged	Tick Wt. (g)	No. Egg Masses	Egg Mass Wt. (g)	% Conversion[3]
			INFESTATION 1		
Immunized[4]	90	0.44	81	0.27	54
Nonimmunized[5]	90	0.67	90	0.40	59
			INFESTATION 2		
Immunized[4]	84	0.39	61	0.26	45
Nonimmunized[5]	119	0.52	114	0.28	50

[1] For each variable at each infestation there was no significant difference ($P<0.05$) between the means of immunized and nonimmunized calves.

[2] Each calf infested with 50 pairs of adult *A. americanum* in each infestation.

[3] See text for explanation

[4] Data from 3 calves immunized with 3 mg/kg of male-female *A. americanum* extract.

[5] Data from 3 nonimmunized calves.

contained at least 20-24 proteins or protein subunits of varying concentrations.

Results of the PHA test were negative in both control and treated animal groups. No antibodies were detectable in either group. Differences in skin responses between treated and control calves were similar to those observed in Experiment 2. In this experiment immunized calves produced lesions within 24 h postattachment and reached maximal diameters of approximately 4 mm (measured at right angles) by 36 h. Lesions on nonimmunized calves measured approximately 2 mm during the same period.

When adult ticks were fed on immunized and nonimmunized calves, the respective numbers of engorged ticks fed on immunized calves was significantly ($P<0.05$) less than ticks that were fed on nonimmunized calves (43 vs 65, Table V). This difference was also reflected in mean engorgement weights of ticks fed on immunized versus nonimmunized hosts (0.43 vs 0.72 g, Table V).

The numbers and mean weights of egg masses of females fed on immunized calves were significantly ($P<0.05$) less compared to females fed on nonimmunized calves (36 vs 64 and 0.26 vs 0.38 g, Table V). There were no significant ($P>0.05$) differences in percent conversion of blood meal to eggs regardless of host treatment. However, the decrease in egg mass weights from female ticks fed on immunized calves resulted in a significant ($P<0.05$) difference in numbers of larvae produced when comparing immunized versus nonimmunized hosts (2208 vs 3597, Table V).

Fluorescent Antibody

Cross-sections of nymphal ticks which had fed on a nonimmunized host exhibited oval-strutured gut diverticulae with intact membranes and

TABLE V

EFFECTS OF HOST TREATMENT ON FEMALE *A. AMERICANUM* FED ON IMMUNIZED AND NONIMMUNIZED HEREFORD CALVES[1]

No. Ticks[4] Engorged	Tick Wt. (g)	No. Egg Masses	Egg Mass Wt. (g)	% Conversion[5]	No. Larvae[5]
		IMMUNIZED CALVES[2]			
43 a	0.43 a	36 a	0.26 a	47 a	2208 a
		NONIMMUNIZED CALVES[3]			
65 b	0.72 b	64 b	0.38 b	52 a	3597 b

[1] Means in the same column followed by different letters are significantly different ($P<0.05$).

[2] Data from 2 calves immunized with 18 mg/kg of male-female *A. americanum* extract.

[3] Data from 2 nonimmunized calves.

[4] Each calf infested with 50 pairs of adult *A. americanum*.

[5] See text for explanation.

little or no rabbit immunoglobulins were observed (Figure 6A). In contrast cross-sections of nymphal ticks which had fed on an immunized host exhibited disrupted gut diverticulae and a complete lack of membrane structural integrity (Figure 6B).

There were no discernible differences between cross-sections of adult ticks regardless of whether the tick had fed on an immunized or a nonimmunized host. All adult tick cross-sections had intact diverticulae and showed no evidence of differences due to a treatment effect.

Immunodiffusion

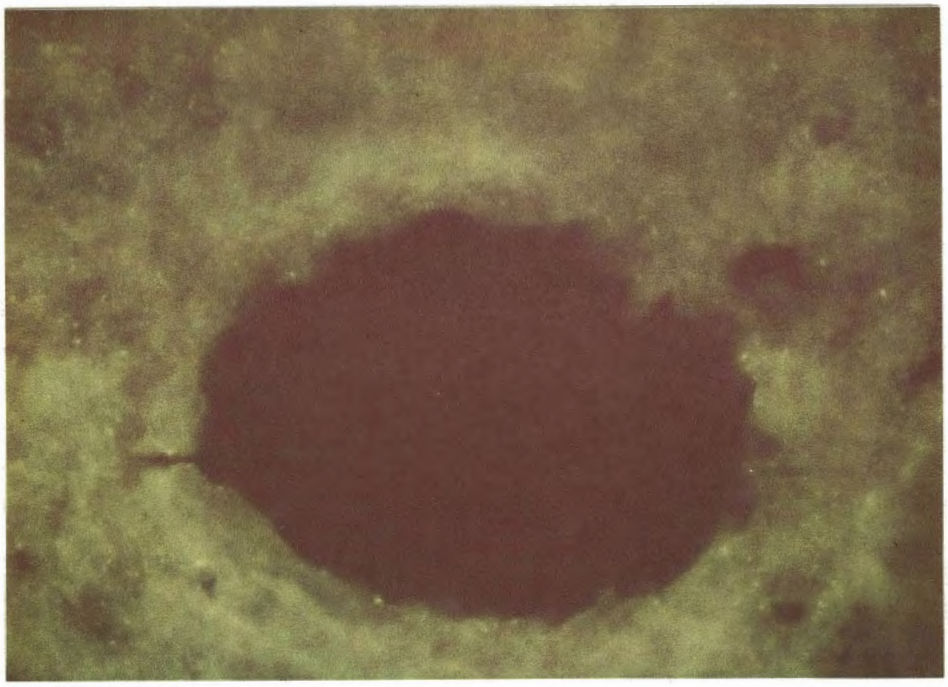
Test 1

In Figure 7A, serum from a rabbit immunized with male A. maculatum extract (Experiment 1) contained antibodies against the antigen as evidenced in the formation of a precipitin band (curved line). This same serum also contained antibodies which reacted with proteins found in an extract of unfed whole adult female A. maculatum. Apparently sera from a nonimmunized rabbit (Experiment 1) and an immunized calf (Experiment 3) contained no antibodies against these tick proteins as there was a complete absence of precipitin band formation. No detectable reaction between agarose and polyacrylamide gels occurred.

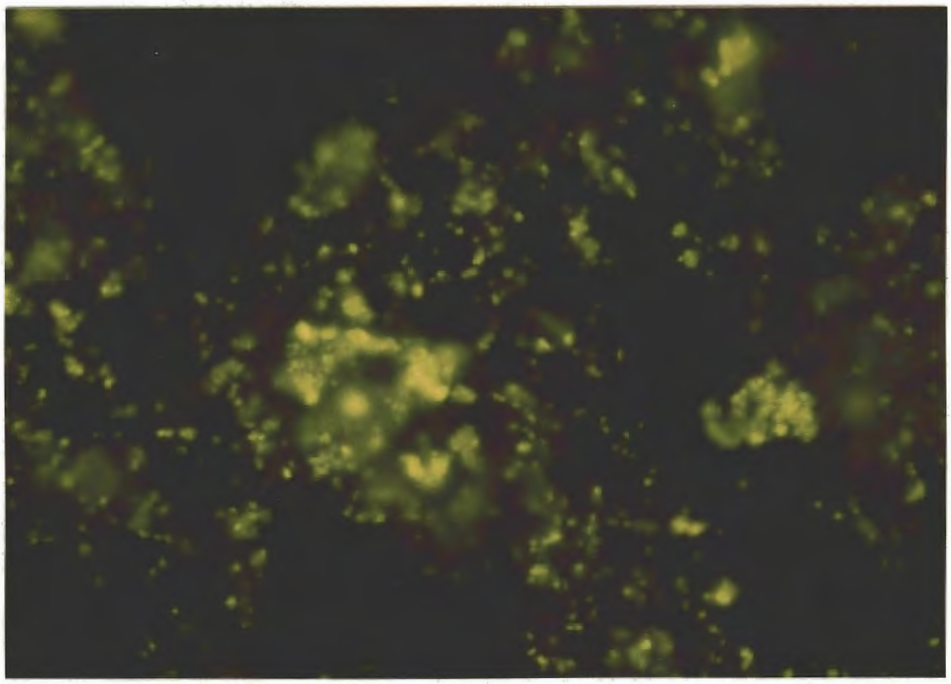
Test 2

In Figure 7B, serum from a rabbit immunized with male A. maculatum extract (Experiment 1) contained antibodies against protein(s) in the extract as evidenced by precipitin band formation. The serum also contained antibodies formed against a protein(s) in an extract from a mix-

Figure 6. Photographs of the Results of Immunofluorescence in Nymphal Amblyomma maculatum. A. Intact Diverticulae in a Nymph Fed on a Nonimmunized Rabbit (125x). B. Fluorescing Complexes Representing Disrupted Diverticulae in a Nymph Fed on an Immunized Rabbit (125x).



A



B

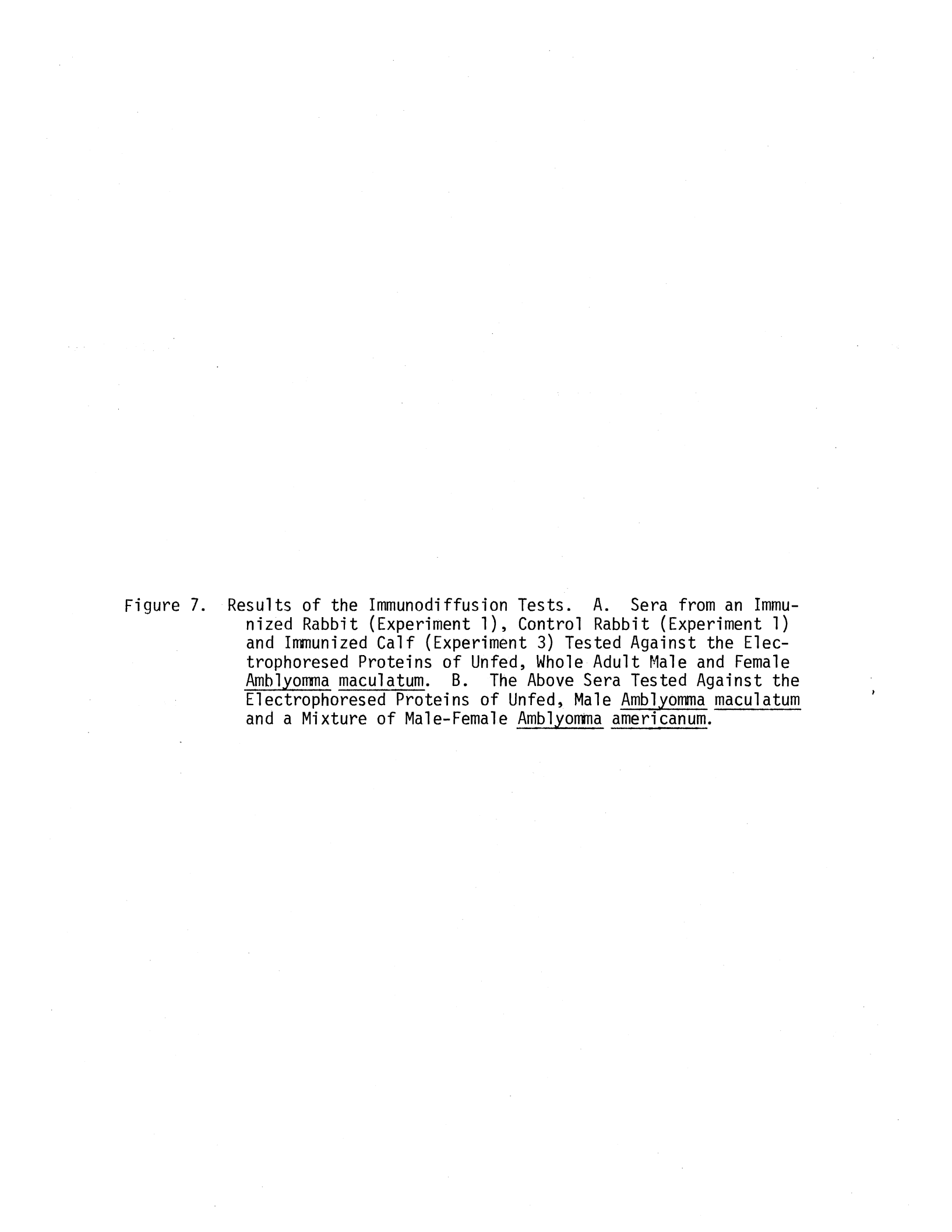
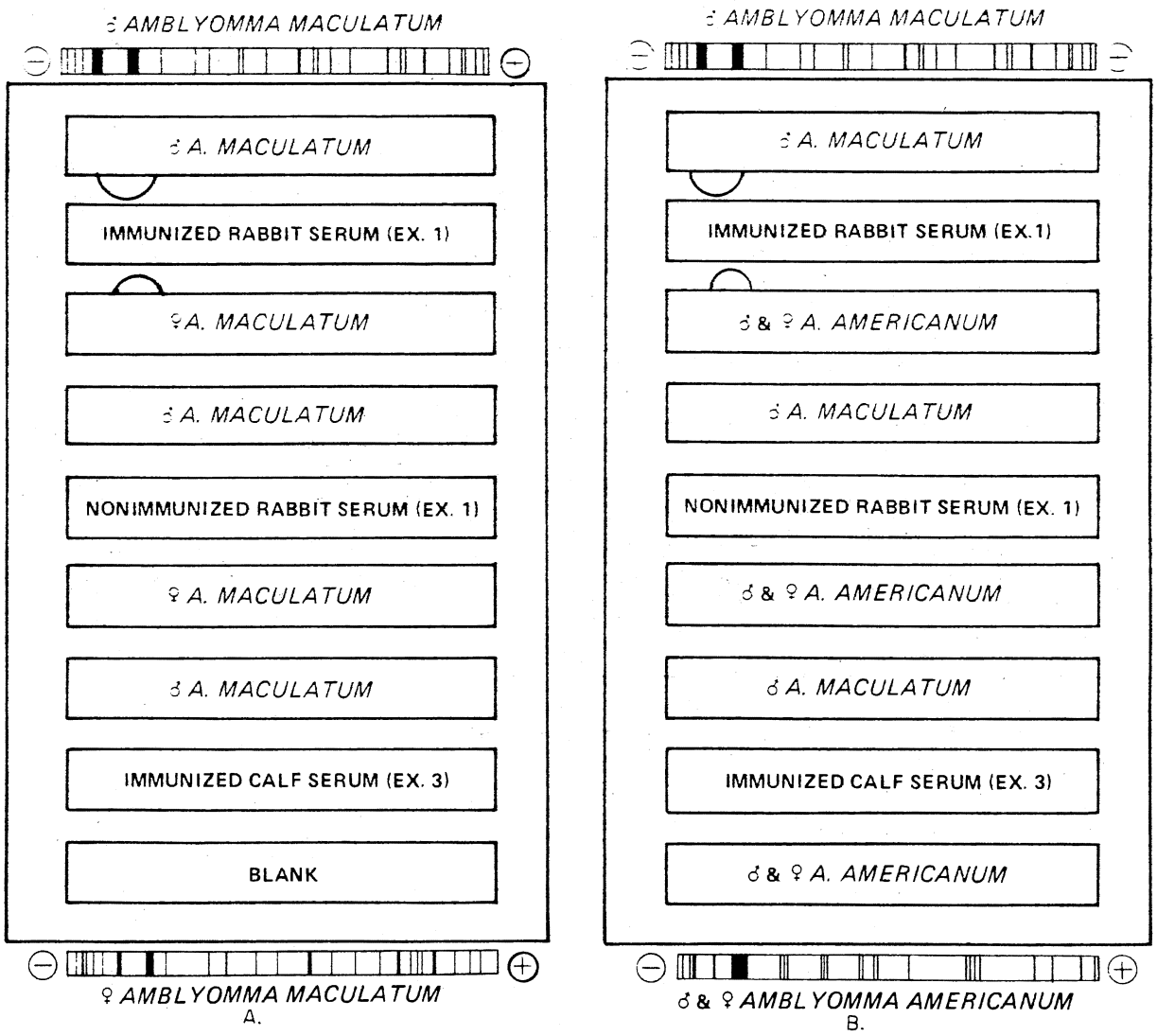


Figure 7. Results of the Immunodiffusion Tests. A. Sera from an Immunized Rabbit (Experiment 1), Control Rabbit (Experiment 1) and Immunized Calf (Experiment 3) Tested Against the Electrophoresed Proteins of Unfed, Whole Adult Male and Female Amblyomma maculatum. B. The Above Sera Tested Against the Electrophoresed Proteins of Unfed, Male Amblyomma maculatum and a Mixture of Male-Female Amblyomma americanum.


♂ AMBLYOMMA MACULATUM
♂ A. MACULATUM
IMMUNIZED RABBIT SERUM (EX. 1)
♀ A. MACULATUM
♂ A. MACULATUM
NONIMMUNIZED RABBIT SERUM (EX. 1)
♀ A. MACULATUM
♂ A. MACULATUM
IMMUNIZED CALF SERUM (EX. 3)
BLANK
♀ AMBLYOMMA MACULATUM
A.
♂ AMBLYOMMA MACULATUM
♂ A. MACULATUM
IMMUNIZED RABBIT SERUM (EX.1)
♂ & ♀ A. AMERICANUM
♂ A. MACULATUM
NONIMMUNIZED RABBIT SERUM (EX. 1)
♂ & ♀ A. AMERICANUM
♂ A. MACULATUM
IMMUNIZED CALF SERUM (EX. 3)
♂ & ♀ A. AMERICANUM
♂ & ♀ AMBLYOMMA AMERICANUM
B.

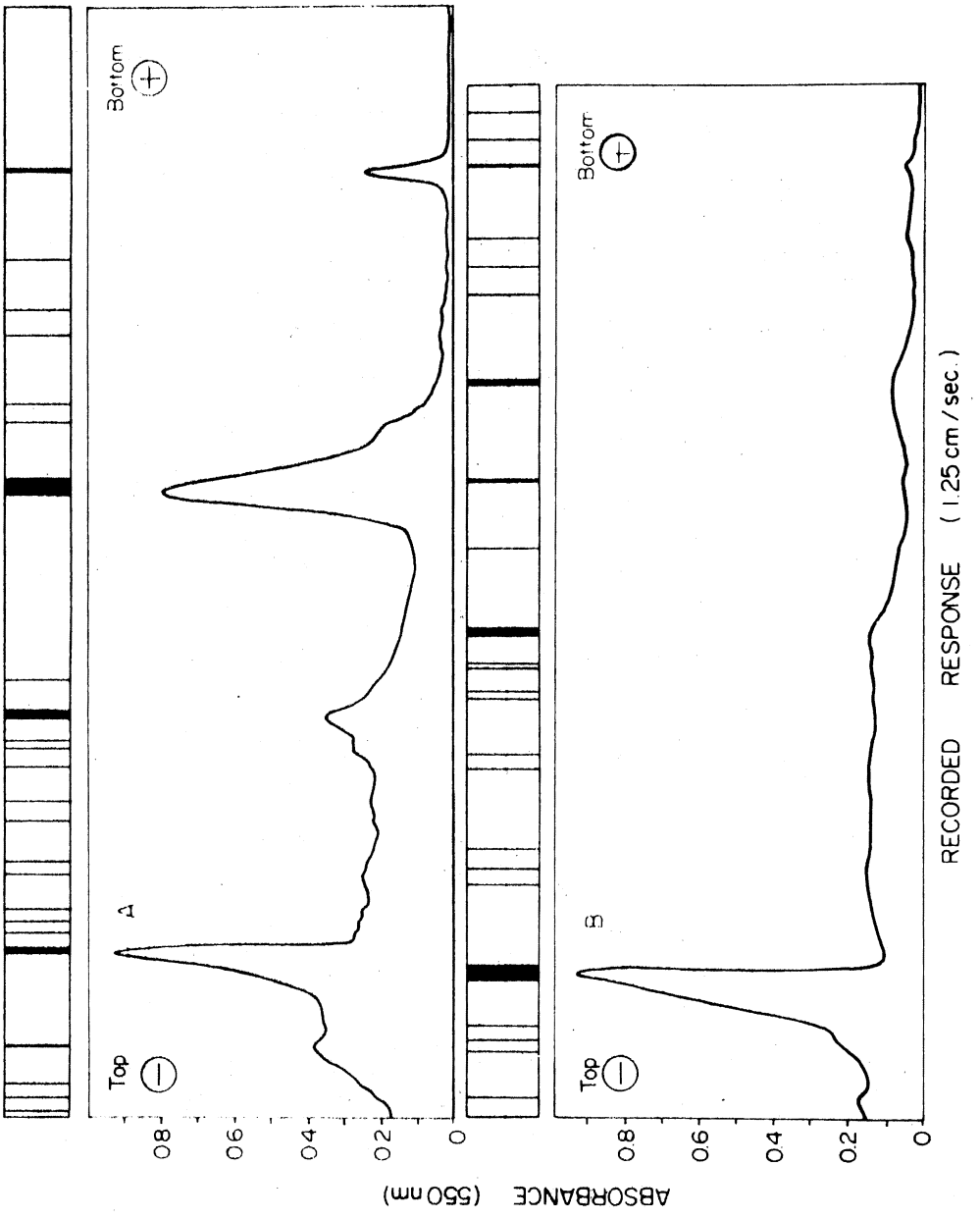
ture of unfed male and female A. americanum. Sera from a nonimmunized rabbit (Experiment 1) and a calf immunized with an extract of male and female A. americanum (Experiment 3) contained no antibodies as evidenced by the absence of precipitin band formation.

Test 3

As seen in a comparison of Figure 8, the extract from male A. americanum fed five days prior to homogenization (Figure 8A) contained several different or at least a higher concentration of proteins than the extract of unfed male A. americanum (Figure 8B).

In Figure 9, precipitin bands were formed between antibodies contained in the serum of a rabbit immunized with male A. maculatum immunogen (Experiment 1) and protein(s) in both male A. americanum, fed five days prior to homogenization, and an extract of male-female A. americanum. Sera from a nonimmunized rabbit (Experiment 1) and an immunized calf (Experiment 2) contained no detectable antibodies formed against proteins in the extracts. There were no apparent reactions between agarose and polyacrylamide gels.

Figure 8. Disc Polyacrylamide Gel (7.5%) Electrophoresis of Whole Adult Male Amblyomma americanum. A. Five-Day-Fed Male Extract. B. Unfed Male Extract (pH 8.9, 2 mA/tube).


Top
Bottom
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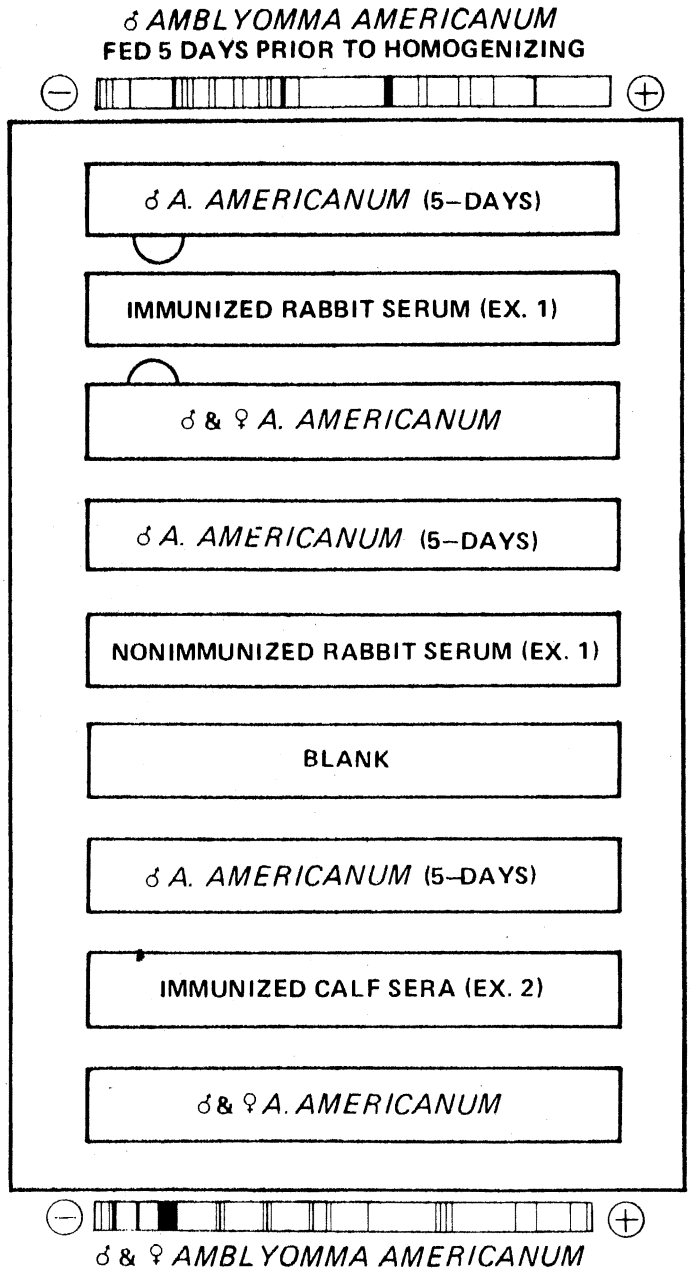
B
0
0.2
0.4
0.6
0.8
ABSORBANCE (550 nm)
RECORDED RESPONSE (1.25 cm/sec.)

Figure 9. Results from the Immunodiffusion Test. Sera from an Immunized Rabbit (Experiment 1), Control Rabbit (Experiment 1) and Immunized Calf (Experiment 2) Tested Against the Electrophoresed Proteins of Five-Day-Fed Male Amblyomma americanum and a Mixture of Unfed Male-Female Amblyomma americanum.


♂ AMBLYOMMA AMERICANUM
FED 5 DAYS PRIOR TO HOMOGENIZING
♂ A. AMERICANUM (5–DAYS)
IMMUNIZED RABBIT SERUM (EX. 1)
♂ & ♀ A. AMERICANUM
♂ A. AMERICANUM (5–DAYS)
NONIMMUNIZED RABBIT SERUM (EX. 1)
BLANK
♂ A. AMERICANUM (5–DAYS)
IMMUNIZED CALF SERA (EX. 2)
♂ & ♀ A. AMERICANUM
♂ & ♀ AMBLYOMMA AMERICANUM

CHAPTER V

DISCUSSION

The immunogens used in these studies were extracts from whole tick homogenates which contained at least 22-24 proteins or protein subunits identifiable by disc gel electrophoresis. This appears to be a rather small number of proteins. However, Krasnobaeva et al. (1971) reported that whole tick homogenates made from Argasid and Ixodid ticks contained only 1-5 large protein bands when separated using electrophoresis in agarose. Riek (1959a) reported only 1-6 distinct proteins in eggs and larvae of Boophilus microplus. Riek also showed that protein composition changed with tick age.

Few authors have used immunodiffusion as an in vitro technique in testing the antigenicity of various tick extracts. Boese (1969, 1974), using the method of double diffusion in two dimensions (Ouchterlony), found that sera from rabbits immunized with whole-tick extracts of Haemaphysalis leporispalustris formed precipitin bands when tested against the extract. However sera from rabbits which had an increased immune response due to infestations alone did not form precipitin bands when tested against the whole-tick extract. Using the Ouchterlony test Fujisaki (1978) found precipitating antibodies in the sera of rabbits resistant to the bite of H. longicornis and concluded that the rabbit antibodies were of the 7S class (IgG). Riek (1959a) using gel diffusion found that sera from rabbits immunized with extracts made from the eggs and

larvae of Boophilus microplus formed 1-7 distinct precipitin bands when tested against the extracts. Riek concluded that host skin hypersensitivity was due to an antigen bound to a fast-moving gamma-globulin and was distinct from antigens responsible for the immune state.

Analyses of these reports suggested that one or more of the 22-24 proteins injected into rabbits and cattle in my research could be producing both humoral and cell-mediated reactions. Therefore, immunodiffusion was utilized in an attempt to separate those protein antigens which may have induced the humoral response in the host animals.

The results indicated that only immunized rabbits produced circulating antibodies in response to extract injections and that there appeared to be a common antigenic protein(s) in males and females of A. maculatum and between the tick species A. maculatum and A. americanum. The antigenic proteins in the extracts appear near the stacking gel in all extracts and have a high affinity for the analine blue-black dye. These results indicate that the protein(s) is of fairly large molecular weight(s) and is apparently an integral component of the tick body. Future research should be directed to identifying the origin and distribution of this protein(s) in nymphal and adult ticks.

Previous investigators (Trager 1939a, b, Boese 1969, 1974, Allen and Humphreys 1979, and others) have reported that ticks fed on animals immunized with tick antigens or subjected to previous tick infestations often fail to engorge, or suffer a reduction in engorgement weight or egg mass weight.

In general my observations of adult tick responses to immunized rabbits and calves support these studies. Results in Experiments 1 and 3 show significant reductions ($P<0.05$) in all measured tick feeding para-

meters when adult ticks were fed on immunized hosts. These reductions were not as substantial in Experiment 2 where the observed significance level (OSL) for mean engorgement weights equaled 0.058. However, in Experiment 2 there appeared to be a trend in the decrease of mean engorgement weights. In Table IV, adult ticks fed on nonimmunized calves had a mean engorgement weight of 0.67 g. Upon reinfestation the calves' immune systems evidently affected tick feeding and resulted in a decreased mean engorgement weight (0.52 g). A similar decrease was recorded in mean engorgement weight of ticks fed on immunized calves. In the first infestation the mean weight was 0.44 g and after the second infestation, this weight was subsequently reduced to 0.39 g. Therefore despite the lack of statistical significance at the 5% level, the OSL indicates a practical significance; especially when the trend of engorgement weights is highest in the first infestation on a nonimmunized calf and lowest in the second infestation on an immunized animal. The difference in the levels of significance between the results in Experiments 2 and 3 may have been due to the larger amount of the male-female A. americanum antigen injected into calves in Experiment 3 (18 mg/kg) than the amount of antigen calves in Experiment 2 (3 mg/kg) received. Allen and Humphreys (1979) reported significant reductions of engorgement weights on Hereford-cross calves immunized with an extract of female D. andersoni which fed on Holstein steers for five days prior to antigen preparation. They reported that there was an apparent increase in antigenicity in extracts of previously fed ticks. An attempt was made to test for the reason for this increase and to evaluate what difference exists between extracts from unfed and fed ticks.

Male A. americanum were fed on a sheep for five days prior to antigen preparation. Results of a disc gel electrophoresis separation indicated the presence of a higher concentration of material in the extract. This increase would be expected as protein synthesis is occurring during tick feeding. Therefore an extract of five-day-fed ticks may be more antigenic than an extract of unfed ticks and may prove useful in future studies of tick induced immunity in cattle.

An immunodiffusion test was run to check for the presence of antibodies in the sera from immunized rabbits and calves (Figures 7 and 9). The results indicated that sera from rabbits immunized with male A. maculatum were cross-reactive to antigens in both an extract of male-female A. americanum and five-day-fed male A. americanum. No antibodies to any tick antigen were apparent in immunized calf sera. Future research should investigate calf responses to injection of an extract made of previously fed ticks.

Nymphal ticks did not exhibit a significant reduction ($P>0.05$) in mean engorgement weights or percent eclosion in Experiment 1. However, long-term effects of host resistance in nymphal ticks that fed on immunized rabbits in Experiment 1 were apparently carried over to the adult female tick stage. This was reflected in a further depression of female engorgement weights when compared to females that were fed as nymphs on nonimmunized rabbits. Possible mechanisms of long-term effects could be manifested in a decrease in female tick feeding, digestion of the blood meal, and/or male mating capabilities.

Brossard (1976) and Bowessidjaou et al. (1976) employed the fluorescent antibody test to detect the presence of host antibodies directed against salivary glands of ticks. Brossard found that there was a

causal relationship between antibody titer and development of resistance in cattle to heavy infestations of Boophilus microplus. Bowessidjaou et al. produced immunity to Ixodes ricinus in rabbits via sequential experimental infestations of ticks. Titers of circulating antibodies in the rabbits were measured by indirect immunofluorescence.

The above studies as well as those of most researchers in the area of tick-host interactions have been concerned primarily with the host response and with the effect of that response on readily measured parameters of tick feeding and fecundity. A technique which proved useful in studying host effects on ticks was immunofluorescence.

Results from the fluorescent antibody tests suggest that antibodies are binding to gut immunogens in nymphal ticks that fed on immunized rabbits, resulting in changes in gut morphology. The fluorescing complexes seen in Figure 6B represent the binding sites where it is hypothesized that rabbit immunoglobulins "recognized" and coupled with the tick gut membrane proteins. The antigen-antibody complexing appeared to cause a collapse of the tick gut membrane. Although the loss of gut structural integrity apparently did not effect nymphal tick engorgement or eclosion, it may have resulted in a partial loss of the capacity of the diverticulae thereby resulting in smaller blood meals taken as adults. Therefore these changes in gut morphology may have been responsible, in part, for the apparent long-term effects expressed as decreased adult female tick engorgement weights and egg mass weights. These findings suggest the sharing of nymphal and adult antigens. However, there was no apparent difference in fluorescence in sections of adult ticks regardless of whether the ticks fed on immunized or nonimmunized hosts.

The lack of significant differences in nymphal engorgement weights

and percent eclosion in Experiment 1, may be due to a short feeding period and ingestion of blood meals containing few anti-tick antibodies, or due to the fact that the immunogen was made from adult male A. maculatum, which may share few or reduced amounts of immunogens with nymphs. Further studies utilizing immunodiffusion are needed to determine if common antigens are present in nymphal and adult ticks.

In all experiments it was observed that some adult male ticks feeding on immunized hosts died prematurely, or became immobilized in the exudates from the lesions which formed at feeding sites. The deaths of these males prior to mating may have been responsible for the failure of a number of female ticks to become replete and produce eggs.

The developmental changes observed in ticks fed on immunized hosts are assumed to be manifestations of acquired resistance caused by the host's specific immune response to tick antigens. Passive transfer studies suggest that both humoral and cell-mediated factors are involved in the expression of this resistance, although the relative importance of each is still unclear (Trager 1939a, Wikel and Allen 1976a, b, Allen et al. 1979; and others).

While the present investigation was not concerned primarily with resistance mechanisms, I believe that the observed alterations in tick development are caused by immunological reactions in the host and/or tick. The combination of tick immunogens with sensitized lymphocytes or circulating and/or cell-bound antibody may produce an unsuitable environment for feeding at the site of attachment. Conversely, antibodies or lymphocytes ingested in the tick blood meal could react with immunogens associated with the tick digestive tract, altering digestive or absorptive properties. In either case the effect may be to reduce the amount

and quality of nutrients with resultant alterations in development.

Alteration of the feeding site due to the host's immune response has been reported by several authors (Trager 1939a, Schleger et al. 1976, and others). Bagnall and Rothwell (1974) and Allen (1973) have reported the deaths of ticks when fed on sensitized guinea pig hosts. Associated with these tick deaths were the accumulation and degranulation of basophils in the lesions. Histamine is one of the major components of basophils and Riek (1962) and Kemp (1978) have shown the presence of measurable amounts of histamines in the lesions of resistant cattle following tick feeding. Kemp (1978) also reported that only the early detachment of ticks appeared to be correlated with histamine release.

In immunized rabbits (Experiment 1) the immunogen elicited moderately high $\log_2$ titers of antibody (10-12, as measure by PHA) which persisted over the course of the experiment (Figure 4). The lack of a classical secondary response may have arisen because the second injection may have been given at a time of maximum antibody production in the rabbits or once the immune response was stimulated, suppressor T cells in the host animal may have been stimulated to suppress competent B and/or T cells. The activity of suppressor cells may also explain why no antibodies were discovered in the sera from immunized calves in Experiments 2 and 3. Weigle (1975) has suggested that the lack of antibody production may also be due to the use of antigens that are rapidly catabolized resulting in a failure of a persistent stimulation of antigen-reactive cells.

Schleger and Lincoln (1976) reported the presence of enzymes in oral secretions of larval B. microplus which effected the dermis of

cattle previously unexposed to ticks. This study and a review of the literature on studies of acarine salivary glands and components of oral secretions (Sauer 1977) show that research is still needed in the area of immunological characterization of tick antigens.

The presence of circulating antibody and the appearance of feeding lesions in immunized rabbits 8-12 h after nymphal tick attachment is suggestive of a Type III or Arthus reaction, as reported by Culbertson (1935). Despite an apparent lack of circulating antibody, calves in Experiment 3 and during the initial infestation in Experiment 2 also exhibited what may have been a Type III reaction. Complexing of antibody with antigens contained in the salivary secretions could have led to fixation of complement. The accompanying pharmacologic and cellular events may have reduced the suitability of the site for tick feeding. Wikel and Allen (1977) have reported that the expression of acquired tick resistance can be blocked by the use of an agent to deplete complement.

These data also suggest that at least some nymphal and adult tick immunogens, probably contained in salivary secretions, are shared. This is in agreement with the findings of Musatov (1967b) who noted reductions in tick size and engorgement weights as well as eclosion in nymphs fed on rabbits previously infested with adult R. bursa.

The rapid formation of the feeding lesions (2 h) in immunized rabbits and in calves during the second infestation in Experiment 2 following attachment of adult ticks is suggestive of a Type I, immediate hypersensitivity reaction, mediated by a homocytotropic antibody, probably IgE. Boese (1974) has reported the development of such an antibody in approximately 75% of rabbits repeatedly infested with nymphal ticks. In a field study, McGowan et al. (1979) correlated the resistance of rabbits to tick

feeding with the rabbits' development of skin-sensitizing antibodies. The sensitization evidently caused an increase in grooming activity and ticks were thereby removed. This increase has also been observed by a number of other authors (Trager 1939a, and others). Riek (1958) found these antibodies associated with tick resistance in laboratory animals injected with tick extracts. The biologically active factors released by the Type I reaction may contribute directly to the alteration of the local environment and also lead to the accumulation of antibody-containing fluids.

From this study and the studies of many other authors, it appears that the host immune response is reliant upon both humoral and cell-mediated components. Although cell-mediated immunity was not measured in the present study the work of Wikel et al. (1978) has shown the importance of a cellular component of the resistance response.

In summary, the hypothesis is that homogenized gut cells will be one of the major components of the tick extracts. Upon injection into the host, antibodies should be produced in response to one or more of the materials comprising the tick's digestive system. Upon tick attachment and initiation of feeding, antibodies could be imbibed with the blood meal. Once entering the tick's digestive tract, the antibodies could "recognize" and attach to, specific antigenic determinants on the cells of the digestive system; thereby causing cell lysis or affecting cell permeability and digestion of the blood meal. These reactions could then result in smaller egg masses and subsequent suppression of the tick population.

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VITA[2]

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